Executive Summary for CRCV 3.3.2 (GWRDC CRV99/16b)

'Role of growth substances in berry development'

PhD Project of Susan Wheeler

ABA and berry ripening

Hormones control plant development by coordinating changes in the expression of numerous genes at crucial times in a tissue and organ-specific manner. They have been implicated in controlling various aspects of grape berry development, in particular, the important process of ripening and are used in some crops to control growth and ripening. Previous studies have implicated Abscisic acid (ABA) in the response of grapevine to water stress, particularly in the control of water loss from leaves. There is also some evidence that ABA, perhaps in conjunction with other plant growth regulators may also have a role in the initiation and perhaps progression of grape berry ripening. This project was conducted to examine the metabolism of ABA in berries, to further explore the role of ABA in initiating berry ripening and to investigate in some depth the mechanism of ABA action during berry development.

It was shown over three seasons that ABA levels in Cabernet Sauvignon berries increase dramatically at veraison, consistent with it being involved either as a trigger for ripening or as a response to the increase in sugars that occurs at this time. It is possible for example, that ABA may be produced as a response to the osmotic changes (stress?) caused by the rapid influx of sugars at veraison. Indeed it was demonstrated that there is an interaction between ABA, sugars and changes in gene expression. Net ABA accumulation doesn't occur until veraison, the decrease in ABA concentration in the first phase of berry development being due to berry expansion. This means that ABA only begins to be synthesised, or imported, at veraison. The decrease in ABA that occurs later in development is likely to be due to a combination of catabolism, as the overall level of free ABA decreases and sequestration into the bound form as there is a rise in the proportion of conjugated ABA later in berry development. Two genes crucial to ABA synthesis, 9-cis-epoxycarotenoid dioxygenase (*NCED*) and zeaxanthin epoxidase (*ZEP*), were expressed throughout berry development and no clear correlation was found between their levels and that of ABA. This may suggest a complex mechanism of control over the expression of the

ABA biosynthetic pathway and later steps not yet identified in grapevine may be important.

Replicated field trials clearly showed that ABA treatments can be effective in significantly enhancing ripening when applied in at or near the end of the first period of berry expansion. Colour accumulation in the skins commenced earlier in ABA-treated fruit than in control fruit as did the increase in sugar levels. ABA treatment also advanced the timing of the second phase of berry expansion as it appeared to eliminate the lag phase of berry growth. Taken together these data demonstrate that ABA is likely to play some part in the control of berry ripening and can be used to advance the timing of ripening. Further investigation into the characteristics of ABA-treated fruit will be needed to investigate the compositional character of treated fruit and to gauge its suitability for winemaking. An ability to control the timing of ripening may provide considerable benefits to the wine industry in terms of wine style/quality and for winery scheduling.

Some of the significant findings from this work, which have added to our understanding include:

- ABA concentration increased reproducibly at veraison at roughly same time as sugar, colour and berry weight change
- Net accumulation of ABA doesn't occur until veraison
- A substantial amount of bound ABA is present at all stages but there is no evidence of conversion to the free form within the berry
- Total and free ABA concentration decreased 2-3 weeks after veraison during each season, due to both breakdown and sequestration of free ABA into the bound form
- Free ABA levels in skin and flesh were fairly similar throughout development, levels in seeds were often a little higher
- The ABA biosynthetic pathway genes *VvNCED1*, *VvNECD2* and *VvZEP* were expressed throughout berry development, the *NCED* genes were normally expressed more highly before veraison but no clear pattern of transcriptional activity was relatable to changes in ABA level
- ABA applied to pre-veraison berries in the vineyard can promote ripening as measured by an increase in sugar levels, skin colour and berry size

Implications for grape growing and winemaking

While the work here is of considerable interest from a physiological perspective, the greater immediate importance is how this new knowledge might impact upon grape production and ultimately on wine style. The outcomes of this research indicate that the application of exogenous ABA to developing Cabernet Sauvignon berries, although it hastened the onset of veraison, did not have a noticeable influence on the final total soluble solid or anthocyanin content at harvest time, compared to the control berries. There are a number of possible reasons why this may be so and it might be expected that increases in these parameters might be achieved with further experimentation.. Fruit did, however, begin to ripen earlier which may have advantages in controlling harvest time and promoting ripening in cool climates. There may be other berry metabolites which are influenced by the ABA application but were not measured within the relatively narrow constraints of this project, such as flavonols, tannins and flavour and aroma compounds. The experiments described here did not include making winemaking and metabolic and/or sensory analysis which would determine the effect of ABA on changes in metabolite levels. Although the total anthocyanin content was similar between the ABA-treated and the control berries at harvest, the relative extractability and stability of different classes of anthocyanins during winemaking was not determined.

There may be ways of altering ABA levels, and hence ripening, that don't involve the direct application of ABA. Some viticultural practices, for example, reduced water applications may increase berry ABA levels with consequent changes in berry development. Given the link between ABA and ripening the effect of viticultural practices on berry ABA levels should be of interest. Finally, as the timing, extent and nature of the ripening process is under strong genetic control and as there is considerable variation in these parameters within *vinifera* it should be possible to develop vines with a range of desirable ripening characteristics, some of these might be expected to have altered hormone levels, e.g. ABA around the time of veraison.

During the course of this project other research has indicated that ethylene and the brassinosteroid castasterone, two other growth regulators in grapes, may also be implicated in the control of berry ripening. These findings are exciting as the combination of action of the three hormones described may offer flexible solutions to alter grape berry ripening in a useful manner.

The Role of Abscisic Acid in Grape Berry Development

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Doctor of Philosophy
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Abstract

Hormones control plant development by coordinating changes in the expression of numerous genes at crucial times in a tissue and organ-specific manner. They have been implicated in controlling various aspects of grape berry development, in particular, the important process of ripening and are used in some crops to control growth and ripening. Abscisic acid (ABA), is associated in grapevine with the response to water stress but may also have a role in berry ripening.

We have shown over three seasons that ABA levels in Cabernet Sauvignon berries increase dramatically at veraison, consistent with it being involved either as a trigger for ripening or as a response to the increase in sugars that occurs at this time. Net ABA accumulation doesn't occur until veraison, the decrease in ABA concentration in the first phase of berry development being due to berry expansion. The decrease in ABA that occurs later in development is likely to be due to a combination of catabolism and sequestration into the bound form. The genes crucial to ABA synthesis, 9-cis-epoxycarotenoid dioxygenase (*NCED*) and zeaxanthin epoxidase (*ZEP*), were expressed throughout berry development and no clear correlation was found between their levels and that of ABA.

Laboratory studies have shown that isolated berries respond to the presence of sucrose through an increase in ABA biosynthesis pathway gene expression (*NCED* and *ZEP*). This resulted in *de novo* synthesis of ABA as inhibition of the carotenoid synthesis pathway by a phytoene desaturase inhibitor prevented ABA accumulation.

Replicated field trials clearly showed that ABA treatments can be effective in significantly enhancing ripening when applied in at or near the end of the first period of berry expansion. Colour accumulation in the skins commenced earlier in ABA-treated fruit as did the increase in sugar levels. ABA treatment also advanced the timing of the second phase of berry expansion as it appeared to eliminate the lag phase of berry growth. Taken together these data demonstrate that ABA is likely to play some part in the control of berry ripening and can be used to advance the timing of ripening. Further investigation into the characteristics of ABA-treated fruit will be needed to investigate the compositional

character of treated fruit and to gauge its suitability for winemaking. An ability to control the timing of ripening may provide considerable benefits to the wine industry in terms of wine style/quality and for winery scheduling.

Declaration

This work contains no material that has been accepted for the award of any other degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Susan Wheeler
October 2006

Abbreviations

1-MCP	1-methylcyclopropene
A_{520}	absorbance at 520nm
AAO	AB aldehyde oxidase
ABA	abscisic acid
ABA2	ABSCISIC ACID DEFICIENT2
ACO	1-aminocyclopropane-1-carboxylic acid oxidase
ASR	abscisic, stress and ripening
BLAST	basic local alignment search tool
bp	base pairs
cDNA	complementary DNA
DNA	deoxyribonucleic acid
DPA	dihydrophaseic acid
dNTP	deoxynucleotide triphosphate
dsDNA	double-strand DNA
EDTA	ethylenediamine-*tetra*-acetic acid
EST	expressed sequence tag
Fruc	fructose
FW	fresh weight
g	gram(s)
g	relative centrifugal force
GA	gibberellin
GC/MS	gas chromatography/mass spectrometry
GE	glucosyl ester
Glu	glucose
GRIP	grape ripening induced protein
h	hour(s)
HPLC	high performance liquid chromatography
IAA	indole-3-acetic acid
IPTG	*Iso*-propyl-β-D-thiogalactopyranoside
kb	kilobase pairs
L	litre(s)
LB	Luria broth

M	molar
Man	mannitol
min	minute(s)
MOPS	3-N-morpholinopropanesulfonic acid
mRNA	messenger RNA
Nor	norflurazon
NAA	naphthaleneacetic acid
NCED	9-cis-epoxycarotenoid dioxygenase
nt	nucleotide
PA	phaseic acid
PCR	polymerase chain reaction
qRT-PCR	quantitative Real-Time PCR
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
rpm	revolutions per minute
RT-PCR	reverse transcription-PCR
s	second(s)
suc	sucrose
SDS	sodium dodecyl sulphate
TBE	tris-borate-EDTA
Tm	temperature of DNA disassociation (melt)
Tris	tris(hydroxymethyl)aminomethane
V	volt(s)
w/v	weight per volume
Wpf	weeks postflowering
ZEP	zeaxanthin epoxidase

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Chapter 1: Introduction

1.1 Preamble

In an evolutionary sense, the flesh of fruit is designed to aid seed dispersal. The seed itself contains both the embryo and the endosperm which acts to provide nourishment for the seedling. Part of the process of fruit development is the growth and maturation of the seed but ripeness is thought of as the stage when the fruit is suitable for eating and this occurs when the seed is developmentally mature. The processes associated with ripening include modification of cell wall structure, the importation of a storable energy source, frequently sugars, alterations in pigment biosynthesis, a visual indicator of ripeness, frequently a decline in acidity and poisonous or astringent compounds and an increase in flavour and aroma compounds. All of these changes impart desirable traits which make the fruit attractive to the dispersal agent. These changes also make the fruit attractive to humans and many crops have been specifically bred to increase fruit size and other traits such as sugar and colour levels.

This literature review focuses on the role which plant growth regulators, in particular the plant hormone abscisic acid (ABA), have in grape berry ripening.

1.2 Physiology of fruit ripening

The development of fruit tissues usually occurs after there has been successful pollination. The exception to this is the phenomenon of parthenocarpy which is the successful production of fruit without fertilisation and therefore, no seed formation (Kadioglu *et al*., 1999). The development of fruit tissue represents the final phase of floral development and involves both cell division and cell expansion (O'Neill, 1997). During anthesis there is little cell division, but when the fruit becomes an active importer of photosynthate, many of its tissues become meristematic and growth commences. At this point, the fruit is defined as being "set". If expansion does not occur e.g. due to lack of pollination, the undeveloped fruits generally abscise (Coombe, 1973).

The fleshy part of fruit can arise from a range of organs and tissue types, almost any part of the inflorescence can give rise to fruit flesh. These include the peduncle in the case of figs, the mesocarp in peaches and the pericarp in grapes. All these tissues are able to accumulate water and organic compounds and are bulky parenchymatous tissues (Coombe, 1976).

There are two broad classifications of fruit based on their ripening physiology. Climacteric fruit (e.g. tomato, banana, avocado, peach, plum and apple) exhibit an increase in respiration and a peak in ethylene production during ripening whereas non-climacteric fruit (e.g. strawberry, grape, and citrus) do not. The respiration rate of non-climacteric fruit declines slowly during ripening (Coombe and Hale, 1973). Non-climacteric fruit do not appear to require ethylene to initiate or coordinate the processes of fruit ripening (Giovannoni, 2001). This does not, however, mean that ethylene may not be involved in some way in berry ripening (Chervin *et al*., 2004).

1.3 Role of plant growth regulators

Plant growth regulators play an integral role in controlling the growth and development of plants. Some growth regulators fit the definition of a "classical" plant hormone i.e. an organic compound synthesised in one part of the plant and translocated to another part, where at low concentrations it elicits a physiological response (Salisbury and Ross, 1992). However, this definition is somewhat restrictive as ethylene has been shown to bring about a change in the same tissue and even in the same cell where it was produced (Chang and Stadler, 2001). Also, there are other compounds such as sucrose (Smeekens, 2000), and even inorganic compounds such as phosphates (Stitt, 1999) and nitrates (Sadka *et al*., 1994) that may fit the classical definition and can elicit physiological responses.

There are five "classical" plant hormone categories; these are auxins, cytokinins, gibberellins, abscisic acid and ethylene. Other more recently recognised plant growth regulator compounds include brassinosteroids, salicylates, the jasmonates and polyamines.

Plant hormones must meet three criteria to elicit a response. They need to be present in the correct location at the correct concentration, to be recognised and bound by a specific receptor molecule, and once bound to the receptor molecule, to trigger some metabolic change that results in amplification of the growth regulator signal.

In animals steroid hormones are perceived by nuclear receptors, however, in plants plasma membrane receptors are employed, for example the BRI1 protein from Arabidopsis (Russinova *et al*., 2004). The binding of the ligand to its receptor that is located on the plasma membrane, causes a conformational change that triggers an intracellular cascade, which in turn changes gene expression and alters metabolic activity (Arteca, 1996). Cytokinins also act through a pathway such as this (Yamada *et al.,* 2001).

Recent research has shown that the signalling pathways of many plant growth hormones interact in both synergistic and antagonistic ways. Due to this the "action" of any single hormone must be considered in context with the presence of other potential influences.

1.4 Grape berry ripening

1.4.1 Physiology of grape berry ripening

Vitis vinifera L. is the species predominantly used for wine production in most commercial vineyards in the world. Its fruit are classified as berries and consist of a thin skin surrounding a fleshy pericarp and one to four seeds (Fig. 1).

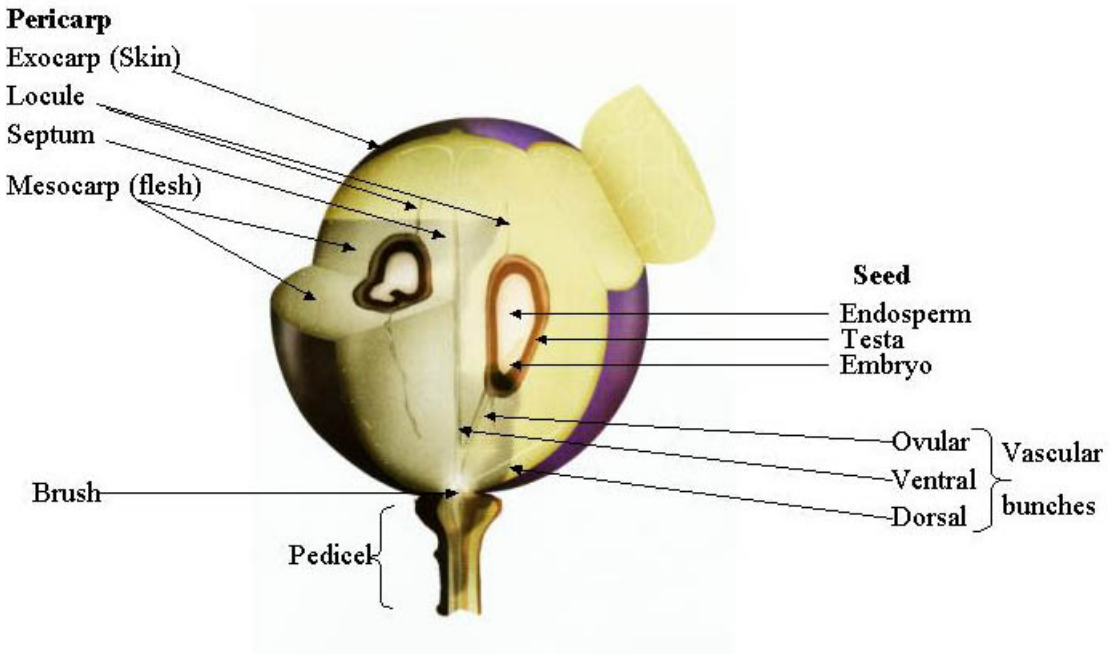


Figure 1.1: Grape berry structure. (From Coombe, 1987)

The pericarp can be divided into exocarp (skin), mesocarp and endocarp (both are flesh). The mesocarp is outside the peripheral vascular bundles, whereas the endocarp is inside. The epidermal and subepidermal layers of the exocarp contain anthocyanins, and the highest concentration of aroma and flavour constituents of the mature berry (Winkler, 1974). Water contributes the greatest proportion of weight to the flesh, but there are a large number of other compounds that also accumulate. These accumulate mainly in the cell vacuole which becomes vastly enlarged during ripening.

The development of grape berries can be described as having three distinct phases using the parameter of volume (or weight). These are (1), a period of rapid growth, (2), a period

of slow growth, (3), a second and final phase of size increase. The increase in berry size can therefore be described by a double sigmoidal curve (Fig.2).

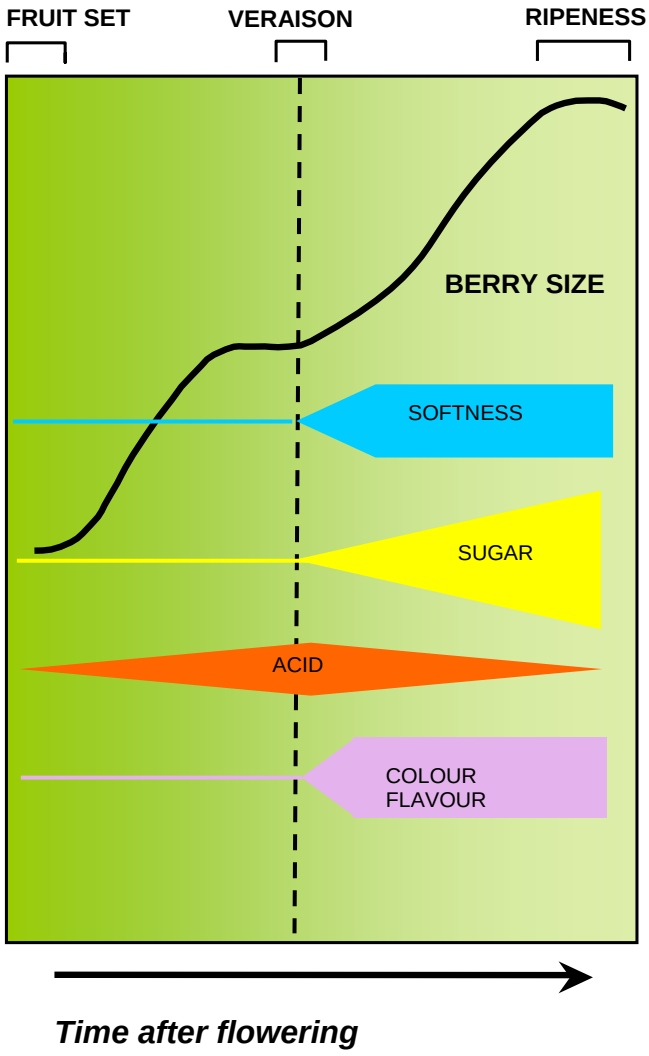


Figure 1.2: Grape berry development. (From Robinson and Davies, 2000)

The first stage begins at anthesis and lasts 45 to 60 days. This period is marked by two to three weeks of rapid cell division which is followed by cell enlargement. Most of the cell divisions take place in five-ten days after anthesis (Harris *et al.*, 1968). The main increase in the size of the berries is due to the large increase in the cell volume, of approximately 300-fold, whereas cell number increases only four-fold (Coombe, 1976). During the first stage the berries still have high levels of chlorophyll and this is the time when organic acids, malic and tartaric, accumulate.

The second stage is known as the "lag" phase. The extent of the lag period varies depending on parameters such as cultivar, time of flowering and the vines' environment

(Lavee and Nir, 1986). During the lag period the berries do not increase markedly in size but the embryos develop rapidly and reach their maximum size by the end of this stage. Also at the end of this stage, the berries begin to soften and to lose their chlorophyll. These are the first indicators of the transition into the third stage of development.

The beginning of the third stage is known as "veraison", a French word meaning "colour change". In the grape berry this is a distinct event and marks the beginning of sugar accumulation, softening, skin colouring, renewed cell expansion and a reduction in organic acid levels (Coombe, 1976). During ripening cell wall disassembly via wall modifying enzymes plays a major role in fruit softening (Barnavon *et al*., 2000; Nunan *et al*., 1998). The levels of sugars and total amino acids increase significantly (Giovannoni 2001). Most of the sugars are synthesised in the leaves and move through the phloem to the berries mainly as sucrose, which is the major carbohydrate used for long distance transport in grapes (Kanellis *et al.,* 1993). From the phloem, sucrose may be unloaded into the apoplast and cleaved by an apoplastic invertase. The present evidence in favour of intense phloem unloading through the apoplastic pathway is fully consistent with the induction of various sucrose and hexose transporters at the plasma membrane at the onset of ripening (Davies *et al.,* 1999; Sarry *et al*., 2004). Sucrose, and the hexoses glucose and fructose obtained from the hydrolysis of sucrose, can be taken up by the mesocarp (Ageorges *et al*., 2000) and during ripening glucose and fructose accumulate in roughly equal amounts in the vacuole (Ageorges *et al*., 2000). Many of the berry secondary metabolites may be made *in situ* from imported sugar or other precursors (Gholami *et al*., 1995).

Veraison is distinguished by a large change in gene expression (Davies and Robinson, 2000; Waters *et al.,* 2005). Therefore, it seems that a considerable part of ripening is due to a coordinated change in transcriptional control. Davies and Robinson (2000) carried out differential screening on Shiraz berry cDNA libraries with pre and post-veraison cDNA probes. Many ripening-associated cDNAs were identified using this approach and many of the genes that are up-regulated at veraison have been linked to stress responses by homology with genes from other species (Davies and Robinson, 2000). Recently transcriptional profiling by da Silva *et al*. (2005) was carried out on the expressed sequence tags from various *Vitis* species from public databases. Of particular focus was the group of genes predicted to be differentially expressed during grapevine flower and berry development. This analysis identified 87 differentially expressed transcripts and the

authors grouped these into eight functional categories based on their homology to *Arabidopsis* proteins or to previously published work in grapes. The eight categories were (1) pathogenesis-related proteins, (2) abiotic stress/cellular redox balance proteins, (3) primary metabolism, (4) secondary metabolism, (5) berry growth, expansion, and water relations, (6) ethylene metabolism, (7) allergenic peptides and seed-specific proteins, and (8) other proteins of interest. These results indicate that berry development is controlled by the carefully coordinated regulation of a suite of genes which is only just beginning to be understood.

The suite of changes that occur with the onset of veraison is coordinated within each berry, but not all berries on the same bunch ripen synchronously. This lack of synchrony suggests that individual berries have some autonomy over the rate and timing of their development and that a generalized, plant wide, ripening signal is not the trigger for individual berry ripening (Kanellis, 1993).

1.4.2 Role of plant hormones during grape berry ripening

The best studied plant growth regulators in grape fall into the five "classic hormones" categories and the more recently investigated brassinosteroids.

Auxin

The term auxin is derived from the Greek word auxein which means to grow. Auxins are characterized by their ability to induce cell elongation in stems, and this characteristic is used to define the hormone (Arteca, 1996). Auxins have been shown to influence many plant growth processes, the differentiation of phloem and xylem, stimulation of root initiation on stem cuttings and delay of leave senescence are just examples (Davies, 1995). Strawberries are considered to be nonclimacteric. During fruit development auxin produced by the achenes has been shown to stimulate growth and development (Perkins-Veazie, 1995). Later in fruit development, when the berry is hard and green (the stage is known as the middle green stage), the auxin levels decline in the berry and this invokes the ripening process (Given *et al.,* 1988). The application of exogenous auxin to strawberries which are at the middle green stage delays the onset of softening and colour development (Veluthambi and Poovaiah, 1984). Auxin levels were studied in Concord, a *Vitis labrusca*, Bailey, variety by Cawthon and Morris (1982). They showed that the concentration of indole-3-acetic acid in the ripening berry was highest at the beginning of berry development. From the beginning of berry development the level continuously decreased.

The HPLC method used in this work, however, is not as reliable as more modern methods. More recently gas chromatography-mass spectrometry-selected ion analysis using a labelled internal standard has been used to study IAA levels in Cabernet Sauvignon (Symons *et al.*, 2006). In contrast to the earlier study this work indicated that IAA levels are low and largely constant throughout berry development which may mean that a reappraisal of the pattern of endogenous IAA accumulation may be required. These differences may be due to the methods used or may reflect genotype differences. Whatever the pattern of IAA accumulation in grape berries the application of exogenous auxins has been shown to affect berry ripening depending on the time of application. Dipping clusters in 2,4-dichlorophenoxyacetic acid at full bloom has been suggested to enhance sugar accumulation and later applications delay ripening (Weaver, 1962). Application of another auxin-like compound, benzothiazole-2-oxyacetic acid, to Shiraz grapes just prior to veraison resulted in berry ripening being delayed by several weeks (Davies *et al*., 1997). It has also been demonstrated that the application of naphthaleneacetic acid (NAA) to bunches of Cabernet Sauvignon fruit at veraison suppresses the mRNA accumulation of *Vvmyb1* (a putative regulatory gene of anthocyanin biosynthesis) and all the genes of the anthocyanin biosynthesis pathway (Jeong *et al*., 2004). The NAA treated bunches also had a lower concentration of soluble solids and higher titratable acidity than that of the control berries (Jeong *et al*., 2004). Extensive studies in strawberry, another non-climacteric fruit, have shown that ripening occurs when auxin levels decrease to below a certain threshold (Given *et al*., 1988). These results suggest that auxin may be implicated in berry development, particularly in non-climacteric fruit, and may retard the timing of the onset of ripening. However, changes in IAA during grape berry ripening may be minimal (Symons *et al*., 2006) and the effect of exogenous auxins may not reflect their true role in 'normal' berry development.

Cytokinins

Cytokinins are compounds which promote cell division and have been shown to stimulate morphogenesis (shoot initiation/bud formation) in tissue culture, as well as stimulating the growth of lateral buds and leaf expansion (Davies, 1995). One study has suggested that cytokinin levels are low in developing grape berries throughout the first growth stage, but increase sharply at the onset of the lag phase (Inaba *et al*., 1976). At the initiation of the third stage (veraison) the concentration decreased. However, this data must be treated with caution due to the experimental system used to detect cytokinins. Cytokinin application at

full bloom has been reported to increase fruit size (Roubelakis and Kliewer, 1976). It seems that cytokinins may be important for fruit set and early cell division; however, there is no evidence of any effect on ripening and an accurate determination of cytokinin levels throughout flowering and berry development is required.

Gibberellins

Gibberellins (GAs) are widespread and ubiquitous in both angiosperms and gymnosperms as well as ferns. Their physiological effect is dependent in the type of gibberellin present as well as the species of plant. There are currently 136 gibberellins identified from plants, fungi and bacteria. Some physiological processes stimulated by gibberellins are the bolting/flowering response to long days, delay of senescence in leaves and citrus fruits, stem elongation and cessation of seed dormancy in some plants (Davies, 1995). In grape berries the content of gibberellin-like substances is directly related to the number of seeds (Scienza *et al*., 1978). In seeded berries there are high concentrations of gibberellin-like substances at the early fruit set stage, persisting for about two weeks post flowering then decreasing to very low levels (Scienza *et al*., 1978; Symons *et al.,* 2006). It is a common commercial practice to apply gibberellin to seedless cultivars to control both bunch size and berry size. It has been shown that application of GAs at full bloom causes thinning of bunches, while post bloom application greatly increases berry size (Weaver and McCune, 1959). This suggests that gibberellins may have a number of roles in grapevine development but the low and unchanging levels around veraison shown by a recent study (Symons *et al*., 2006) suggest that they are unlikely to influence berry ripening.

Ethylene

Ethylene is a gaseous hormone and like abscisic acid is the only member of its class. It is produced in all higher plants and is usually associated with fruit ripening, however, it has also been shown to affect dormancy, leaf and fruit abscission and flower opening (Davies, 1995). Early studies showed that, as might be expected of a non-climacteric fruit, grapes do not exhibit a large increase in ethylene production during ripening (Coombe and Hale, 1973). 2-chloroethylphosphonic acid (or ethephon, registered name Ethrel, a compound which releases ethylene) was shown to advance veraison a little or to retard veraison depending on the timing of application (Hale *et al*., 1970). Ethylene could also advance ripening a little if applied in a suitable timeframe.

Recently a moderate (2-3 fold), transient increase of ethylene production occurring over a three week period just prior to veraison was detected in Cabernet Sauvignon berries (Chervin *et al*., 2004). This increase although small may well be significant and influence the ripening of the developing grape berry. There was also a corresponding increase in expression of the 1-aminocyclopropane-1-carboxylic acid oxidase gene (*ACO*), which encodes the enzyme responsible for the final step in the ethylene biosynthesis pathway. Application of an ethylene receptor inhibitor, 1-methylcyclopropene (1-MCP), prior to veraison, at the time predicted for the ethylene peak, delayed the increase in berry diameter and anthocyanin accumulation. This seems to implicate ethylene in cell enlargement and colour development (sugar levels were not reported in this study, Chervin *et al*., 2004).

Brassinosteroids

Brassinosteroids are the only example of a steroid acting as a hormone in plants, which is a common occurrence in animals and fungi. Brassinosteroids are found in gymnosperms, monocotyledonous and dicotyledonous plants, and in algae, and the highest concentrations are found in the reproductive organs and growing tissues such as pollen, immature seeds and shoots (Khripach *et al*., 2000). Brassinosteroids are involved in the process of cell enlargement through their effects in gene expression and on enzyme activity (Khripach *et al*., 2000).

Recent data published by Symons *et al*. (2006) showed the first published data for brassinosteroid levels in a Cabernet Sauvignon berry developmental series. The levels of the bioactive brassinosteroid castasterone were elevated in flowers and during early berry development (two weeks postflowering) but decreased after this reaching a minimum (ten fold less) by the six weeks postflowering sampling time point. The level of castasterone remained low until between eight and ten weeks postflowering when its levels increased by 13 fold, as did that of its direct precursor 6-deoxycastasterone by nine fold. This increase in the brassinosteroid and its precursor coincided with the onset of ripening in the Cabernet Sauvignon berries as indicated by the increase in berry weight and soluble solids. The application of exogenous epi-brassinolide to preveraison Cabernet Sauvignon berries was shown to significantly promote veraison by the increase in the percentage of berries that were coloured as well as sugar accumulation, where as the application of brassinazole, a brassinosteroid biosynthesis inhibitor, significantly delayed veraison. These results

suggest that endogenous brassinosteroid levels influence the onset of ripening in grape berries.

Abscisic Acid

Abscisic acid is the single representative of this class, unlike the auxins, gibberellins, cytokinins and brassinosteroids. It was called "abscisin II" originally because it was thought to play a major role in abscission of fruits. It has also been shown to be involved in the inhibition of shoot growth, induction of seeds to synthesize storage proteins and the induction and maintenance of dormancy (Davies, 1995). It has been proposed that ABA may be associated with or induce ripening in some non-climacteric fruits, including grape (Coombe, 1976). Kobashi *et al*., (2001) demonstrated that the total sugar content in peach fruit increased with an exogenous ABA application to the developing fruit, implicating ABA in the accumulation of sugar in the peach fruit flesh.

In grape berries a role for ABA in ripening seems likely due to the rapid increase in ABA levels which occurs at ripening initiation. Earlier work has suggested that the ABA concentration in grape berries declines during the first stage of growth, from a high level at anthesis to its lowest point seven to ten days prior to the onset of veraison (Scienza *et al*., 1978,;Cawthon and Morris, 1988; Davies *et al*., 1997). At around the time of veraison ABA level increases and this increase continues until the ripening events are well established after which levels decline. Coombe (1976) showed that this increase in ABA at veraison was an approximately six-fold increase in the skin and approximately eight fold increase in the flesh over the preversion berry ABA level. The timing of increase in ABA levels at veraison correlates closely with the increase in sugar concentration and anthocyanins. The decrease in concentration in the early stages of berry growth may be due to the rapid berry expansion during this phase resulting in dilution of the ABA pool rather than ABA catabolism. If the data are recalculated on a content per berry basis, it becomes apparent that the total amount of ABA does not decrease markedly during the lag phase (Fig. 4) i.e. net accumulation does not occur until veraison.

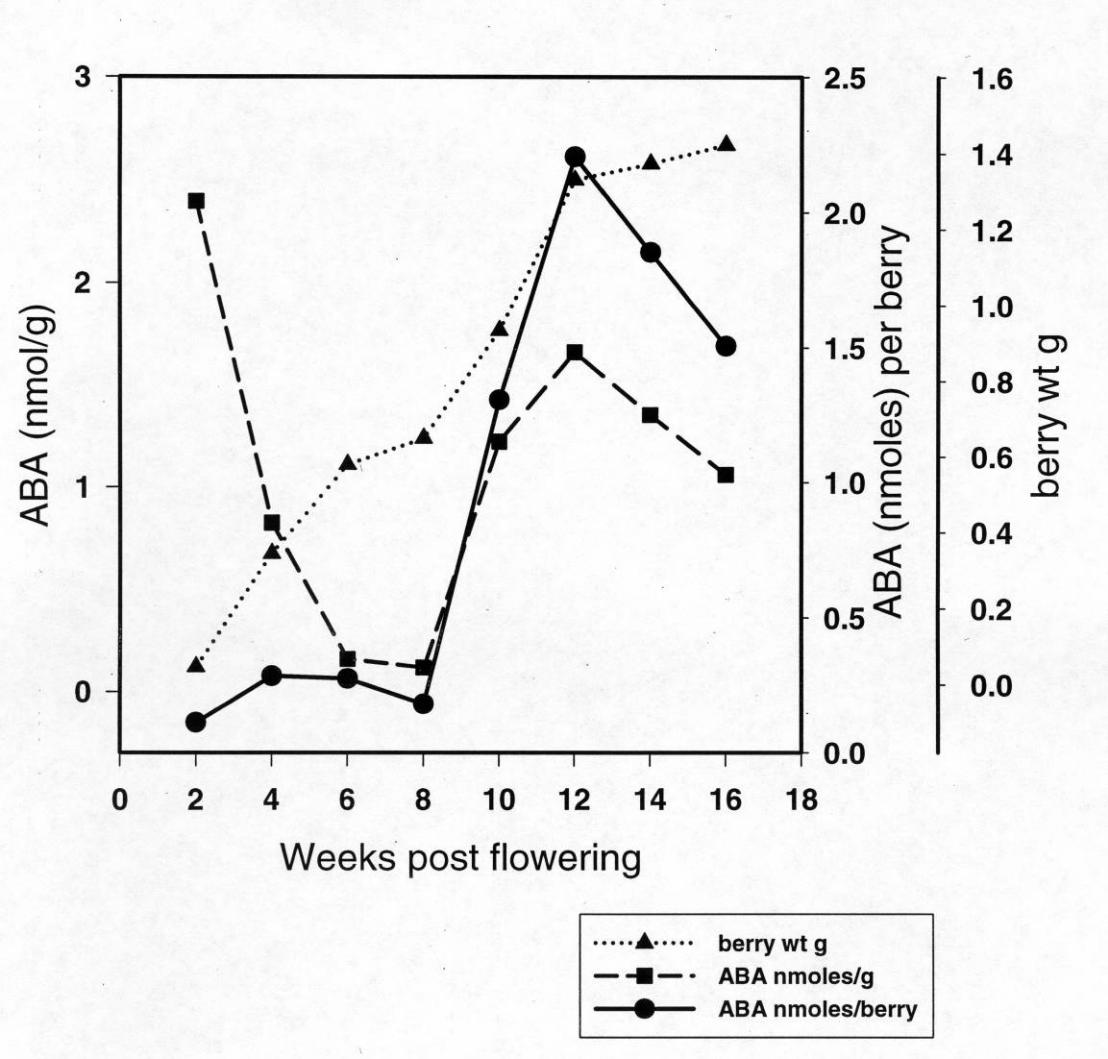


Figure 1.3: ABA concentration in ripening 'Cabernet Sauvignon' grape berries. (Modified from Davies *et al*., 1997)

Not only is ABA present in a free form but bound or conjugated forms are also present**.** Recently, data has been published detailing the relationship of free and bound ABA (ABA-glucose ester) in *Vitis vinifera* L. cv. Cabernet Sauvignon tissue (Koussa *et al*., 2004). This work showed that the concentration of free ABA was high at flowering then decreased through to berry set. The level of bound-ABA was higher than free ABA only in flower buds. However, this paper only measured the ABA and ABA-bound up until the berry set stage and so does not include veraison.

The application of exogenous ABA has been used to study the potential role of ABA in grape berry ripening. In one field trial exogenous application of ABA was shown to stimulate an increase in sugar levels (Kataoka *et al*., 1982). In another experiment the accumulation of anthocyanin was enhanced by ABA treatment at veraison and suppressed

by NAA and shading (Jeong *et al*., 2004). The ABA treated berries ripened earlier and also had a higher concentration of soluble solids and lower titratable acidity than the control berries (Jeong *et al*., 2004). Matsushima *et al.* (1989) also showed that the biosynthesis of anthocyanins was promoted by the application of ABA to postveraison 'Olympia' berries. It appears that the timing of ABA applications designed to hasten the initiation of ripening is crucial and has to occur in a window of opportunity just prior to veraison. The application of exogenous ABA to grapes hastened ripening if the ABA was applied at one week prior to veraison, that is, once the endogenous ABA had reached its lowest concentration (Coombe and Hale, 1973). If the ABA was applied earlier when endogenous concentrations were still high then the treatment proved ineffective (Hale and Coombe, 1974). Palejwala *et al*. (1985) demonstrated that supplementing grape berry slices with ABA can lead to an increase in sugar concentration and in the production of anthocyanins in berries selected just prior to veraison (Inaba *et al*., 1976).

The experiments described above were done using a range of cultivars and some of the apparently conflicting results may be due to these cultivar differences. Also more accurate measurement techniques with internal standards have been developed which may mean that some of the older data regarding ABA levels may be viewed as less reliable.

Possible interactions between different hormones that may be involved in the control of grape ripening have been suggested. As described in section 2.2.1 it has been shown that treating berries with the compound benzothiazole-2-oxyacetic acid, a synthetic auxin-like compound, not only delays ripening by approximately two weeks when applied prior to veraison, but also delays the corresponding increase in ABA levels (Davies *et al*., 1997).

The origin of the ABA that accumulates in the berry and the stimulus that initiates its accumulation are yet to be determined. Some researchers suggest that berry ABA is made in the leaves and is transported to the ripening berry where it accumulates (Antolin *et al*., 2003). The evidence for this is somewhat circumstantial and is based on the similarity of ABA accumulation in leaves and berries during berry development. ABA is high at flowering in leaves and flowers, then decreases only to rise again at veraison (Antolin *et al*., 2003).

The increase in ABA level at veraison and the possible influence on sugar and anthocyanin accumulation indicate that ABA may be a crucial mediator of the ripening process in

grapes and further research needs to be done to elucidate its role. As mentioned above there is now credible evidence for the three plant hormones to be involved in grape berry ripening. The relative roles of these three hormones and the interactions between them are yet to be determined. It has been difficult thus far to determine what factor, or factors, are responsible for the initiation and maintenance of ripening. The lack of suitable grapevine mutants, the time consuming and expensive nature of grapevine transformation and an incomplete array of hormone perception and biosynthesis inhibitors have made the analysis of ripening control difficult in grape berries. The new information regarding ethylene and brassinosteroids was not available at the commencement of this study. ABA was thought likely to have an important influence on ripening but further experimental work needed to be done to better define the role of ABA during ripening and its mechanism of action. For these reasons the focus of this study is to gain a better understanding of the likely role of ABA during berry development.

1.4.3 ABA biosynthesis and mechanism of action

Introduction

The chemical structure of ABA was determined in 1965 and it was identified as a sesquiterpene (Fig. 5) (Liontenberg *et al*., 1999).

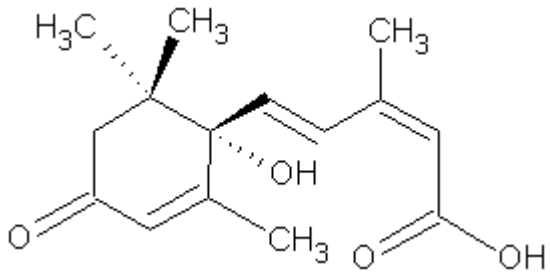


Figure 1.4: Chemical structure of S-(+)-abscisic acid

ABA is involved in the triggering of many of the physiological adaptive responses plants have developed to cope with adverse environmental conditions such as drought, high salinity and even herbicide toxicity (Grossmann *et al*., 1996). ABA is also involved in regulating seed maturation and germination.

Many ABA induced effects result from modification of gene expression through ABA-responsive gene promoters. There has been a large amount of research done on the biosynthesis pathway, mostly using mutants affected in ABA biosynthesis.

ABA responses

ABA is an essential mediator of a plants response to various environmental stresses. For example, during water stress of vegetative tissues in *P. sativum* the endogenous ABA levels increase several hours after wilting. The increase in ABA levels is due to the synthesis of new enzymes as the expected rise in ABA is blocked by the protein synthesis inhibitor cyclohexamide (Guerrero and Mullet, 1986).

Increased ABA levels result in closure of the stomata thus limiting the water loss of the plant through transpiration (Zeevaart and Creelman, 1988). ABA has been shown to cause rapid alterations in the anionic and K^+ ionic channels of the guard cell plasma membranes (Assmann, 1993).

ABA is also implicated in the response to a variety of other stress conditions including wounding (Pena-Cortes *et al*., 1995). ABA-deficient mutants have been shown to be affected in the regulation of numerous genes when stressed by drought, salt or cold, although there are ABA-independent pathways also involved in all these responses (Shinozaki and Yamaguchi-Shinozaki, 1996). Lower water activity may be the common link between all these responses.

Apart from ABA being involved in responses to external stimuli such as drought it is also involved in "normal" plant development. ABA has been shown to be involved in the maturation of seeds and the acquisition of desiccation tolerance which prevents precocious germination (McCarty, 1995).

As ABA is involved in a number of important plant responses its levels are tightly regulated. ABA in plant cells is continually synthesised and degraded. By using fluridone, an inhibitor of carotenoid synthesis, it was demonstrated that continued ABA synthesis is required for maintaining ABA concentrations, as well as preventing precocious germination of seeds (Yoshioka *et al*., 1998).

ABA anabolism

Through the identification of ABA-deficient mutants over the last 15 years the probable ABA biosynthesis pathway in plants has been determined (Fig. 5).

ABA-deficient mutants have been invaluable in elucidating the ABA biosynthesis pathway. ABA-deficient mutants have been easy to isolate as the resulting phenotypes are readily identifiable. The phenotypes include precocious seed germination, poor response to water stress (Koornneef *et al*., 1982) and inability to cope with specific environmental stresses such as low temperature (Heino *et al*., 1990).

As ABA levels were found to be reduced in plants where carotenoid biosynthesis was inhibited by fluridone (which inhibits phytoene desaturase) it was deduced that ABA was formed from a carotenoid precursor (Zeevaart and Creelman, 1988). ABA synthesis is a minor branch of the carotenoid pathway and thus they share the same early biosynthesis enzymes. The ultimate precursors of ABA in plants are pyruvate and glyceraldehyde-3-phosphate which are converted to isopentenyl pyrophosphate (IPP) C5 building blocks (Cutler and Krochko, 1999) in the plastids of cells (Lichtenthaler *et al*., 1997). The ABA biosynthesis pathway diverges from that of the carotenoids at the step of the hydroxylation of two β-carotene rings to yield zeaxanthin, mutants downstream of this enzyme have ABA, rather than carotenoid, deficiency phenotypes (Taylor, 1991).

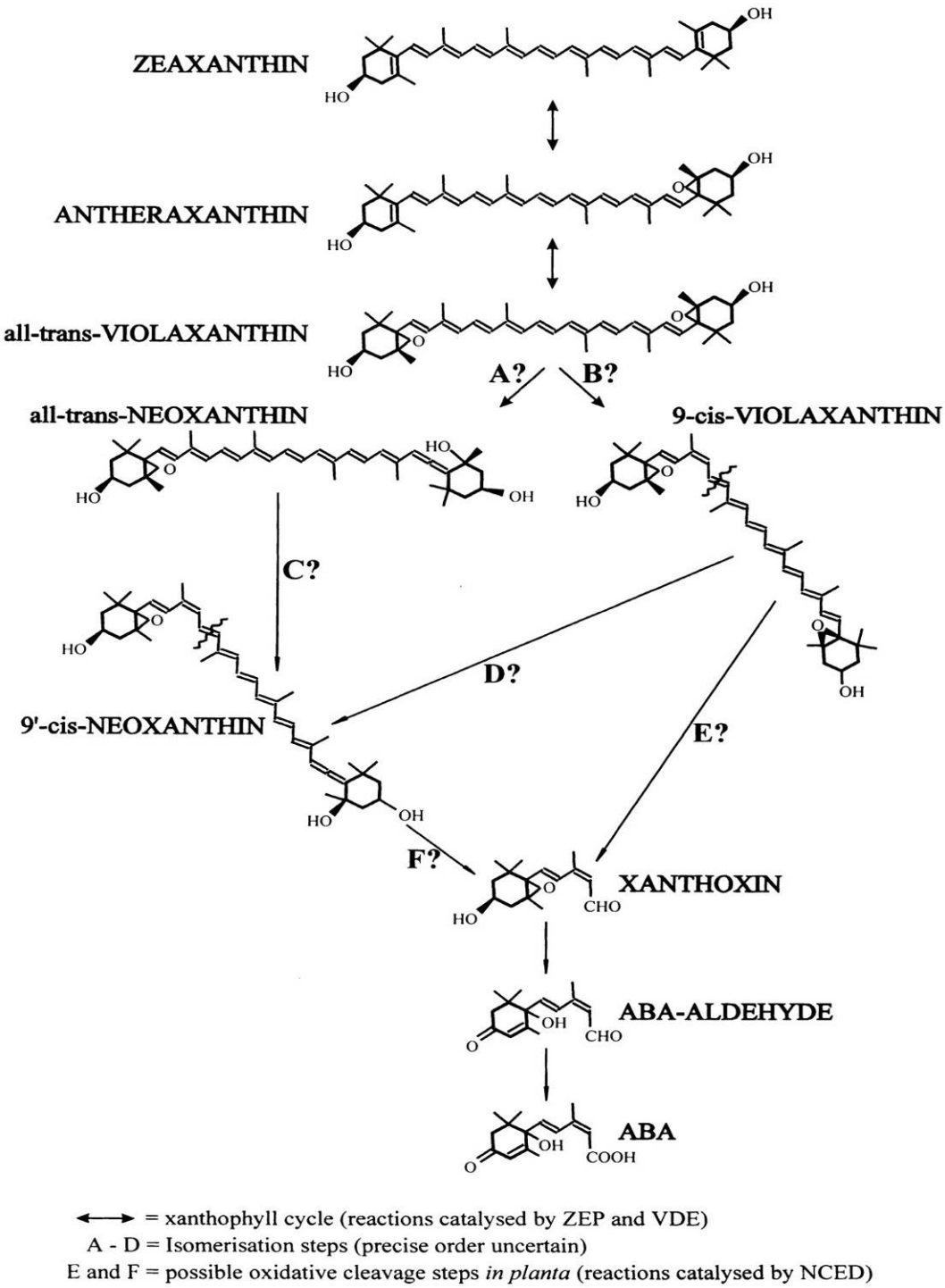


Figure 1.5: ABA biosynthesis pathway. (From Taylor *et al*., 2000)

ABA biosynthesis and mechanism of action

Zeaxanthin epoxidase (ZEP)

Zeaxanthin epoxidase catalyses the epoxidation of zeaxanthin to trans-violaxanthin via antheraxanthin as an intermediate (Liontenberg *et al*., 1999) (Fig. 6). The gene (*ABA1*) encoding this protein was cloned via non-targeted transposon mutagenesis in *Nicotiana plumbaginifolia* (Marin *et al*., 1996). The stable *N. plumbaginifolia* mutant plant exhibited a wilty phenotype due to increased transpiration and was shown to accumulate high levels of zeaxanthin and had low levels of the epoxyxanthophylls (Marin *et al*., 1996). *NpZEP* mRNA levels do not increase in response to dehydration treatments and as the metabolites downstream of ZEP are present in high levels in steady-state leaves it is thought that this is not a rate-limiting step (Audran *et al*., 1998).

ZEP appears to catalyse the rate-limiting step for ABA biosynthesis in non-photosynthetic tissues such as roots and developing seeds in *Pisum sativum* L. (Zdunek-Zastocka *et al*., 2004).

9-cis-epoxycarotenoid dioxygenase (NCED)

9-cis-epoxycarotenoid dioxygenase converts the final C40 precursor (9-*cis*-violaxanthin and/or 9'-*cis*-neoxanthin) to the first C15 intermediate xanthoxin, (Fig. 6). This is thought to be the rate-limiting step of ABA synthesis in photosynthetic tissues, as well as the first committed step of the ABA biosynthesis pathway (Qin and Zeevaart, 1999). In maize, non-targeted mutagenesis was used to generate mutations in this gene (Taylor *et al*., 2000). *NCED* mRNA levels increased within an hour of leaf detachment, which is so rapid a response it suggests that the reduction in turgor of the guard cells almost instantly triggers expression of this gene (Taylor *et al*., 2000).

In *Arabidopsis* there are five *NCED* genes that have been identified and studied for expression (Lekanne Deprez *et al.,* 2002). In well-watered plants *AtNCED2* and *AtNCED3* are the genes that account for NCED activity in roots, whereas it appears only one of these genes, *AtNCED3*, is the major stress-induced NCED in leaves. These expression results indicate that developmental control of ABA synthesis involves localized patterns of *AtNCED* gene expression.

The control of *NCED* expression seems to be carefully regulated as it has been shown that drought and osmotic stress cause the accumulation of the *NCED* transcript (Leon and

Sheen, 2003). It has also been suggested that the epoxycarotenoid cleavage reaction catalysed by NCED might be the key regulatory step of drought-induced ABA biosynthesis (Iuchi *et al*., 2001).

Short-chain dehydrogenase/reductase (ABA2)

The conversion of xanthoxin to AB-aldehyde is a three step, ring modification process (Fig. 6). The gene catalysing this step is a short-chain dehydrogenase/reductase (SDR1) known as *ABA2* (Gonzalez-Guzman *et al*., 2002). *ABA2* is a member of a large family of SDR genes which are known to be NAD- or NADP-dependent oxidoreductases. Based on sequence analysis in *Arabidopsis ABA2* is likely to be a single copy gene and has a non-redundant function (Hsing *et al*., 2002). Through cellular and biochemical analyses it has been demonstrated that ABA2 catalyses the conversion of plasmid-derived xanthoxin to abscisic aldehyde in the cytosol (Hsing *et al*., 2002). Activation of the *ABA2* gene has been shown to be induced by glucose, but not by drought or osmotic stress (Leon and Sheen, 2003). This is the opposite to the induction of NCED and indicates the complicated control system plants have developed for ABA biosynthesis.

ABA2 expression has a highly restricted localization to vascular tissues and the spatial and temporal expression patterns of *ABA2* seemed to be distinct from other ABA biosynthesis genes, such as *ZEP* (Hsing *et al*., 2002). These results would appear to suggest that inter-organ, inter-cellular and inter-organelle transport of ABA and/or its precursors is required to produce the strictly defined localization of ABA at the physiologically functional sites (Koiwai *et al.,* 2004).

AB aldehyde oxidase (AAO)

The final step in the synthesis of ABA is the oxidation of AB-aldehyde (Fig. 6). Aldehyde oxidase belongs to the family of molybdenum hydroxylases and catalyses the oxidative hydroxylation of a number of diverse aldehydes (Zdunek-Zastocka *et al.*, 2004).

There are many aldehyde oxidases present in plants, but recently there has been one identified in *Arabidopsis* that appears to be specific for ABA biosynthesis (Seo *et al*., 2000). This gene, *AAO3*, was demonstrated to produce ABA in transgenic yeast cells when supplied with its precursor abscisic aldehyde. The transgene was also shown to restore the wilty phenotype of the *aao3* mutant in *Arabidopsis* (Seo *et al*., 2000).

The *Arabidopsis aao3* mutant (mutated in the putative AB-aldehyde oxidase gene) has significantly reduced levels of ABA in its leaves when compared to wild type plants under well watered conditions (Seo *et al*., 2000). Recently it was demonstrated, using *AAO3* promoter:AAO3-GFP transgenic *Arabidopsis* plants, that the expression of GFP-fluorescence was detected in root tips, vascular bundles of roots, hypocotyls & inflorescence stems and along leaf veins (Koiwai *et al*., 2004). There was significant immunofluorescence in phloem companion cells and xylem parenchyma cells as well as signal observed in the leaf guard cells. These results indicate that the ABA synthesized in vascular systems may be transported to various target tissues and cells and also that guard cells themselves are able to synthesize ABA. *AAO3* mRNA is abundant in leaves and may be necessary for drought-inducible ABA accumulation as it has been shown that the *aao3* mutant exhibits a wilty phenotype and there is almost no increase in the endogenous ABA level after drought treatment (Koiwai *et al*., 2004).

ABA biosynthesis inhibitors

Inhibitors of ABA biosynthesis have been used in attempts to study the function of ABA in plants (Seo and Koshiba, 2002). Fluridone and norflurazon have been used widely. These both inhibit phytoene desaturase, which converts phytoene to phytofluene in the carotenoid biosynthesis pathway. As carotenoids are precursors for ABA, inhibitors that affect the carotenoid biosynthesis will also inhibit the biosynthesis of ABA. However, inhibiting carotenoid biosynthesis causes more wide ranging effects than just a reduction of ABA biosynthesis. Carotenoids protect plants against photo-oxidative damage and inhibiting their production can cause lethal damage to the plant (Zdunek-Zastocka *et al*., 2004). Recently an inhibitor specific for the NCED enzyme of the ABA biosynthesis pathway has been reported (Zdunek-Zastocka *et al*., 2004; Han *et al.*, 2004). The compound has been shown to inhibit stomatal closure in guard cells in response to drought stress. Future experiments utilizing this inhibitor will be useful to further identify the ABA specific effects on plant physiology.

ABA catabolism and efflux

The ABA concentration in tissues and cells can also be regulated by efflux or degradation. Efflux can be accomplished by passive or carrier-mediated efflux from the cells (Cutler and Krochko, 1999). Degradation occurs through the catabolic inactivation of ABA by hydroxylation. The predominant pathway is through the unstable intermediate 8'-

hydroxyABA, which then cyclises to form (-)-phaseic acid (Fig. 7). However, recently several other minor catabolic pathways were identified in *Brassica napus* siliques, where the hydroxylation of ABA occurs at the 9`and 7`-methyl groups (Zhou *et al*., 2004).

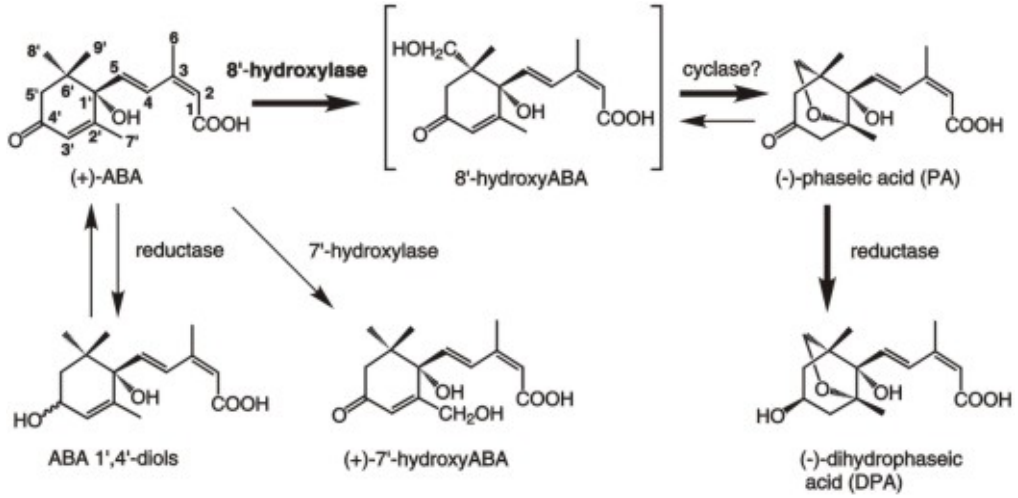


Figure 1.6: ABA Catabolism (From Cutler and Krochko, 1999)

ABA 8'-hydroxylase activity is present at high levels in plants recovering from abiotic stress, as well as in roots, seedlings, and developing and germinating seeds (Zeevaart and Creelman, 1988). The gene encoding the 8`-hydroxylase has been identified in *Arabidopsis* and is a member of the cytochrome P450 family of genes belonging to the *CYP707A* sub-family (Saito *et al*., 2004). It has been shown that the transcript accumulation of all four *CYP707A* family members was greatly increased by the application of exogenous ABA to Arabidopsis plants (Saito *et al*., 2004). The expression of the CYP707A3 gene is not ubiquitous throughout the plant, it is more highly expressed in roots, rosette leaves and flowers than in mature siliques and inflorescence stems. However, all four CYP707A family members show increased transcript accumulation when Arabidopsis plants are treated with exogenous ABA, salt (NaCl) or mannitol, treatments that induce ABA synthesis (Saito *et al*., 2004).

The enzymes responsible for further ABA catabolism downstream of (-)-ABA 8'hydroxylase and the genes which encode them are yet to be identified.

ABA glycosylation

Apart from hydroxylation the other main route through which active ABA can be metabolized to apparently inactive compounds is conjugation (Kondo and Kaiwai, 1998).

Conjugation is the simple process of converting ABA to ABA-glucosyl ester (-GE) or ABA-glucosyl ether (-GS). The physiological significance of ABA glycosylation in plants remains unclear. A gene catalysing the conversion of racemic ABA and UDP-D-glucose to ABA-GE has been identified in Adzuki bean seedlings (Cutler and Krochko, 1999). It is an abscisic acid-specific glucosyltransferase (ABA-GTase), and the mRNA level of this gene was shown to be increased by ABA application or by water stress or wounding. This suggests that ABA-GTase takes part in hormonal homeostasis to reduce the amount of free ABA. Other research suggests that free ABA is not released from ABA-GE during stress response, so the conjugation may not be reversible (Zeevaart and Creelman, 1988).

ABA perception and receptors

Studies with optically pure ABA analogues revealed that the stereochemical requirements of ABA are not identical in all ABA responses, which indicates that there may be several types of ABA receptors (Walker-Simmons *et al*., 1992). Recent reports by Razem *et al*., (2006) identify an *Arabidopsis* protein which is a nuclear RNA-binding protein that binds ABA. This protein however, appears to mediate only a subset of ABA's actions, and there are likely other ABA receptors to be found. It has been shown through the utilization of biotinylated ABA that ABA is perceived on the plasma membrane of stomatal guard cells in *Vicia faba* (Yamazaki *et al*., 2003). However, there is also evidence that ABA can also be perceived intracellularly by the application of ABA to the cytosol of guard cell protoplasts and the resulting inhibition of inward K^+ channels that prevents stomatal opening (Schwartz *et al.,* 1994).

The signal transduction pathway for ABA is largely unknown. However, it has been shown that ABA can alter the transcription of genes, such as the *Rab* genes (response to ABA) (Mundy and Chua, 1988). Promoters of some genes have been shown to contain ABA-responsive regions, and when these abscisic acid response complexes are added to a minimal promoter, they can confer a substantial ABA induction (Skriver *et al.,* 1991).

Some genes involved in ABA signal transduction have been discovered through mutant screening. The *ABI1* to *ABI5* loci were identified in *Arabidopsis* by screens that selected seeds capable of germinating in the presence of ABA at concentrations normally inhibitory to the germination of wild type seeds (Koornneef *et al*., 1984; Bonetta and McCourt, 1998). *ABI1* and *ABI2* genes code for serine/threonine protein phosphatase 2C enzymes, which are highly homologous and may indicate that they have overlapping functions

(Koornneef *et al*., 1998). *ABI3* encodes a protein that is most likely to be a transcriptional activator (Giraudat *et al*., 1992). *abi3* mutants have a lack of seed dormancy, reduced sensitivity to the ABA inhibition on seed germination and a disturbed seed maturation due to the reduction of seed-maturation specific transcripts (Parcy *et al*., 1994).

There are also mutants, (*era1-era3*), that exhibit an enhanced response to ABA and these fail to germinate on ABA levels that permit the germination of wild type seeds (Cutler and Krochko, 1999 Koornneef *et al*., 1998). *ERA1* codes for a β subunit of a farnesyl transferase and the mutant *era1* lacks peptide farnesylation activity. ERA1 may act as a negative regulator modifying ABA signal transduction proteins (Koornneef *et al*., 1998).

ABA and sugar signalling

Apart from their roles in general metabolism and carbon transport and storage sugars play an integral role in plants by acting as signalling molecules that can modulate metabolism, development and other physiological important processes. It has been recently discovered that several sugar-insensitive mutants affect genes known to be involved in hormone biosynthesis, and ABA in particular (Arroyo *et al*., 2003). There are also sugar-insensitive mutants that reveal that components of ABA signalling also are involved in sugar responses, for example the sugar insensitive mutants *gin6*, *sun6*, *sis5* and *isi3* are allelic to *abi4*, an ABA-insensitive mutant. ABI4 is a transcription factor of the APETALA family (Arroyo *et al*., 2003). Also the application of glucose to Arabidopsis seedlings has been shown to induce *ABI4* expression, which is thought to affect the sensitivity of tissue to endogenous ABA (Gibson, 2004).

The application of exogenous glucose to very young *Arabidopsis* seedlings has been demonstrated to cause increased levels of ABA and even cause the induction of some of the ABA biosynthesis genes and retard the rate at which endogenous ABA declines (Leon and Sheen, 2003).

There has also been an *ASR* (abscisic, stress and ripening) gene identified in grapes that has been suggested to function in response to both sucrose and ABA as part of a transcriptional regulating complex (Gibson, 2004). All these interactions would suggest that sugar and ABA signalling and the responses induced are very closely interconnected and controlled.

1.5 Conclusion

ABA is a plant growth regulator that has very important effects on a plants response to environmental stresses such as osmotic stress and temperature extremes. It also has a critical role in seeds involving their acquisition of desiccation tolerance and in preventing precocious germination. Mostly through mutant analysis, many of the genes involved in the ABA anabolism and catabolism have been identified in a variety of plant species. In grapes, three plant hormones have now been implicated in the control of berry ripening i.e. ethylene, the brassinosteroid castasterone and ABA. However, the role of ABA in grape berry development, and ripening in particular, is not well understood. Among the questions that remain unanswered are-

- Is NCED the rate limiting step in the synthesis of ABA in ripening berries?
- Is ABA synthesised in the berry, or is the ABA that is measured after the onset of veraison imported into the berry from vegetative tissues (leaves, shoots, stem, roots)?
- What processes are influenced in the grape berry as a result of the increase in ABA post veraison?
- Which ripening-related genes are regulated by ABA?
- Is ABA the trigger at veraison for the onset of ripening?
- Does the increase in sugar concentrations in ripening grape berries cause an increase in ABA levels? i.e. Is ABA concentration increased due to stress or developmental cues?
- Are there interactions between ABA and the two other hormones implicated in the control of berry ripening?

The question we have with regard to the role of ABA in ripening grapes which has particular significance to the grape and wine industries is of course whether the levels of ABA are related to berry quality, i.e. does ABA influence the accumulation of such compounds as anthocyanins, tritratable acids, flavour and aroma compounds and of course sugars. To answer these questions further work needs to be done elucidating ABA's role in grape berry. The work described in this thesis is intended to develop our insight into the synthesis and role of ABA during grape berry development

Chapter 2: ABA Accumulation During Grape Berry Development

2.1 Introduction

As described in the General Introduction (Chapter 1) grape berry development is characterized by three distinct phases of size increase (Fig. 1.2). Measurements of berry weight, soluble solids and colour have been used previously for describing and determining the progress of the ripening process and are used in this study to follow berry development..

There is some evidence that berry ABA levels increase at veraison and that this increase may relate to the initiation of ripening, or at least influence the progress of berry ripening (Hale and Coombe, 1974; Inaba *et al*., 1976; Davies *et al*., 1997). Much of the published data reporting ABA levels in berries was determined using methods made obsolete by the development of high performance liquid chromatography (HPLC) and gas chromatography combined with mass spectrometry (GC/MS) technologies. It is timely therefore to re-evaluate the possible connection between ABA and ripening. A recent report by Koussa *et al*. (2004) presented data for ABA levels in flowers and berries at 13 days after fruit set but not beyond this. This paper also reported ABA-GE levels but again, only in very young berries, up to 10 days after full flowering.

As stated previously ABA is a signalling molecule present at low concentrations in all higher plants. For ABA measurements, plant tissues are commonly extracted using boiling water, followed by ethyl acetate partitioning from the aqueous phase and HPLC separation (Soar *et al*., 2006). Modern, accurate and reliable methods for determining ABA utilize GC/MS techniques to measure the ABA methyl ester. ABA is methylated prior to the gas chromatography step to allow ion detection. An internal standard, [$^2H_6$]ABA, is employed in the quantitation by GC/MS (Abrams *et al.*, 2003). The ethyl acetate partitioning step of the ABA purification process used in the past is both laborious and generates a large volume of solvent waste. In this chapter the development and use of an alternative purification method using cation exchange sorbent columns is described. This method is cheaper, quicker and produces less waste material than the ethyl acetate portioning method and is suitable for the simultaneous preparation of large numbers of samples.

This chapter also describes the measurement of ABA levels in developing Cabernet Sauvignon berries over three seasons and changes in ABA levels that are related to changes in berry phenotype and composition. ABA was extracted and purified using the modified method outlined above and measured using the most sensitive analytical procedures currently available. Furthermore, in the fruit from one year the levels of all forms of ABA released by alkaline treatment were also determined, to give an indication of the size of the "bound" and "free" pools.

2.2 Materials and methods

2.2.1 Plant tissue

The berries used in this study were sampled over three growing seasons from *Vitis vinifera* L. cv Cabernet Sauvignon vines grown at the Slate Creek vineyard in Willunga, South Australia. The vines were planted in 1988 and grown on modified Scott-Henry trellises. Sampling dates for the three seasons are presented in Table 2.1. Flowering was defined as the time when approximately 50% of the opercula had fallen from the flowers (50% cap-fall). Bunches were collected randomly from vines spread across two to four rows. Samples were taken at fortnightly intervals after flowering (except during the 2003-04 season, when samples were collected weekly) and immediately frozen in liquid nitrogen in the field. In 2000-2001 and 2002-2003, all berries except the samples taken two weeks post-flowering were deseeded prior to being frozen. Berries from the 2003-04 season were frozen whole and the separate skin, flesh and seed samples were obtained by peeling the frozen fruit and separating the seeds and flesh prior to extraction. All samples were stored at -80^{o}C awaiting further analysis.

Table 2.1: Sampling dates for Cabernet Sauvignon berries Slate Creek Vineyard

Tissue (Location, Developmental Stage as wpf)	Collection date
2000-2001 season	
Willunga, berries 2wpf	1/12/2000
Willunga, berries 4wpf	14/12/2000
Willunga, berries 6wpf	27/12/2000
Willunga, berries 8wpf	10/01/2001
Willunga, berries 10wpf	23/01/2001
Willunga, berries 12wpf	7/02/2001
Willunga, berries 14wpf	23/02/2001
Willunga, berries 16wpf	7/03/2001
Willunga, berries 18wpf	23/03/2001
2002-2003 season	
Willunga, berries 2wpf	10/12/2002
Willunga, berries 4wpf	23/12/2002
Willunga, berries 6wpf	7/01/2003
Willunga, berries 8wpf	21/01/2003
Willunga, berries 10wpf	4/02/2003
Willunga, berries 12wpf	17/02/2003
Willunga, berries 14wpf	6/03/2003
Willunga, berries 16wpf	17/03/2003
2003-2004 season	
Willunga, flowers open	20/11/2003
Willunga, berries 1wpf	27/11/2003
Willunga, berries 2wpf	4/11/2003
Willunga, berries 3wpf	11/12/2003
Willunga, berries 4wpf	18/12/2003
Willunga, berries 5wpf	24/12/2003
Willunga, berries 6wpf	31/12/2003
Willunga, berries 7wpf	8/01/2004
Willunga, berries 8wpf	15/01/2004
Willunga, berries 9wpf	22/01/2004
Willunga, berries 10wpf	29/01/2004
Willunga, berries 11wpf	5/02/2004
Willunga, berries 12wpf	12/02/2004
Willunga, berries 13wpf	19/02/2004
Willunga, berries 14wpf	26/02/2004
Willunga, berries 15wpf	4/03/2004
Willunga, berries 16wpf	11/03/2004
Willunga, berries 17wpf	18/03/2004
Willunga, berries 18wpf	25/03/2004

To define the stage of berry development, 50 randomly selected berries were measured for individual berry weights and an average berry weight calculated. These berries were squeezed collectively and measured for soluble solids (^{o}Brix) with a temperature-compensated refractometer (model 10430: Reichert, Vienna, Austria). Sugar accumulation (total soluble solids, measured in ^{o}Brix) in the berries was used to determine the time of veraison. Having determined the base levels of total soluble solids during the earlier stages of development, veraison was defined as the date of harvesting of the last sample prior to a significant increase in the ^{o}Brix.

2.2.2 Free abscisic acid analysis

Quantitative determination of ABA levels was carried out by stable isotope dilution analysis (Loveys and van Dijk, 1988). The established protocol involved an aqueous extraction of the ABA from the tissue, followed by ethyl acetate extraction prior to HPLC separation, methylation and then quantification using GC-MS. This method was time consuming and limited the number of samples that could be purified at any one time. An alternative to the ethyl acetate extraction was to use column purification, and so this approach was evaluated to determine whether it was suitable for ABA extractions from grape berry tissue. This modified method was developed in collaboration with Ms Susan Maffei at CSIRO, Plant Industry, Adelaide.

ABA measurement by the ethyl acetate method

To prepare samples for HPLC purification the protocol from Soar *et al*. (2006) was used. Frozen berries were ground in liquid nitrogen using an electric coffee grinder. A 100-200 mg subsample of the homogenised tissue was used for ABA analysis. The frozen powder was extracted with 5 ml of water at 100^{o}C for 10 minutes. The sample was snap-cooled and the internal standard of 79.66 ng $^{2}H_{3}$-(±)ABA was added at a rate of 1 mg/g fresh weight. The extract was spun at 3,000g for 5 minutes. The aqueous phase was removed and the pellet was washed with 5 ml MilliQ water, pelleted again, and the two aqueous supernatants were pooled. This aqueous extract was adjusted to pH 2.5-2.7 with 1N HCl and extracted three times with an equal volume of ethyl acetate. The organic phase was dried overnight over sodium sulphate then reduced to dryness *in vacuo*. The samples, redissolved in 30% (v/v) MeOH and injected directly onto the HPLC (Agilent 1100 series) using an Activon Goldpak Exsil RP C18 ODS 5-µm 25 × 4.6 mm column (Activon,

Thornleigh, NSW, Australia). The sample was eluted using a linear gradient of methanol in aqueous 0.2% (v/v) acetic acid, running from 30–100% (v/v) MeOH over 11.8 min. The HPLC was equipped with a fraction collector that enabled collection of the purified ABA fraction. This fraction which had been previously determined by Soar *et al.*, (2006) was dried *in vacuo* and methylated with ethereal diazomethane, dried *in vacuo* and dissolved in 30µl of acetone. GC-MS was conducted using a Hewlett-Packard HP-GC series 6809. Ions at 162, 165, 190 and 193 were monitored. Quantification was by reference to a calibration curve. Data analysis was carried out using Microsoft Excel for average and standard deviation determination and then standard error values were calculated (standard error = standard deviation/√number of samples). The Sigmaplot program was used to graph these results.

ABA measurement by the Phenomenex strata-X column purification

The method used was the same as described above for the aqueous extraction, up to and including the pH adjustment to 2.5-2.7. Then instead of extracting with ethyl acetate the aqueous extract was passed through a Strata-X 33 µM Polymeric Sorbent Column (Phenomenex Sydney, Australia, Catalogue # 8B-S100-HCH). Columns were pre-treated with 5 ml 100% methanol and centrifuged at 2,000 rpm for 2 minutes. This was followed by a wash with 5 ml MilliQ water and the columns were again centrifuged at 2,000 rpm for 2 minutes. After the samples were added, the columns were spun at 2,000rpm for 2 minutes followed by a wash with 5% (v/v) methanol before the extract was eluted from the columns with 5 mL of 100% methanol. The eluate was reduced to dryness *in vacuo* and then HPLC separation and GC/MS detection were conducted as described in 2.2.2a

2.2.3 Bound abscisic acid analysis

The protocol used was the same as that given for the Phenomenex ion exchange column purification (2.2.2b), however, prior to the column purification the samples were hydrolysed (Loveys and van Dijk, 1988). Briefly, the aqueous extractions were adjusted to pH 11.0 with 1 N NaOH. The samples were then heated to 60^{o}C for one hour, and adjusted to pH 2.5-2.7 with 1 N HCl. The subsequent steps were the same as those given in 2.2.2b.

Anthocyanin extraction

To prepare samples for total anthocyanin determination, 0.1 g of powdered samples were added to 1 mL of methanol containing 1% (v/v) HCl and the anthocyanins extracted at room temperature in the dark on a rotating mixer for one hour. The tissue was pelleted by

centrifugation at 15000 rpm for 15 min and the supernatant retained. Total anthocyanins were measured spectrophotometrically by reading absorbance at 520 nm immediately following centrifugation.

2.3 Results

2.3.1 Abscisic acid purification method development

The purification methods tested (ethyl acetate, Phenomenex columns) both performed well and either would be suitable to use in future experiments (Fig. 2.1). When extracting ABA from grape leaves, the ethyl acetate method gave the higher ABA value at 160 ng/g, with the Phenomenex columns with 142 ng/g. However, when grape berry material was used, the highest ABA level obtained was with the Phenomenex column purification, just over 101 ng/g, whereas the ethyl acetate method yielded slightly less (just below 101 ng/g).

2.3.2 Grape berry development

Data from the measurement of various ripening parameters throughout the development of the Cabernet Sauvignon berries sampled are presented in Figures 2.2 and 2.3. Measurements for the 2000/2001, 2002/2003 seasons were collected each fortnight but the final season, 2003/2004 was sampled on a weekly basis and the data is therefore, more detailed.

Berry weight data was only collected for the 2002/2003 and 2003/2004 seasons. The berry weight curves for both years follow the expected double sigmoidal pattern of berry growth (Fig. 2.3 B,C). The final average berry weights do differ by almost 90 mg (760 mg for 2002/2003 and 852 mg for 2003/2004) and the lag phases appeared to be of different duration.

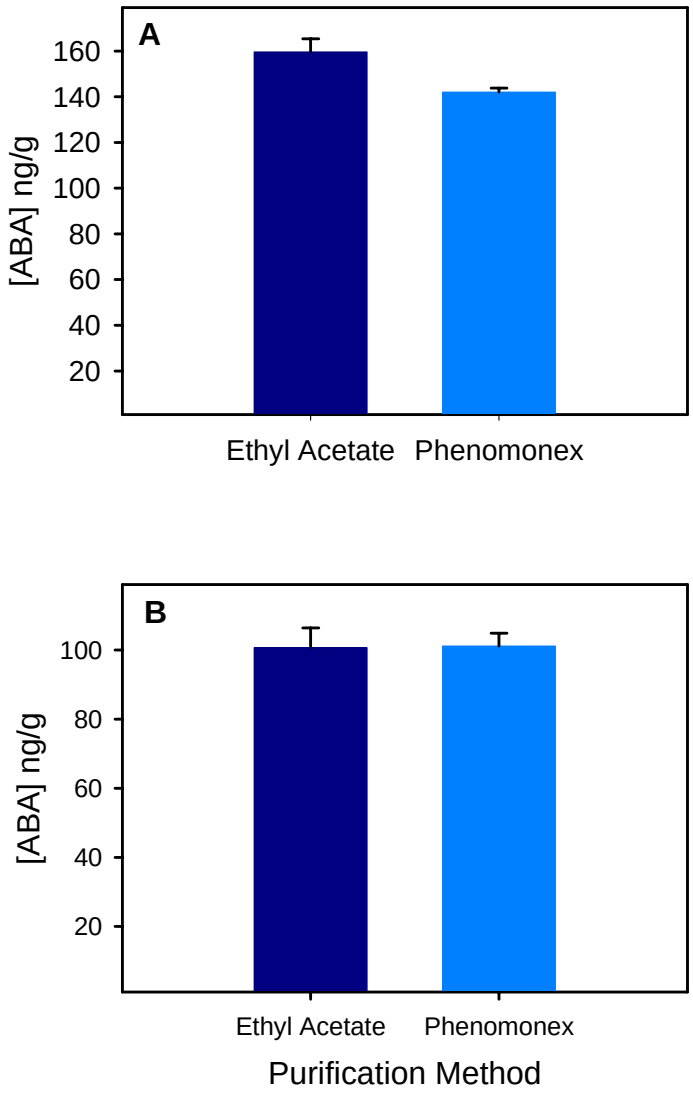


Figure 2.1: Comparison of ABA purification methods on a variety of starting material. A) Cabernet Sauvignon leaves; B) Cabernet Sauvignon berries. Bars represent standard errors.

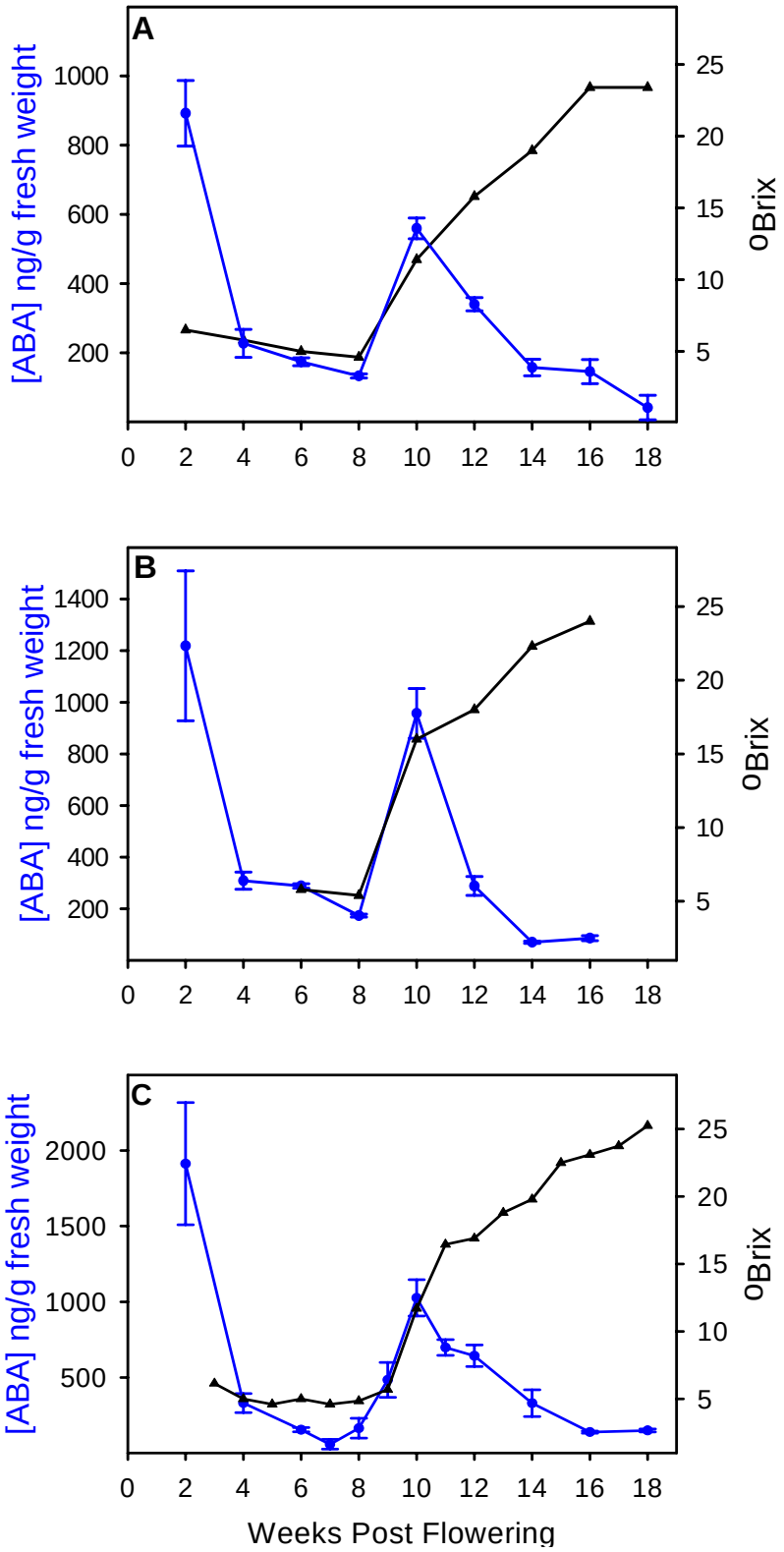


Figure 2.2: Changes in absorbance at A_{520} and average berry weight measured during the development of cv. Cabernet Sauvignon grape berries during three growing seasons. A) 2000-01, B) 2002-03 C) 2003-04. Bars represent standard errors.

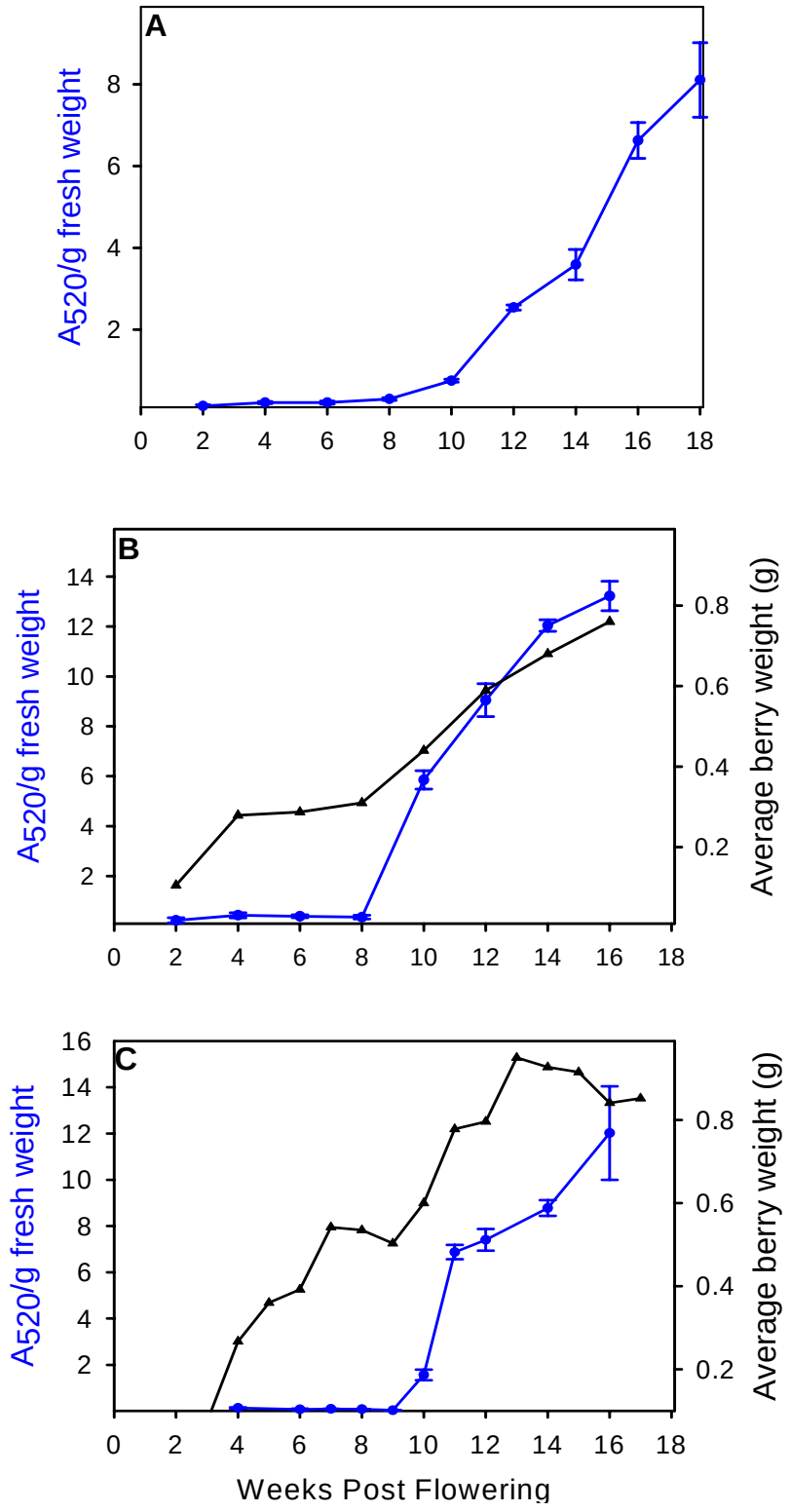


Figure 2.3: Changes in various ABA concentration and Brix measured during the development of cv. Cabernet Sauvignon grape berries during three growing seasons. A) 2000-01, B) 2002-03 C) 2003-04. Bars represent standard errors.

The lag phase as determined by berry weight increase appeared to be of longer duration in the 2002/2003 season than the 2003/2004 season (Fig. 2.3 B,C). The apparent commencement of the second phase of berry growth was estimated to be 8 wpf for the 2002/2003 season and 9 wpf for the 2003/2004 season. However, caution should be exercised when comparing these two sets of data as the different sampling rates may affect the estimation of phase duration and dates of parameter change.

Berry anthocyanin levels were estimated, by absorbance at 520nm, in the fruit from all three seasons (Fig. 2.2). For the 2002/2003 and 2003/2004 seasons the increase in A_{520} coincided with the increase in berry weight described above (i.e. 8 and 9 weeks post flowering respectively. The increase in A_{520} during the 2000/2001 season commenced at the same time as for the 2002/2003 season.

After veraison, total soluble solids as measured by refractive index (Brix units) are a reliable estimation of the increasing sugar levels in berries as most of the soluble solids at this stage are due to the presence of glucose and fructose (Ageorges *et al.*, 2000). The increase in ^{o}Brix in each season coincided with the increase in berry weight and colour which occurs as fruit move from the lag phase into the second stage of rapid berry expansion (Figs. 2.2, 2.3). The final ^{o}Brix reached at harvest (final sample in each series) was similar in all seasons, as were the overall rates of sugar accumulation.

In summary, the data collected for average berry weight, A_{520} and ^{o}Brix describe a similar pattern of development for each of the seasons, where the initiation of the second phase of berry growth coincides with an increase in total soluble solids and anthocyanin accumulation. There appear to be some minor differences in overall levels and rates of accumulation but as stated above, such comparisons are complicated by the different frequency of sampling between the years.

2.3.3 Free ABA accumulation during berry development

Free ABA concentrations (ng/g fresh weight) in deseeded berries were determined for each of the three seasons analysed (Fig. 2.2). Grape seeds mature at about the time of veraison and can accumulate significant amounts of ABA. Therefore, as one of the main goals of this work is to better understand the hormonal control of skin and pericarp ripening rather than changes in ABA that occur due to seed maturation it was decided to concentrate mostly on deseeded berries. The concentration of free ABA was relatively high in the two

weeks post flowering sample in each of the seasons. The weight of the developing fruit at this stage was quite low and therefore, the quantity of ABA present was small. By 4 weeks post flowering, free ABA concentrations had decreased dramatically and they remained low until around the time of veraison, when berry size increase recommenced and sugars and anthocyanins rapidly accumulated. This occurred at 8 weeks post flowering for the 2000/2001 and 2002/2003 seasons and coincided exactly with the increases in berry size, colour and ^{o}Brix.

The data for the 2003/2004 series describe a slightly different pattern, probably because the sampling frequency was twice that of the other two seasons tested. This allowed higher resolution of the timing of the various ripening events. In this series it seems as if the increase in ABA at around veraison pre-empts the increases in ^{o}Brix, A_{520} and berry weight (Figs. 2.2, 2.3). Berry weight, A_{520} and ^{o}Brix only appear to increase significantly after 9 weeks post flowering whereas ABA levels have increased significantly by this time.

The timing of the initiation of flowering between the seasons varied (Table 2.1); however, when the ABA data is plotted with respect to timing postflowering the profiles for the three seasons look remarkably similar (Fig. 2.4). ABA concentrations peaked at 10 weeks post flowering in all three seasons data. For the 2002/2003 season the higher levels were only maintained in one sample but in the 2003/2004 series especially the levels remained elevated for more than one time-point, which gives extra credence to the validity of this temporary rise in ABA levels. After peaking at 10 weeks post flowering ABA concentrations declined steadily until harvest even though the relative rates of decrease varied between years.

When ABA is plotted on a per-berry rather than a per-gram basis it can be seen that there is no net accumulation before the increase that occurs about the time of veraison Fig 2.5. This suggests that the decrease in ABA concentration early in berry development may be at least in part due to the rapid berry expansion that occurs at this stage resulting in the dilution of the existing ABA.

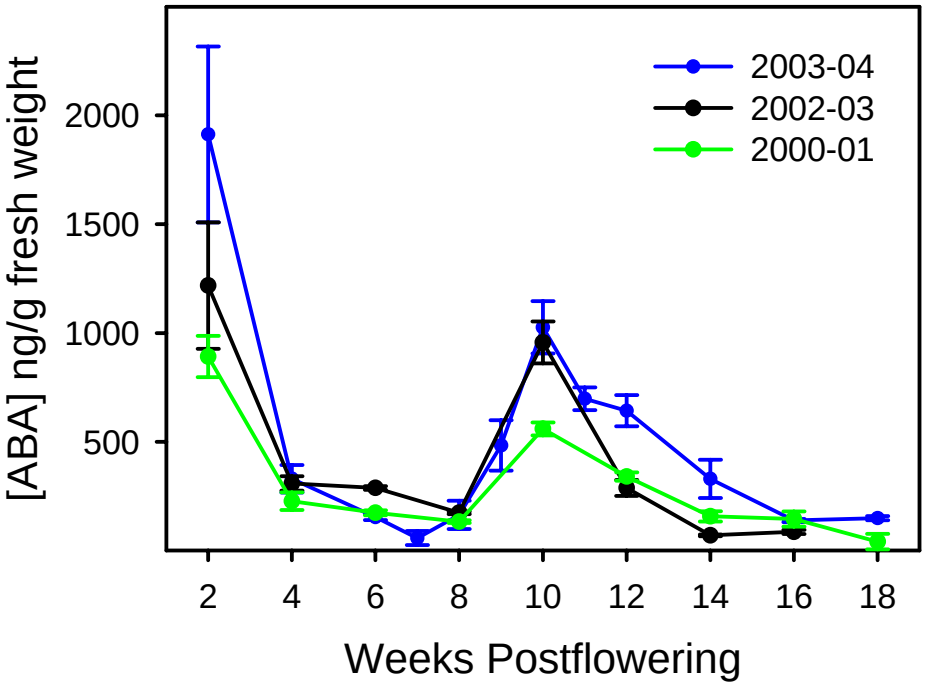


Figure 2.4: Comparison of ABA levels in cv. Cabernet Sauvignon berry developmental series over three seasons. Bars represent standard errors.

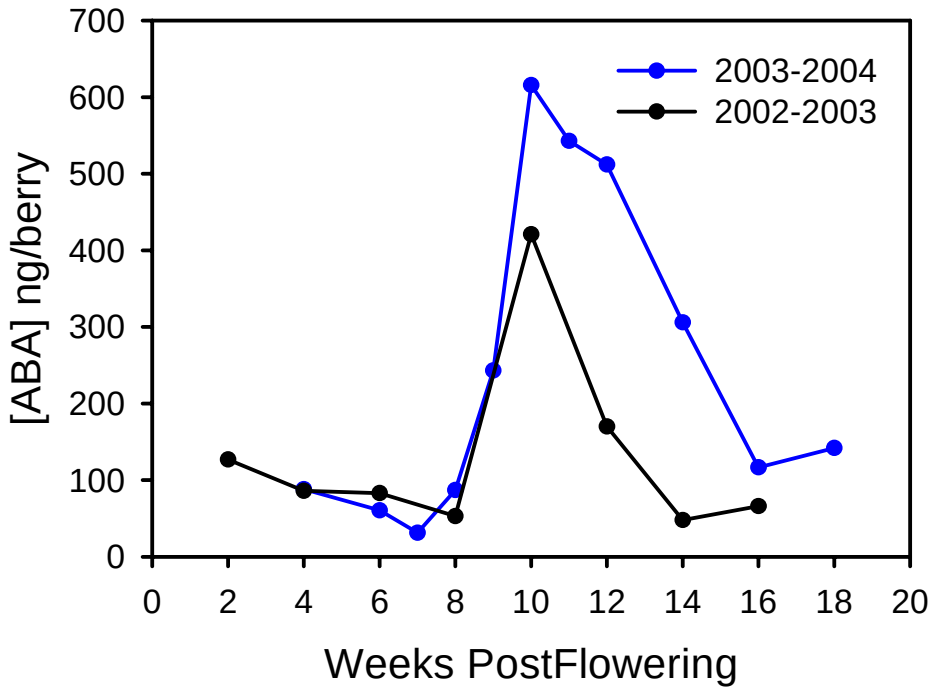


Figure 2.5: Comparison of ABA levels in cv. Cabernet Sauvignon berry developmental series over two seasons on a per berry basis.

2.3.4 Comparison of free and bound abscisic acid levels

The concentrations of bound and free ABA were determined for all three Cabernet Sauvignon berry series. The total ABA levels were calculated from the hydrolysed samples, and the level of bound ABA was calculated by subtracting the free ABA for each time point from the corresponding value obtained for the hydrolysed sample. The trend in all three years is strikingly similar (Fig. 2.6).

From two weeks post flowering until up to and including the peak in free ABA (10 weeks post flowering in all seasons) relatively small amounts of bound ABA are present. As the level of total ABA decreased during ripening, the proportion of total ABA in the bound form increased.

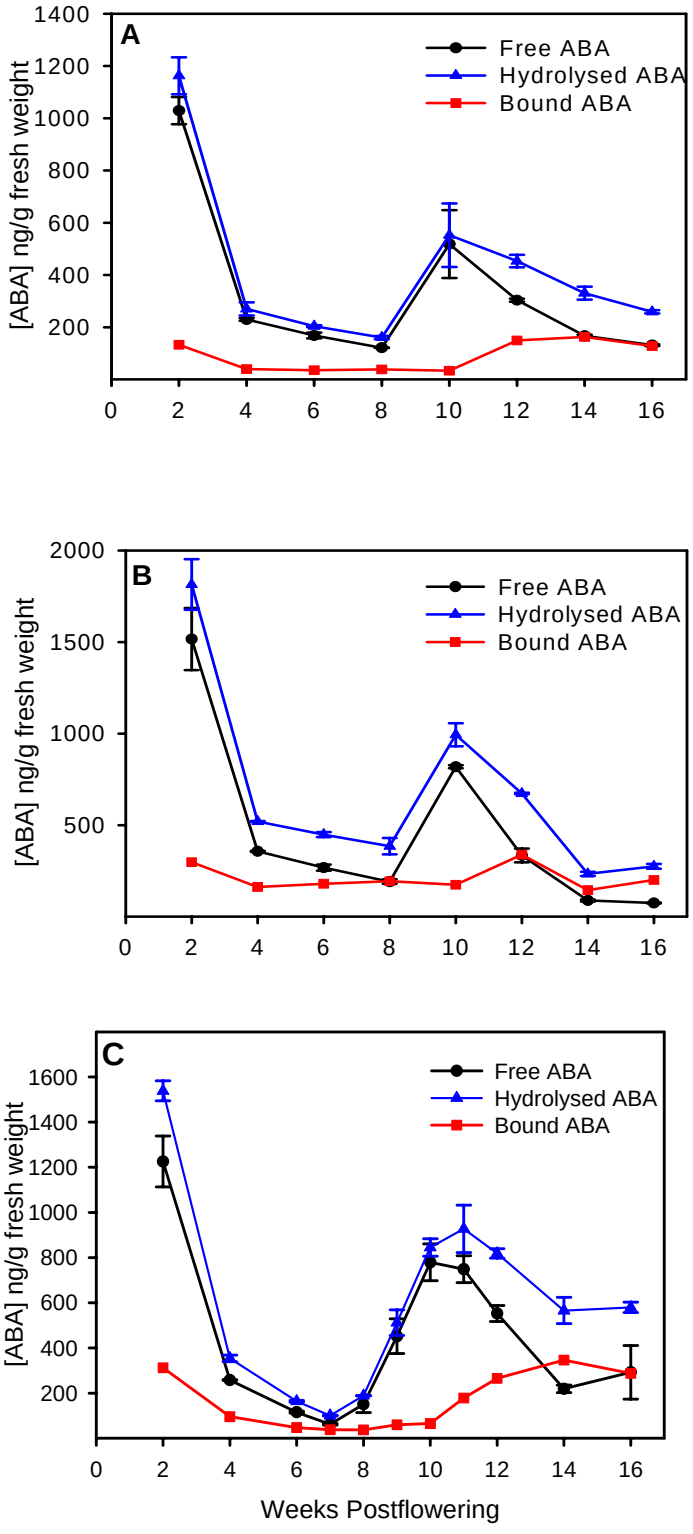


Figure 2.6: Comparison of free and bound ABA levels in cv. Cabernet Sauvignon berry developmental series during the development and ripening during three growing seasons. A) 2000-01, B) 2002-03 C) 2003-04. Bars represent standard errors.

2.3.5 Abscisic acid levels in grape berry skin, flesh and seed

The free ABA levels were determined for a selection of skin, flesh and seed samples that were harvested in the 2003-04 season (Fig. 2.7). The samples were from the four, eight, ten, twelve and sixteen weeks post flowering time points. In all the tissues selected for analysis, detectable levels of free ABA were found. In general the levels of free ABA in the seed tended to be higher than levels in the skin and flesh at the corresponding time. The only exceptions to this are at 10 weeks post flowering where the levels in all three tissues were roughly the same, and at 12 weeks post flowering when the skin and seed levels are nearly equal. As berries increase in size the skin and seeds make up less of the total berry weight, and at maturity they contribute a relatively small proportion of the berry overall weight. For this reason, the flesh contains an increasingly large proportion of the ABA pool as development progresses.

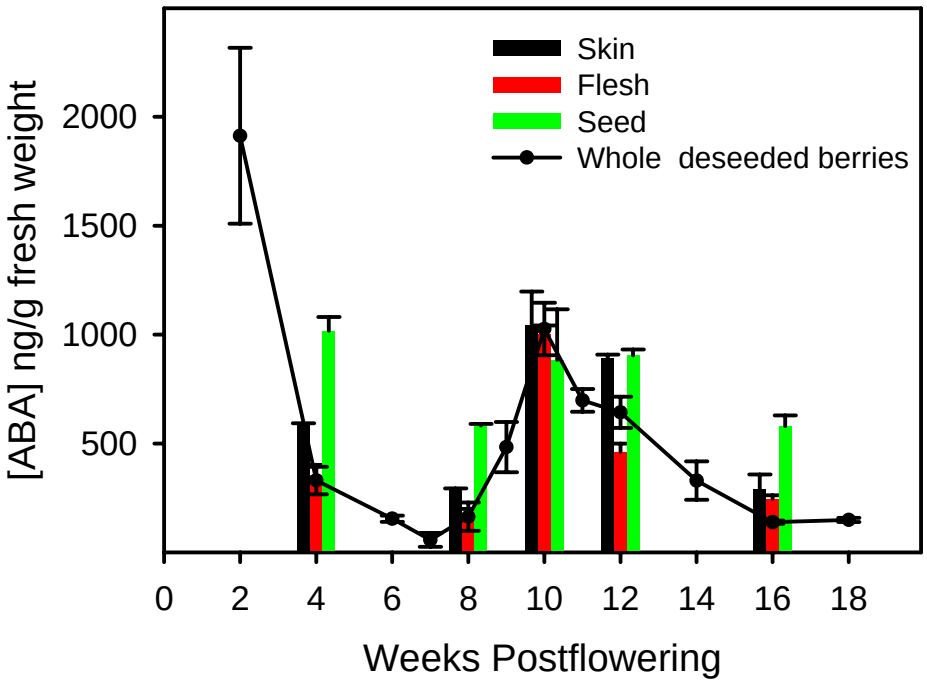


Figure 2.7: Comparison of ABA levels in cv. Cabernet Sauvignon berry skin, flesh and seed tissues for 2003-04 season. Bars represent standard errors.

2.4 Discussion

Although there were minor differences, the berries from the three seasons investigated followed very similar patterns of development. Flowering dates varied up to nine days between the years sampled (17/11/2000, 26/11/2002 and 20/11/2003). However, when the plots were aligned by flowering date, the curves describing berry weight, A_{520}, ^{o}Brix and free ABA concentration were similar (Figs. 2.2, 2.3, 2.4). Veraison occurred at eight to nine weeks post flowering in the three years investigated. The accumulation of soluble solids, especially sugars, is one of the parameters often measured to determine the progress of grape berry ripening. There is no net increase in sugar levels until veraison, after which levels increase rapidly. The total soluble solids values at 10 weeks post flowering for all three seasons were quite different (11.4 for 2000-01, 16.0 for 2002-03 and 11.7 for 2003-04). This may indicate a variation in the rate of sugar accumulation between seasons. However, due to the steep slope of sugar accumulation at this stage of development and the sampling regime these apparent differences should be treated with caution. Small differences in the actual date of veraison, as distinct from the recorded time of veraison, would significantly affect the slope of the curves. The overall rates of sugar accumulation between eight and 16 weeks post flowering were similar (data not shown).

One clear difference between the seasons is that the anthocyanin levels, as measured by $A_{520,}$ were lower for the equivalent stage as determined by ^{o}Brix value for the 200/2001 season than for the other two seasons (Figs. 2.2, 2.3). Anthocyanin accumulation can be affected by a number of influences including, bunch exposure, radiation levels, temperature (Downey *et al*, 2004). The 2000/2001 season was the hottest as measured by the number of days over 35^{o}C (Fig. 2.7A) and this may have had some bearing on anthocyanin levels being lower than in the other two years.

The duration of the lag stage, which varied between the two vintages which were sampled, is dependent on parameters such as cultivar, time of flowering and the vines' environment (Lavee and Nir, 1986). As the same vines were sampled for these two years, the variation is not attributable to cultivar effects but may be due to different weather conditions such as temperature (Fig. 2.7A).

These results suggest that variation in environmental factors or viticultural management influenced the timing of flowering in the three seasons investigated, and may have affected

other developmental parameters to a greater or lesser extent. However, the basic berry developmental plan was fairly stable once commenced. The significant difference in the extent of the lag phase between the 2002/2003 and 2003/2004 seasons contrasts with the quite similar patterns of development post veraison, and may indicate that development is more susceptible to external influences pre veraison rather than post veraison (Hale *et al*., 1970).

The development of a faster and easier ABA extraction system allowed an intensive examination of ABA levels in developing Cabernet Sauvignon berries to be undertaken. Due to the highly similar results obtained with the column purification method compared to the established solvent partitioning method, ABA purifications were done with the Phenomenex ion exchange columns. The columns were preferred as they could be reused up to fifteen times, decreasing the cost of preparing large numbers of ABA purifications. The method was also more rapid, allowing a larger number of samples to be processed. This enabled more replicates to be done for each sample, giving greater statistical confidence in the ABA determinations undertaken.

Although the pattern of ABA accumulation in deseeded berries was the same for all three seasons sampled, the absolute amounts of ABA and the slope of the decline from the 10 weeks post flowering peak differed between the years (Figure 2.2). The ABA concentration for 2000-01 was considerably lower at 10 weeks post flowering (560 ng/g fresh weight) compared to the levels at 10 weeks post flowering for 2002-03 (957 ng/g fresh weight) and 2003-04 (1026 ng/g fresh weight). Again, the frequency of sampling and the differences in frequency between years must be taken into account as discussed above in regard to other developmental parameters. Differences in sugar levels alone cannot explain the differences in ABA concentration between the years. For example, the ^{o}Brix at 10 weeks post flowering in 2000-01 was 11.4 and the ABA concentration was 560 ng/g fresh weight. At 10 weeks post flowering in 2003/2004, however, the level of ABA was roughly double at 1026 ng/g fresh weight even though the ^{o}Brix was similar at 11.7. The profile of ABA accumulation during the post veraison period was somewhat different in the 2003/2004 fruit compared to that in fruit of the other two years. The concentration of ABA in the 12 and 14 weeks post flowering samples for this year were higher than in the other two years and indicating a more gradual decline in ABA levels post veraison (Fig. 2.4).

It is possible that temperature and water stress may influence ABA levels in the berry; in other tissues ABA is frequently important in the stress response to high and low temperatures and drought (Grossmann *et al*., 1996). The vines used in this study were irrigated (soil moisture levels were not measured) and so the variations observed in rainfall during the three growing seasons (Fig. 2.7B) may not have resulted in vine stress but may have merely supplemented what was already an adequate water supply.

High temperatures can cause significant changes to metabolism and may affect berry ABA levels. The three seasons sampled varied considerably in the number of days over 35^{o}C (compare the figures for January over the three years for example). In hot weather grapevines limit water loss through transpiration by closing their stomata (Soar *et al*., 2006), an ABA mediated response. This also interrupts photosynthesis and can delay or reduce the export of photosynthate to the developing berries. As water supply to the berry after veraison appears to occur mainly through the phloem (Bondada *et al*., 2005), changes in phloem loading may also affect berry stress and ABA accumulation.

Bound ABA was determined by alkaline hydrolysis as described by Loveys and van Dijk (1988), under conditions that were reported to convert all bound ABA to free ABA. The physiological significance of ABA conjugations and especially of ABA glycosylation in plants remains unclear (Kondo and Kaiwai, 1998). Plant hormone homeostasis is maintained mostly by biosynthesis, movement, storage and catabolism, but also, to a lesser extent, by conjugation with sugars, amino acids and peptides (Lin *et al*., 2005). The glycosylation of plant hormones such as zeatin and IAA is believed to play an important role in their transportation (Leznicki and Bandurski, 1988). Some researchers have suggested that the bound forms of ABA are utilised as a long-distance transport form (Sauter *et al*., 2001), which can be converted to free ABA by apoplastic hydrolases before being imported into the cell. This statement, however, is disputed by others who have reported that ABA-GE is not directly transported in phloem (Zeevaart and Boyer, 1984). It is possible that the purpose of the conversion of free to bound ABA is to remove active, free ABA from circulation either through sequestration or inactivation. It has been suggested that once conjugated ABA is localised into a vacuole, it is trapped by hydrophilic properties and therefore withdrawn from further metabolism (Bray and Zeevaart, 1986). Some early work suggested that the hydrolysis of ABA conjugates to free ABA may occur *in vivo* (Johnson and Ferrell, 1982). However, more recent work by

Zeevaart (1999) has suggested that the conjugation of ABA to ABA-GE is irreversible. Further to this, experiments investigating the structure-activity relationship of ABA demonstrated that its activity was markedly decreased or even lost when its side chain was modified (Walton, 1983) suggesting that the glycosylated form of ABA is not biologically active.

Free ABA levels were determined in grape berry skin, flesh and seed samples from a selection of time points for the 2003-04 season. ABA was detectable in all tissues at all time points, which indicates that ABA is not strictly compartmentalised. Tissue specific ABA levels have not been widely determined in other fruits, although it has been reported by Cowan *et al*. (2001) that avocado fruit has similar levels of ABA in seed, seed coat and mesocarp tissue over the ripening period. Setha and Kondo (2005) reported that the levels of free ABA in sweet cherry flesh and seed had the same trend as observed here for grape tissues, that is, low early in ripening and then accumulating at the fruit softening stage, and then decreasing until harvest, which may suggest a common mechanism for the initiation of ripening. The levels of ABA in the flesh and seed samples of sweet cherry were similar to each other, which was observed also with the grape berry skin, flesh and seed samples. Setha and Kondo (2005), however, also measured the levels of the ABA catabolic products phaseic acid (PA), dihydrophaseic acid (DPA) and epi-dihydrophaseic acid (epi-DPA); the profiles of the breakdown products were noticeably different between the flesh and seed samples. In the seed the level of DPA was almost 30 fold higher compared to the flesh at 43 days after full bloom. The authors suggested that the amount of DPA may have an active role in the regulation of ABA concentration in the seeds. It is possible that the levels of the ABA catabolism products may vary between the grape berry tissues in a similar manner to those of the sweet cherry; further experiments would be necessary to determine this.

A number of other conclusions regarding the metabolism of ABA in grape berries can be drawn from the data presented. The plot of ABA on a per berry basis (Fig. 2.5) demonstrates that there is no net increase in ABA until veraison. This does not mean that no synthesis or importation occurs during this period as ABA may be synthesised/imported and degraded at the same rate to maintain the status quo. The increase that occurs at veraison cannot be entirely due to the release of free ABA from the pool of bound ABA. Figure 2.6 clearly shows that the levels of bound ABA are relatively low prior to veraison

and even if the bound ABA was fully converted to the free form the amounts released would not account for the increase in ABA levels observed at veraison. Also, as the berry increases in size rapidly after veraison the amount needed to increase the concentration increases with time. After veraison, when the concentration of ABA declines, the amount and proportion of ABA in the bound forms increases. This in part explains the decrease in free ABA but as the overall levels also decline there must also be some anabolism occurring. The conversion of free to bound ABA during this period may be an example of the berry decreasing ABA activity through conjugation. The reason for this conversion is unknown.

The factors affecting ABA accumulation are obviously complex and a better understanding of ABA synthesis and degradation/sequestration is required to better define and predict these interactions.

In general the data presented here agrees with previously published data for grape berries; however, comparisons are difficult for a variety of reasons. First, the techniques used to measure ABA in plant tissues have altered greatly over the years. Previous methods employed ethanol extraction followed by Thin Layer Chromatography (Coombe and Hale, 1973) and ethyl-acetate methanol extraction followed by gas-liquid chromatography (Downtown and Loveys, 1978). The method that was used in determining the ABA concentrations presented in this work was modified from published methods which employ HPLC and GC-MS techniques (Soar *et al*., 2006) and an internal standard to correct for any variability during extraction. These measurements should be the most accurate available.

Another reason that complicates direct comparison of these results to other published ABA grape data is that few researchers have published ABA concentrations using *Vitis vinifera* cv. Cabernet Sauvignon. *V. vinifera* cv. Doradillo has been extensively used in many of the early grape berry ABA experiments (Coombe and Hale, 1973), other varieties used have been *V. vinifera* cv. Shiraz (Hale and Coombe, 1974; Davies *et al*., 1997), *V. vinifera* cv. Delaware (Inaba *et al*., 1976) and Kyoho (*V. vinifera* L X *V. labruscana* Bailey). Therefore differences observed in ABA profiles may be due to varietal variation. The only available data from *V. vinifera* cv. Cabernet Sauvignon berry series shows a similar trend to that determined in this work (Scienza *et al*., 1978). One clear difference between the data presented here and that of Scienza *et al*. (1978) is that the initial high ABA

concentration recorded by them at two weeks post flowering (40 μg/100 g fresh weight) was only slightly higher than the peak observed at the 10 weeks post flowering (34 μg/100 g fresh weight). This ratio is very different to those measured in the 2001/02 and 2003/04 seasons described in the current work, where the ratio between the two weeks post flowering and the 10 weeks post flowering was almost 2:1. It is possible that the reasons for this may be due to differences in climate or viticultural practice between the two sites or in the definition of flowering time as the curve is very steep at this point and a small difference in timing would make a big difference to levels.

Clearly, experiments where the application of exogenous ABA affects sugar accumulation in other fruits such as peach (Kobashi et al., 2001) indicate that ABA may also have a role in grape berry sugar accumulation. The observations made in the experiments described here i.e. the concurrence of the initiation of ripening with a substantial increase in the concentration of endogenous ABA suggest that ABA has some role in 'normal' berry ripening. In this work data has been presented which indicates that the rise in ABA may pre-empt the increase in other ripening correlated processes suggesting a role in ripening initiation. It may also be true that applications of exogenous ABA after ripening has commenced may enhance processes such as anthocyanin accumulation (Jeong *et al*., 2004). Although ABA may be required for the initiation of ripening the high levels present just after veraison soon decline, suggesting that the continued presence of high levels of ABA is not required to maintain the ripening state. It seems that ABA may affect colour and sugar accumulation but it may also influence other ripening-related processes and control the expression of other ripening-related genes, perhaps in concert with the two other hormones that have been implicated in berry ripening, i.e. castasterone and ethylene. The relative roles of these hormones in ripening and the genes and processes that they influence are yet to be determined.

The trigger that signals the commencement of ABA accumulation is unknown. It may be no coincidence that sugars levels rise at about the same time as ABA levels do as sucrose and glucose can also act as signalling molecules. In other plants the increase in osmotically active solutes leads to reduced water activity and results in ABA accumulation (Fricke *et al*., 2006). The rapid increase in hexose levels after veraison may therefore influence ABA accumulation as part of an osmotic stress response. The seeming paradox i.e. that exogenously applied ABA can stimulate sugar accumulation while at the same time the

increase in sugar levels may stimulate ABA accumulation may be the result of the sharing of common elements in the respective signalling pathways. Further evidence for this interaction is presented in a later chapter.

Chapter 3: qReal-Time PCR Analysis of Grape Berry Developmental Series

3.1 Introduction

As described in Chapter 1 phytohormones have a role in affecting or controlling most stages of fruit growth, directing development from fertilization to senescence (Gillaspy *et al.,* 1993). Plant phytohormones are involved in the control of fruit growth through the enhancement of cell division and enlargement during development (Bohner and Bangerth, 1988). Hormones can also affect fruit development in other ways; for example, ethylene plays an important role in tomato fruit development (Lanahan *et al.,* 1994).

Endogenous hormone concentrations (as measured for ABA in the previous chapter) in any tissue are a balance between synthesis, degradation, transport, and conjugation. Therefore, determination of free plant hormone levels alone is often of limited value as it may reveal little about the site of hormone metabolism within the tissue under investigation and tells us little about hormone metabolism and how it relates to changes in net hormone levels. Potentially more meaningful data may be obtained by measurement of the activity of key enzymes involved in phytohormone metabolism, and relating any changes in activity to the measured phytohormone levels. The determination of changes in transcription of the various genes involved in hormone metabolic can also be a useful tool in understanding both hormone metabolism and role during development.

The rate limiting step of ABA biosynthesis in plants is thought to be the cleavage of 9-*cis*-epoxycarotenoids, the first committed step of ABA biosynthesis (Fig. 1.5).(Chernys and Zeevaart, 2000). This step is catalysed by the enzyme NCED and generates the C_{15} intermediate xanthoxin and C_{25}-apocarotenoids. As the carotenoid substrate is abundantly available in photosynthetic tissues (Qin and Zeevaart, 1999), it follows that the cleavage of 9-*cis*-epoxycarotenoids is the rate-limiting step in the ABA biosynthetic pathway. In non-photosynthetic tissues (arguably most of the grape berry is non-photosynthetic for most of its development) changes in *ZEP* expression may also influence ABA accumulation (Zdunek-Zastocka *et al*., 2004). Available evidence indicates that the conversion of xanthoxin to ABA is not rate limiting and that the later steps in the pathway are not up-regulated by water stress (Qin and Zeevaart, 1999). As stated in Chapter 1 the metabolism of ABA in grapevine is of interest because of the likely interaction between ABA and berry ripening.

The expression of the *NCED* genes during ripening has been studied in avocado. Three *NCED* (*Vp14)* homologs were cloned from ripening avocado fruit, and their expression during the ripening process was investigated. Two of these genes (*PaNCED1* and *PaNCED3*) were induced as the fruit ripened. A third gene, *PaNCED2*, exhibited constant expression during fruit ripening (Chernys and Zeevaart, 2000). The tissue-specific differences in expression of the *NCED* genes may have implications for their *in vivo* physiological role in regulating ABA biosynthesis (Chernys and Zeevaart, 2000). No other details of *NCED* gene expression during fruit ripening have been reported.

In this chapter data regarding the expression of the ABA biosynthesis genes *VvZEP* and *VvNCED* (two *NCED* homologues, *VvNCED1* and *VvNCED2* have been identified in grapevine by Dr Jim Speirs, CSIRO Plant Industry, Adelaide) in developing berries over a number of seasons are presented. This information should give insight into the possible location and transcriptional control of ABA synthesis. For example, expression of one or more of these genes in line with the observed ripening-associated increase in ABA might indicate that the ABA accumulation within the berry is due to *in situ* synthesis rather than importation. In contrast no detectable expression of the biosynthesis genes in the berry may indicate that the ABA is transported into the berries from the vine.

Various methodologies have been developed to study changes in gene transcript level. Expression analysis by northern blot hybridization is hindered by probe specificity when applied to closely related members of a gene family. In addition, it is also more difficult to gain quantitative information on relative levels of gene expression using this method. To circumvent these problems, quantitative Real-Time PCR (qRT-PCR) analysis was carried out on developing berries over three seasons in Cabernet Sauvignon. qRT-PCR has greatly improved the ease and sensitivity of quantitative gene expression and is rapidly being adopted as a standard method for gene expression studies.

In addition to studying the ABA biosynthesis gene expression profile in a whole berry developmental series, the fruit from one year was separated into skin, flesh and seed components to determine the tissue localisation of *VvZEP, VvNCED1* and *VvNCED2* transcripts.

3.2 Materials

3.2.1 Solutions

The compositions of the solutions used in this project are shown in Appendix table 1. All components used were analytical or molecular biology grade. Solutions were prepared with nanopure or deionised water and autoclaved where appropriate.

3.2.2 Oligodeoxyribonucleotides

The oligodeoxyribonucleotides used in this study were obtained from GeneWorks (Adelaide, Australia). Nucleotide sequences of these molecules are shown in Appendix Table 2.

3.2.3 Bacterial strains

Escherichia coli strain XL1-Blue (Stratagene; Cedar Creek, TX) was used for all routine cloning work.

3.2.4 Plant tissue

The various grape tissues used in study were obtained from the Slate Creek vineyard, Willunga harvested at various times, the details are listed in Table 2.1.

3.3 Methods

This section outlines general molecular biology methods used throughout this project, which are essentially as described by Sambrook and Russell (2001) or according to the manufacturer's instructions. Methods which have been significantly modified from their published form are outlined. Specific protocols are outlined in the relevant chapters.

3.3.1 Restriction enzyme digestion of DNA

DNA was digested with restriction endonucleases from Roche Diagnostics (Indianapolis, IN), Promega (Annandale, Australia), New England Biolabs (Beverly, MA), and Fermentas (Hanover, MD) using buffers supplied by the manufacturers.

3.3.2 Agarose gel electrophoresis

EasyCast horizontal minigel tanks (OWL Scientific Inc., Cambridge, UK) were used for electrophoresis of DNA. Agarose gels 0.7-2.0% (w/v) were prepared using Type I-A low EEO agarose (Edwards Instrument Company)in TBE buffer (Table 2.1), and contained 0.5 μg/ml (w/v) ethidium bromide. Samples were adjusted to 2× loading dye before applying

to the wells. Gels were electrophoresed at approximately 100 V in TBE running buffer before being visualised and photographed using a short wavelength UV transilluminator.

Electrophoresis of RNA was essentially the same as that described for DNA except gel tanks, trays, and combs were treated with 0.2 M NaOH for approximately 2 h prior to use and washed thoroughly in sterile deionised water. Samples were loaded with Bromophenol blue loading buffer (Table 2.1).

3.3.3 Purification of DNA from agarose gel slices

Purification of specific DNA species from agarose gels after visualisation with ethidium bromide was achieved using a QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions.

3.3.4 3.3.4 DNA amplification by the polymerase chain reaction (PCR)

For amplification of fragments to be cloned, Platinum Taq DNA Polymerase High Fidelity (Invitrogen; Carlsbad, CA) was used, according to the manufacturer's instructions. For all other purposes, PCR reaction mixes were as follows: DNA template, oligonucleotide primers (200-500 nM), 1× reaction buffer (Gibco BRL; Rockville, MD), 200 μM dATP, dCTP, dGTP, and dTTP (Promega), 1.5 μM $MgCl_2$, and 0.5 units of recombinant Taq DNA polymerase (Gibco), in a 20 μl or 50 μl reaction volume. Thermal cycling generally consisted of: 3 min at 95°C (one cycle); 45 sec at 94°C, 30 sec at 50°C, 1 min at 72°C (30 cycles); 7 min at 72°C (one cycle).

3.3.5 Purification of DNA samples following enzymatic reactions

Purification of DNA samples after restriction enzyme digestion (Section 2.2.1) and PCR (Section 2.2.4) reactions was achieved using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions.

3.3.6 DNA ligation

Ligation of PCR products into the T-tailed vectors pGEM T-Easy (Promega) was carried out according to the manufacturer's instructions.

3.3.7 Transformation of bacteria with recombinant plasmids

Electrocompetent *E. coli* XL1-Blue, *E. coli* DH5α, and *A. tumefaciens* C58 cells (Section 2.2.10) were transformed by electroporation using a Gene-Pulser apparatus (Bio-Rad, Hercules, CA). Approximately 1 ng of plasmid or 1 μl of a ligation reaction was mixed

with a 25 µl aliquot of cells and transferred to an ice-cold electroporation cuvette (path length = 1 mm) (Invitrogen). This was then given a single pulse in the Gene-Pulser (1.8 kV, 125 µFD, 200 Ohms), and immediately resuspended in 400 µl of LB (Table 2.1). After incubation at 37°C for 1 h to allow expression of antibiotic-resistance genes, the transformed cells were spread on 1.2% (w/v) LB agar plates (Table 2.1) with appropriate antibiotic selection and incubated at 37°C overnight.

Chemically-competent *E. coli* XL-1 blue cells (Section 2.2.11) were transformed by heat-shock. 1 µl of a ligation reaction was mixed with 100 µl aliquots of cells and incubated on ice for 30 min. Cells were then placed at 42°C for 45 sec, resuspended in 500 µl LB, and incubated at 37°C for 1 h. The transformed cells were spread on 1.2% LB agar plates with appropriate antibiotic selection and incubated at 37°C overnight.

3.3.8 Preparation of electro competent *E. coli* cells

500 ml of LB was inoculated with a 5 ml overnight culture of the *E. coli* strain of interest and grown at 37°C with vigorous shaking to an optical density (OD_{600}) of 0.5. Cells were chilled on ice for 10 min and centrifuged at 5000g for 15 min at 4°C. The cells were resuspended in 500 ml of sterile ice-cold water and centrifuged again. The cells were washed and centrifuged again with 250 ml sterile ice-cold water and resuspended in 10 ml of sterile ice-cold 10% glycerol. The cells were transferred to a new 50 ml falcon tube and centrifuged again. The cells were finally resuspended in 2 ml of ice-cold glycerol. Aliquots of 25 µl were placed into ice-cold Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at –80°C.

3.3.9 Preparation of chemical competent *E. coli* cells

100 ml of LB broth was inoculated with a 1 ml overnight culture of the *E. coli* strain of interest and grown at 37°C with vigorous shaking to an optical density (OD_{600}) of 0.5. Cells were chilled on ice for 20 min and centrifuged at 5000g for 15 min at 4°C. The cells were resuspended in 10 ml of ice-cold TSS solution (Table 2.1). Aliquots of 100 µl were placed into ice-cold Eppendorf tubes, snap-frozen in liquid nitrogen, and stored at –80°C.

3.3.10 Growth of bacteria in liquid cultures

Liquid cultures were set up by inoculating LB containing appropriate antibiotics with a single bacterial colony or a loopfull of frozen glycerol stock. Cultures were incubated at 37°C overnight with shaking.

3.3.11 Preparation of bacterial plasmid DNA

Preparation of plasmid DNA from 1-5 ml of overnight culture was done using a QIAprep Spin Miniprep Kit (Qiagen). Large-scale (20-50 ml cultures) preparation of plasmid DNA was achieved using a Plasmid Midi Kit (Qiagen).

3.3.12 Preparation of DNA samples for sequencing

DNA sequencing reactions were carried out using an ABI PRISM BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Norwalk, CT). Extension products were precipitated by adding 80 µl of 75% isopropanol, incubating for 20 min at room temperature, and centrifuging at 16,100g for 20 min. After discarding the supernatant and adding another 250 µl of 75% isopropanol, the tubes were centrifuged at 16,100g for 5 min. The samples were then aspirated, dried under vacuum for 15 min, and sent to the Institute of Medical and Veterinary Science (Adelaide, Australia) for analysis.

3.3.13 Preparation of bacterial glycerol stocks

Bacterial glycerol stocks were prepared by adding 1 volume of 40% or 80% sterile glycerol to overnight culture, snap-freezing in liquid nitrogen, and storing at –80°C.

3.3.14 Preparation of grape RNA

This protocol is based on the one given in Davies and Robinson (1996). Ground tissue samples had previously been prepared in Chapter 2. One to two grams of frozen ground tissue was mixed with 15 ml Extraction buffer (see Table 2.1) and 150 µl β-mercaptoethanol (BME)(1% (v/v) and then shaken vigorously on an orbital shaker for 30 min at RT. The sample was then passed through a 20 ml syringe plugged with approx. 2 cm^3 of glass wool wrapped in Miracloth (Calbiochem). Fifteen ml of Solution 1 (with 1% (v/v) BME) was added to the syringe, shaken to resuspend the remaining tissue and allowed to drip through. Cold 100% EtOH was added to 50 ml total volume and precipitated at -20°C for at least 2 hr. RNA was pelleted at 5000rpm for 30 min at 4^oC. The supernatant was decanted and the pellet washed with cold 70% (v/v) EtOH and allowed to dry. Each pellet was resuspended in 1 ml TE pH 7.6 containing 20 µl BME

(final concentration 0.2% (v/v) The resuspended pellet was extracted by adding 1 ml of phenol/chloroform/isoamyl alcohol (25:24:1) to the resuspended pellet, vortexing and centrifuging for 5 min. The aqueous phase was collected and transferred to a new Eppendorf tube. The phenol/chloroform extraction was repeated and then followed with a final extraction with 1 ml chloroform/isoamyl alcohol (24:1). The upper, aqueous phase was transferred to sterile 2 ml Eppendorf tube, 2–2.5 vol. cold 100% EtOH and 1/10th vol. Na Acetate were added and the RNA precipitated at -20°C for at least 2 hr. The samples were then centrifuged at 4°C for 10 min. The pellet was rinsed with 80% (v/v) ethanol, allowed to air dry and then resuspended in 100 µl sterile water.

3.3.15 RNeasy purification of grape RNA

Total RNA was quantified using a spectrophotometer (A_{260}) and 100 µg was further purified and DNase treated using RNeasy mini spin columns (Qiagen, #74104) and the RNase-Free DNase set (Qiagen, #79254). Yield was quantified as above and 1 µg run on an ethidium bromide stained agarose gel in 20% formaldehyde buffer to check for quality.

3.3.16 DNA synthesis

cDNA was synthesised in 20 µl reactions from 2 µg of total RNA using the SuperScript First-strand cDNA synthesis system (Invitrogen, #11904-018) and the 3-end, $(dT)_{17}$-adpater primer (Frohman *et al*., 1998) according to the manufacturers instructions. cDNA reactions were diluted 10-fold before use in subsequent experiments.

3.3.17 Degenerate oligo PCR

For PCR using degenerate oligonucleotides Biotaq Red DNA polymerase (Bioline, #BIO-21041) was used with the supplied buffer as described by the manufacturer. Briefly, 2 units of polymerase were used in a 25 µl reaction containing 1 µl diluted mixed berry cDNA, 0.7 µM of each primer (Refer to Appendix 1 for primer sets), 160 µM dNTPs and 2 mM $MgCl_2$ and 2.5 µl 10X PCR buffer. The reactions were run on a thermocycler for 94^{o}C, 2 min, 1 cycle; 94^{o}C 30 sec, 37^{o}C 30 sec, 72^{o}C 1 min 2 cycles; 94^{o}C 30 sec, 55^{o}C 30 sec, 72^{o}C 1 min 28 cycles;72^{o}C 10 min 1 cycle. Reaction products were run on agarose gels and DNA from the bands isolated using the Qiaquick Gel extraction kit (Qiagen, #28706). The purified fragments were ligated into pGEMT-easy (Qiagen) using the PCR Cloning Kit (Qiagen, #231122) followed by electroporation of the ligation products into electrocompetent *E. coli* XL-1 blue cells. Colonies were screened for inserts and overnight

cultures of positives set up according to 2.2.10 and plasmid DNA prepared according to 2.2.11. Insert DNA was sequenced according to 2.2.12.

3.3.18 Quantitative real-time PCR amplification

Expression analysis of *VvNCED1*, *VvNCED2*, *VvZEP* and *VvASR*, *VvACO* and *VvGRIP4* was done by qReal-Time PCR using a Rotor Gene 3000 system (Corbett Research, Australia) and SYBR Green PCR Master Mix (Applied Biosystems, #4309155). Total RNA was extracted, DNase treated and cDNA synthesised as described above. The primers used are given in Table 2.3.

Each reaction contained 1 x SYBR Green PCR Master Mix (Applied Biosystems, #4309155), 2 µl of diluted cDNA, 333 nM of each primer and water to 20 µl. Cycling conditions were: 95°C 10 min, 40 cycles: 95°C 30 sec, 58°C 30 sec, 72°C 30 sec, followed by a melt cycle of 1°C increments from 50°C to 96°C (45 secs first step, 5 secs for each subsequent step) . Each primer pair gave a single product of the expected size and sequence, verified by analysis of the melt curve, agarose gel electrophoresis and DNA sequencing. All reactions were performed in triplicate.

Standard DNA for the calibration curve of qReal-Time- PCR was prepared using each specific primer set as follows: first, amplified DNA, which formed a single band, was purified using a QIAquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions; second, to determine the relative expression values the purified standard DNA was diluted to appropriate concentrations to generate a standard curve for each product. A 10-fold dilution series of each purified PCR product was established, qReal-Time PCR was performed and arbitrary values assigned to each standard according to the dilution factor. The expression value of each gene in each cDNA tested, which was determined by reference to the standard curve, was normalised to the level of expression of ubiquitin in each cDNA.

3.3.19 Preparation of SYBR green reagent for quantitative real-time PCR amplification

Optimisation of expression analysis of the same genes that were stated in 2.2.17 was also done utilising SYBR Green reagent prepared based on the method given in Karsai *et al.* (2002). RNA preparation, DNase treatment and cDNA synthesis were as described above. The cDNA was diluted 25 fold prior to use. Primers used are given in Table 2.3. Each reaction contained 2 µl 1000-fold SYBR Green I (Biowittaker Molecular Applications),

1 µl cDNA, 0.1 µM each primer (20 pM final concentration), 2 µl 10X buffer (100 mM Tris-HCl pH 8.5, 500 mM KCl, 20 mM $MgCl_2$, 1.5% Triton X-100), 200 µM dNTPs, 2 units Taq polymerase and MilliQ water to 20 µl. The cycling conditions and analysis were as described above.

3.4 Results

3.4.1 Degenerate oligo PCR

Using the degenerate oligo PCR primers and cDNA prepared from RNA extracted from berries collected throughout the berry developmental series for 2002-2003, PCR products were obtained and sequenced. Sequences that were homologous to both the previously identified *VvNCED1* (Genebank Accession Number AY337613) and *VvNCED2* (Genebank Accession Number AY337614) genes were obtained, but no novel NCED sequences were identified.

3.4.2 qRT-PCR

The amount of cDNA synthesised for all the RNAs was determined to be uniform as the ubiquitin qReal-Time PCR for the tissues gave very similar threshold cycle detection levels (data not shown).

As the 2000-2001 and 2002-2003 seasons were both sampled fortnightly these results will be compared and discussed, then the 2003-2004 season which was sampled weekly will be analysed separately, as will the 2003-2004 developmental skin, flesh and seed samples.

The transcript abundance profiles of *VvNCED1* for the 2000-2001 and 2002-2003 seasons were similar (compare Fig. 3.1A with Fig. 3.3A). Generally, higher levels of *VvNCED1* mRNA were present in the preveraison samples than in the postveraison samples for both seasons. The level of *VvNCED1* transcripts decreased at around the same time in both seasons, roughly coincident with veraison. In the 2000-2001 season, the eight weeks postflowering sample had the highest level of *VvNCED1* mRNA measured, whereas in the 2002-2003 season the peak in abundance was detected in the six weeks postflowering tissue sample. After decreasing from the preveraison levels *VvNCED1* expression remained low until harvest.

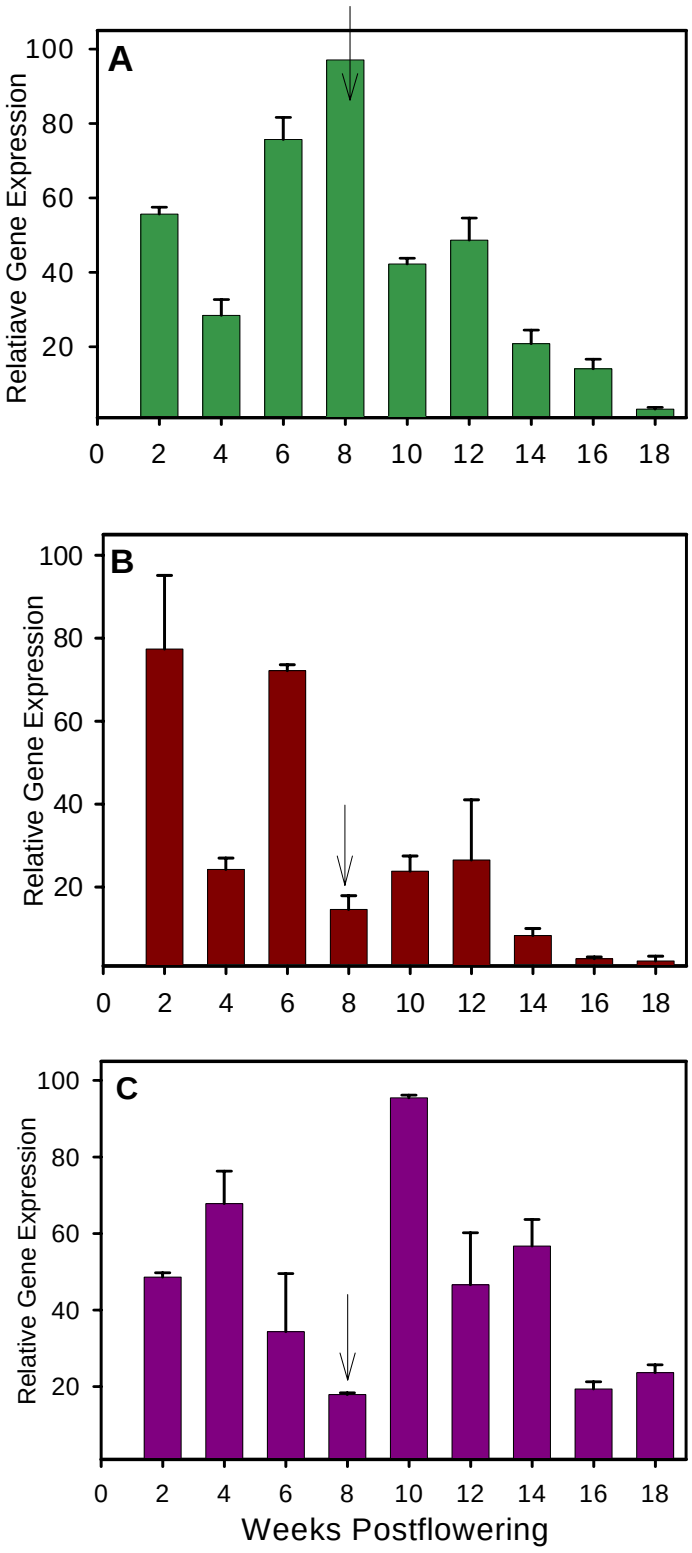


Figure 3.1: Relative gene expression of ABA biosynthesis genes measured during the development of cv. Cabernet Sauvignon grape berries during the 2000-01 season: A) *VvNCED1*; B) *VvNCED2*; C) *VvZEP*. The arrows denote veraison, which is the last sampling time point before an increase in soluble solids.

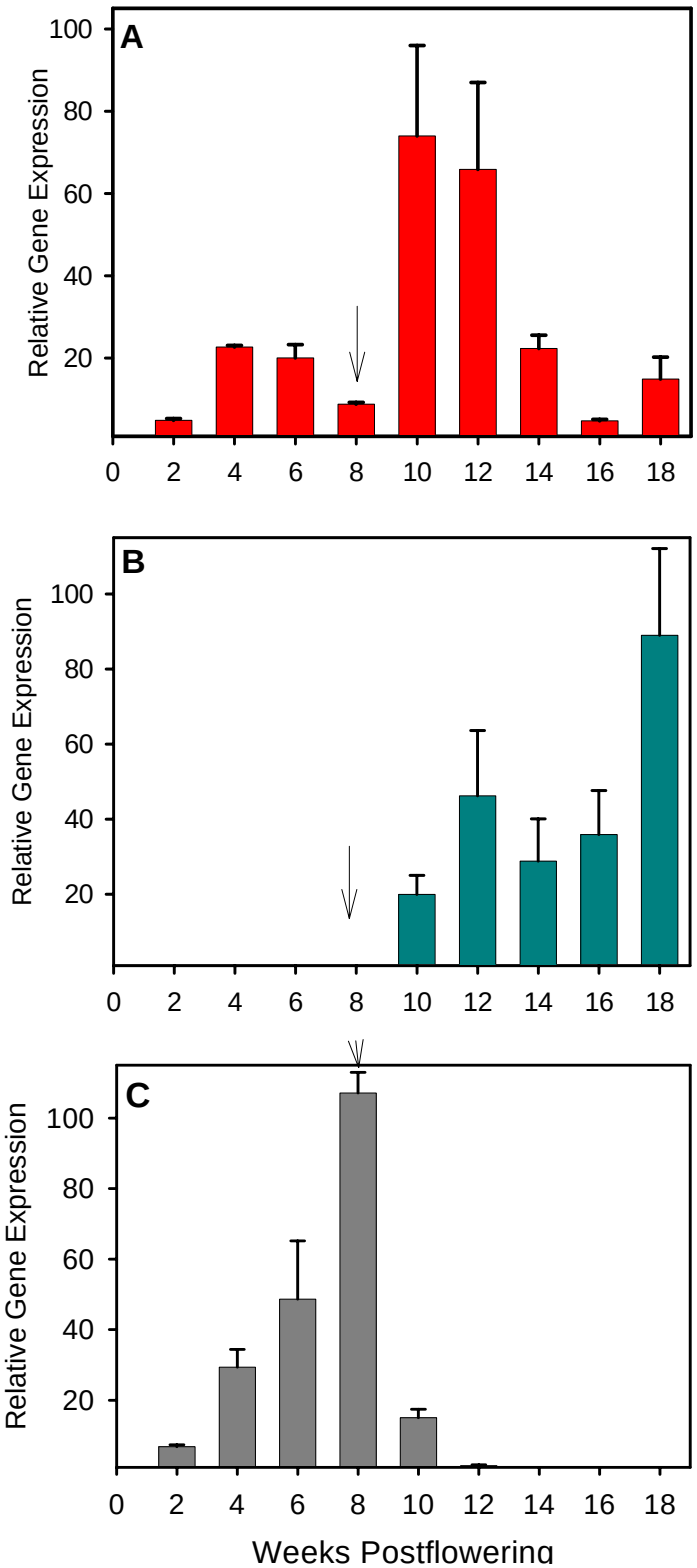


Figure 3.2: Relative gene expression of ripening associated genes measured during the development of cv. Cabernet Sauvignon grape berries during the 2000-01 season: *VvASR*; B) *VvGRIP4*; C) *VvACO*. The arrows denote veraison, which is the last sampling time point before an increase in soluble solids.

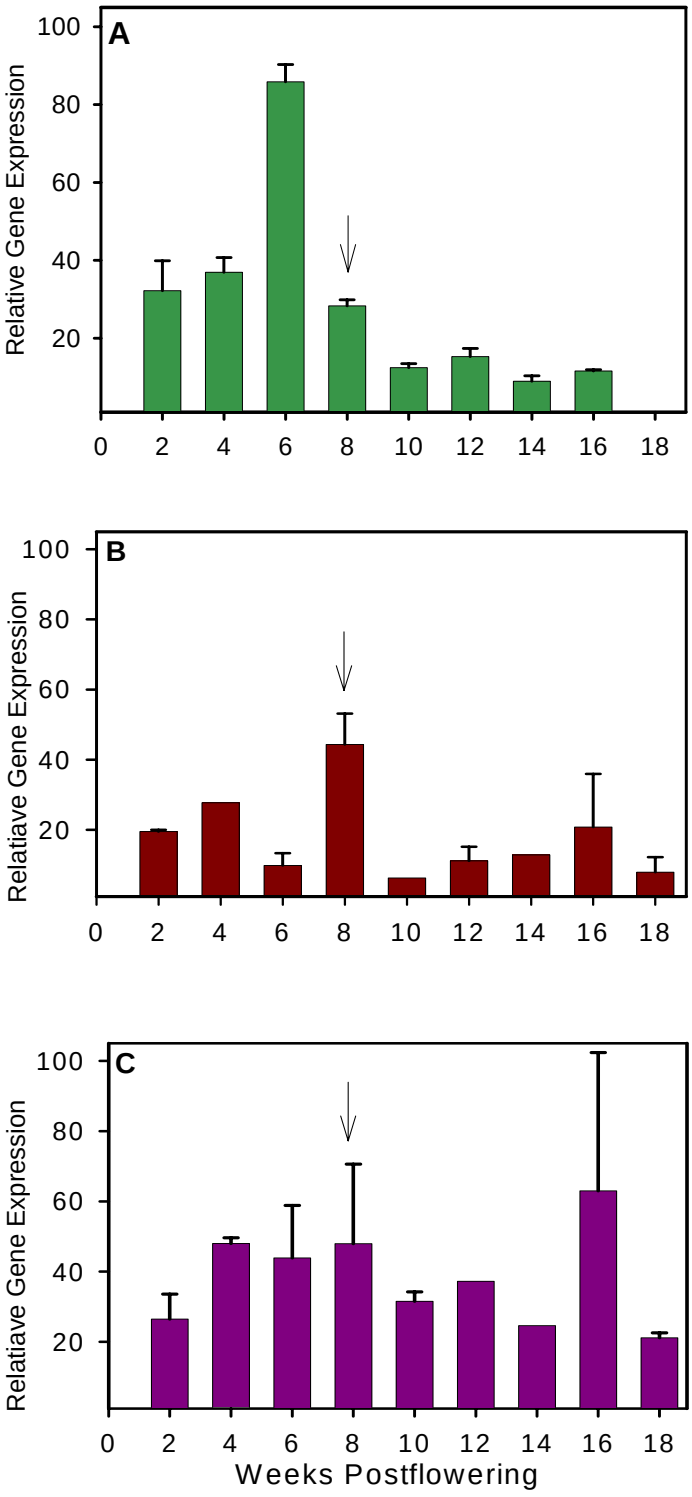


Figure 3.3: Relative gene expression of ABA biosynthesis genes measured during the development and ripening of cv. Cabernet Sauvignon grape berries during the 2002-03 Berry Series: A) *VvNCED1* expression; B) *VvNCED2* expression; C) *VvZEP* expression. The arrows denote veraison, which is the last sampling time point before an increase in soluble solids

The qReal-Time PCR results for the *VvNCED2* mRNA expression in the 2000-2001 and 2002-2003 berry developmental series (Figs. 3.1B, 3.3B) did not show a clearly defined pattern as seen for *VvNCED1*. It might be said that the preveraison samples generally had higher relative gene expression than the postveraison samples but the levels fluctuate considerably between successive samples. *VvNCED2* relative gene expression for the 2000-2001 season was highest in the two and the six weeks postflowering tissues compared to the 2002-03 season where the highest relative gene expression was measured in the eight weeks postflowering tissue sample.

Like the profile seen for *VvNCED2* there was no clear pattern of relative transcript abundance for *VvZEP* (Figs. 3.1C, 3.3C) as was seen for *VvNCED1* and the profiles of *VvZEP* expression were not consistent between the years. In the 2002-2003 season, there was fairly consistent levels of relative expression in all the samples. The levels in consecutive samples during the 2000-2001 season varied considerably with a bimodal distribution. One peak occurred in the four weeks postflowering sample, the level decreased in the six and eight weeks postflowering samples and then increased to a maximum relative gene expression in the ten weeks postflowering sample. Transcript levels in the 12 and 14 weeks postflowering postveraison samples were approximately two fold higher than in the later postveraison samples taken at 16 and 18 weeks postflowering.

The qReal-Time results for the *VvASR* gene are very similar for the two seasons 2000-2001 and 2002-2003 (Figs. 3.2A, 3.4A). The highest relative gene expression level was detected at around veraison in both seasons; at ten weeks postflowering for the 2000-2001 season and 12 weeks postflowering for the 2002-2003 season. This high relative gene expression was maintained in both seasons for another sampling time point (12 weeks postflowering for 2000-2001 and 14 weeks postflowering for 2002-2003), after which the level decreased dramatically.

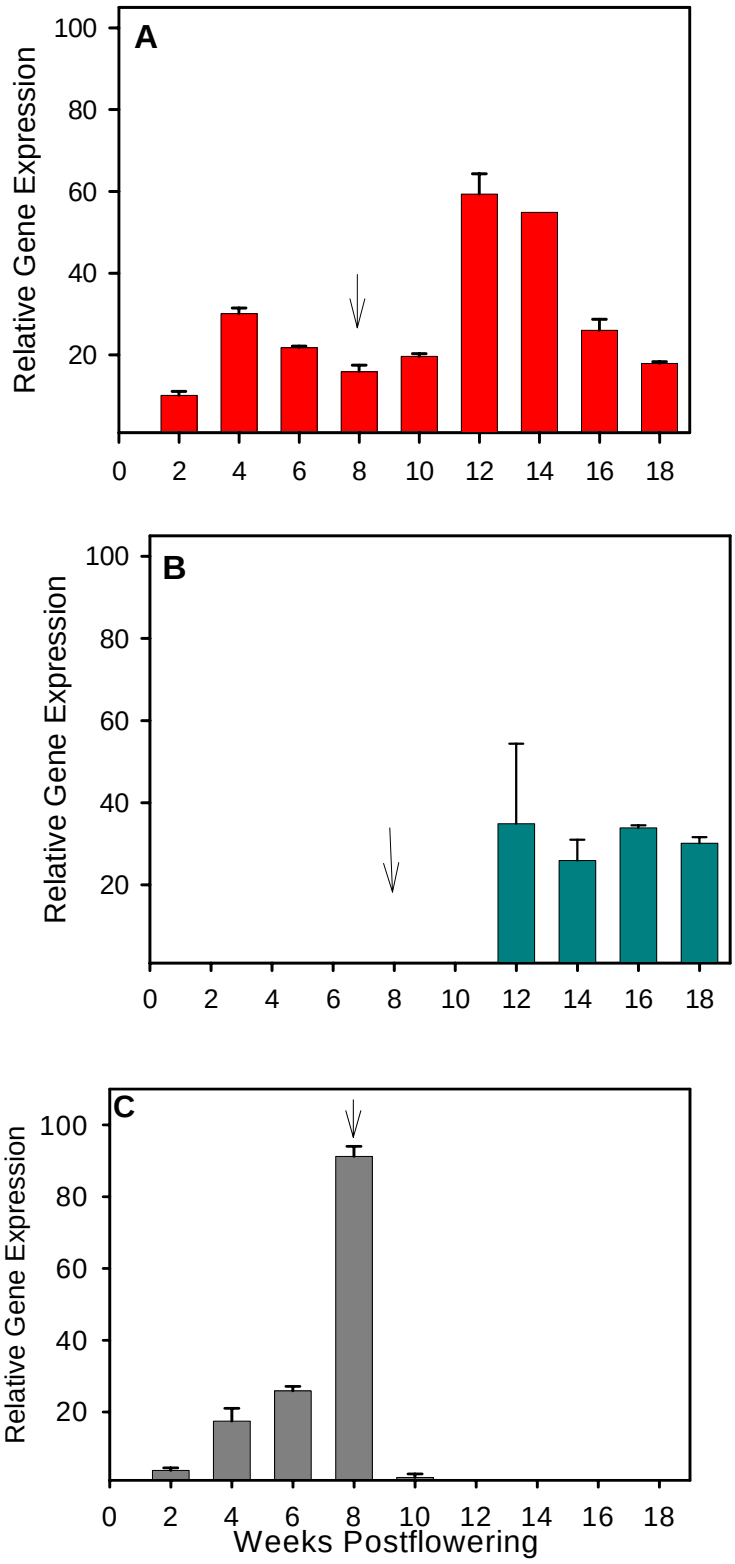


Figure 3.4: Relative gene expression of ripening associated genes measured during the development and ripening of cv. Cabernet Sauvignon grape berries during the 2002-03 Berry Series: A) *VvASR* expression; B) *VvGRIP4* expression; C) *VvACO* expression. The arrows denote veraison, which is the last sampling time point before an increase in soluble solids.

The expression pattern for the *VvGRIP4* gene was striking as there was no detectable gene expression in any preveraison tissues for either of the 2000-2001 or 2002-2003 seasons. Transcripts were only detected in the veraison and postveraison samples for the 2000-2001 and 2002-2003 seasons. The qRT-PCR data obtained for these developmental series of Cabernet Sauvignon berries was verified by previously published work by Davies and Robinson (2000) with *VvGRIP4* expression detectable at high levels in the postveraison berries.

The *VvACO* expression profiles for the 2000-2001 and 2002-2003 seasons were very similar, both showed a sequential increase in relative gene expression from 2 weeks postflowering up to a maximal level in the eight weeks postflowering sample. The ten weeks postflowering samples both had very low levels of relative gene expression, and the later samples contained no detectable transcripts.

The 2003-2004 berry series was sampled at weekly time points starting at one week postflowering. The more frequent sampling time points give a more complete picture of the gene transcript profiles (Figs 3.5A, B, C, 3.6A, B, C). Again, *VvNCED1* mRNA was generally higher in the preveraison samples than in the postveraison and the level of *NCED1* transcript decreased shortly after veraison (Fig. 3.5A).

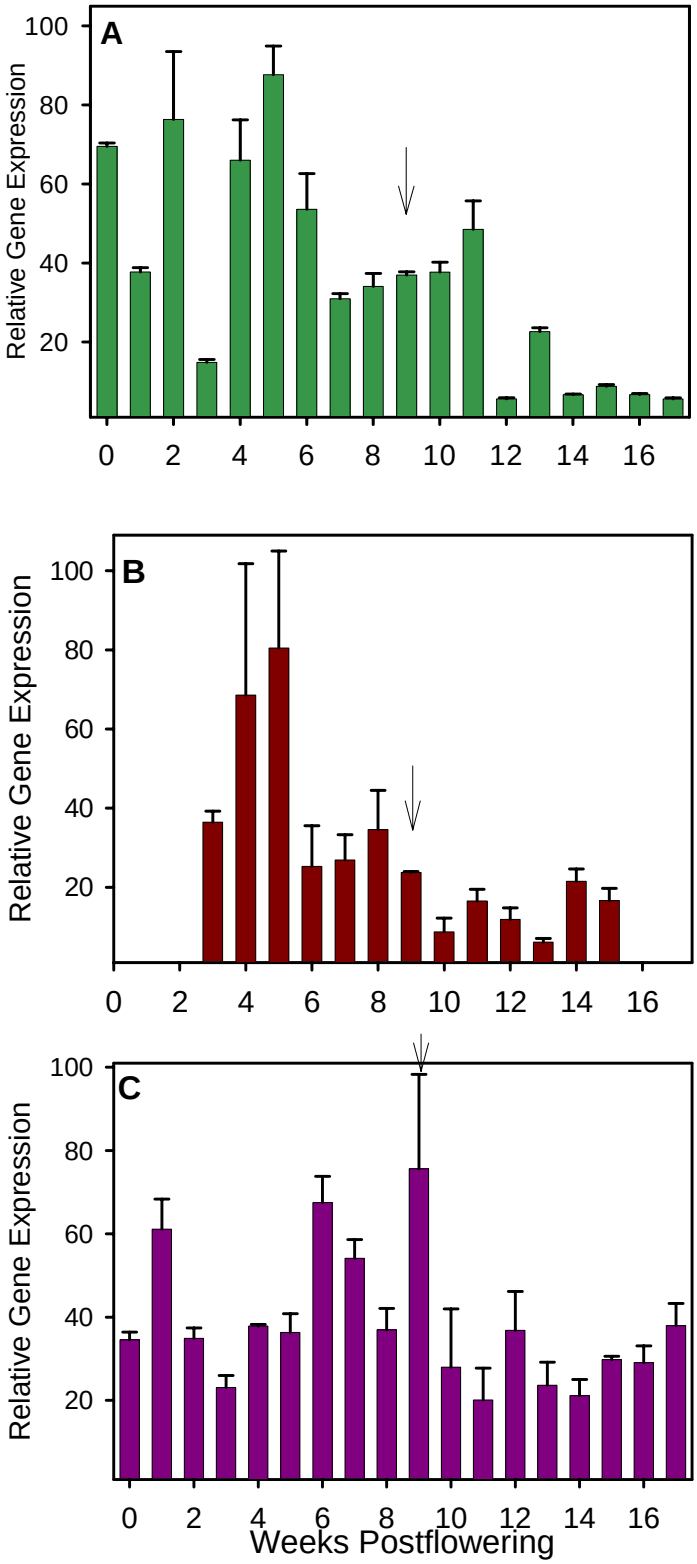


Figure 3.5: Relative gene expression of ABA biosynthesis genes measured during the development and ripening of cv. Cabernet Sauvignon grape berries during the 2003-04 Berry Series: A) *VvNCED1* expression; B) *VvNCED2* expression; C) *VvZEP* expression. The arrows denote veraison, which is the last sampling time point before an increase in soluble solids.

The qReal-Time PCR results for the *VvNCED2* mRNA expression in the 2003-2004 berry developmental series (Figs. 3.5B) had a clearer pattern than the other two berry development series sampled. The preveraison samples had higher relative gene expression than the postveraison samples, especially the three, four and five weeks postflowering tissues. The *VvNCED2* relative gene expression for the 2003-2004 berry series was lower in the postveraison samples compared to the preveraison berry samples.

The pattern of relative transcript abundance for *VvZEP* (Fig. 3.5C) was not as apparent as that seen for *VvNCED1* or *VvNCED2* and in the 2003-2004 season, there was fairly consistent levels of relative expression in all the samples. The levels increased in the six week postflowering sample to a maximum relative gene expression in the nine weeks postflowering sample. Transcript levels then decreased in the ten weeks postflowering postveraison samples and the remaining postveraison samples had slightly lower levels than those measured in the preveraison samples.

The qReal-Time results for the *VvASR* gene in the 2003-2004 are quite different to the other seasons described in this chapter (Fig. 3.6A). In the 2003-2004 season is a bimodal distribution, with peaks at six and twelve weeks postflowering. The six weeks postflowering is the lone high relative gene expression sampling point with regard to the first peak in expression, however, although the 12 week time point has the highest relative *VvASR* gene expression detected in the 2003-2004 season, the eight through to 11 samples all have relatively high levels of expression. At both the early preveraison time points and the late post veraison time points the level of *VvASR* gene transcript detected is quite low.

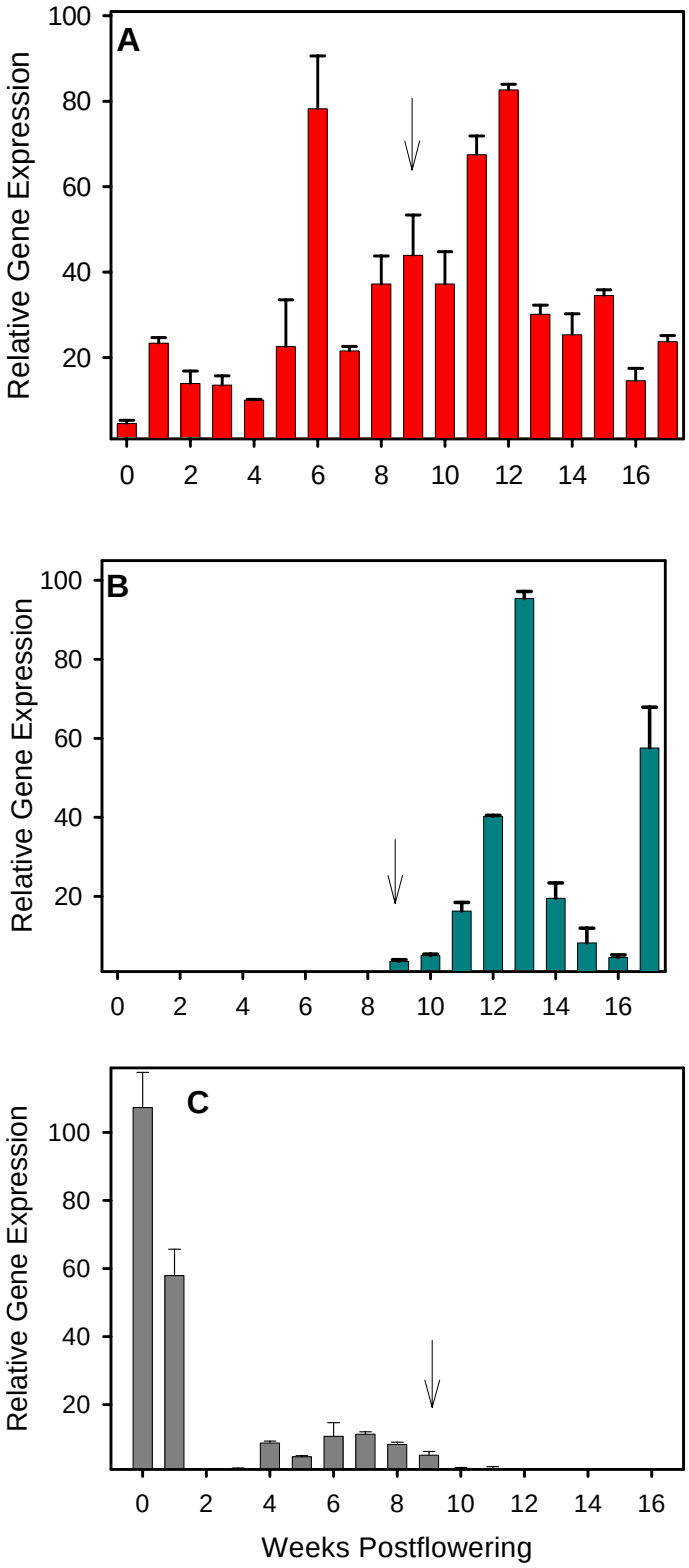


Figure 3.6: Relative gene expression of ripening associated genes measured during the development and ripening of cv. Cabernet Sauvignon grape berries during the 2003-04 Berry Series: A) *VvASR* expression; B) *VvGRIP4* expression; C) *VvACO* expression. The arrows denote veraison, which is the last sampling time point before an increase in soluble solids.

The expression pattern for the *VvGRIP4* gene was striking as there was no detectable gene expression in any preveraison tissues in the 2003-2004 season, which was the same as the previous seasons sampled (Fig. 3.6B). Transcripts were only detected in the veraison and postveraison samples.

The *VvACO* expression profile for the 2003-2004 season was similar to that found in previous seasons with regard to the time points after two weeks postflowering, that is a sequential increase in relative gene expression from two weeks postflowering up to a peak in the seven weeks postflowering sample (Fig. 3.6C). The ten weeks postflowering sample had very low levels of relative gene expression, and the later samples contained no detectable transcripts. However, when the first two time points are included on the graph, (the closed flowers and one week postflowering berry sample) the *VvACO* transcript levels in these samples are very high and make the subsequent peak around the nine weeks postflowering sample appear much less significant.

Due to difficulty in preparing RNA from the 2003-2004 12 and 16 weeks postflowering seed tissues there was insufficient RNA to prepare cDNA and so these samples were not included in the qReal-Time PCR analysis for the various grape berry tissues (Figs. 3.7A, B, 3.8A,B,C). The *VvNCED*2, *VvZEP, VvASR, VvGRIP4* and *VvACO* transcript levels were determined in the separate skin, flesh and seed samples as an indication of the distribution of these genes. Transcripts of both *VvNCED2* and *VvZEP* genes were detectable in all the berry tissues sampled, for each of the time points tested. Due to the lack of postveraison seed RNA it is difficult to draw many conclusions regarding the expression patterns in seeds. *VvNCED2* mRNA levels were higher in preveraison skin samples compared to the postveraison samples. In contrast, the *VvNCED2* transcript levels in flesh were high in the two week postflowering sample, decreased prior to veraison and reached a second peak in the 10 weeks postflowering sample. *Vv*ZEP transcript levels were highest at two weeks postflowering with regard to the seed and flesh tissues, whereas the eight weeks postflowering sample was the highest for the skin tissue. Except for in the two weeks postflowering sample the levels of *VvNCED2* and *VvZEP* transcripts changed in concert relative to each other (Fig. 3.7A).

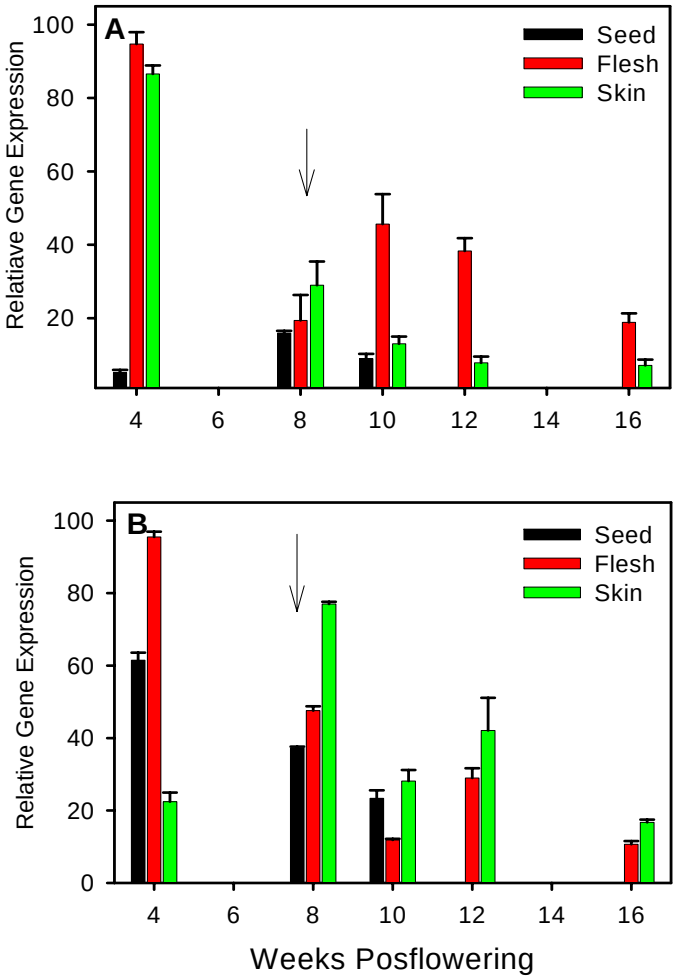


Figure 3.7: Relative gene expression of ABA biosynthesis genes measured during the development and ripening of cv. Cabernet Sauvignon grape berries skin, flesh and seed tissues during the 2003-04 Berry Series: A) *VvNCED2* expression; B) *VvZEP* expression. The arrows denote veraison, which is the last sampling time point before an increase in soluble solids.

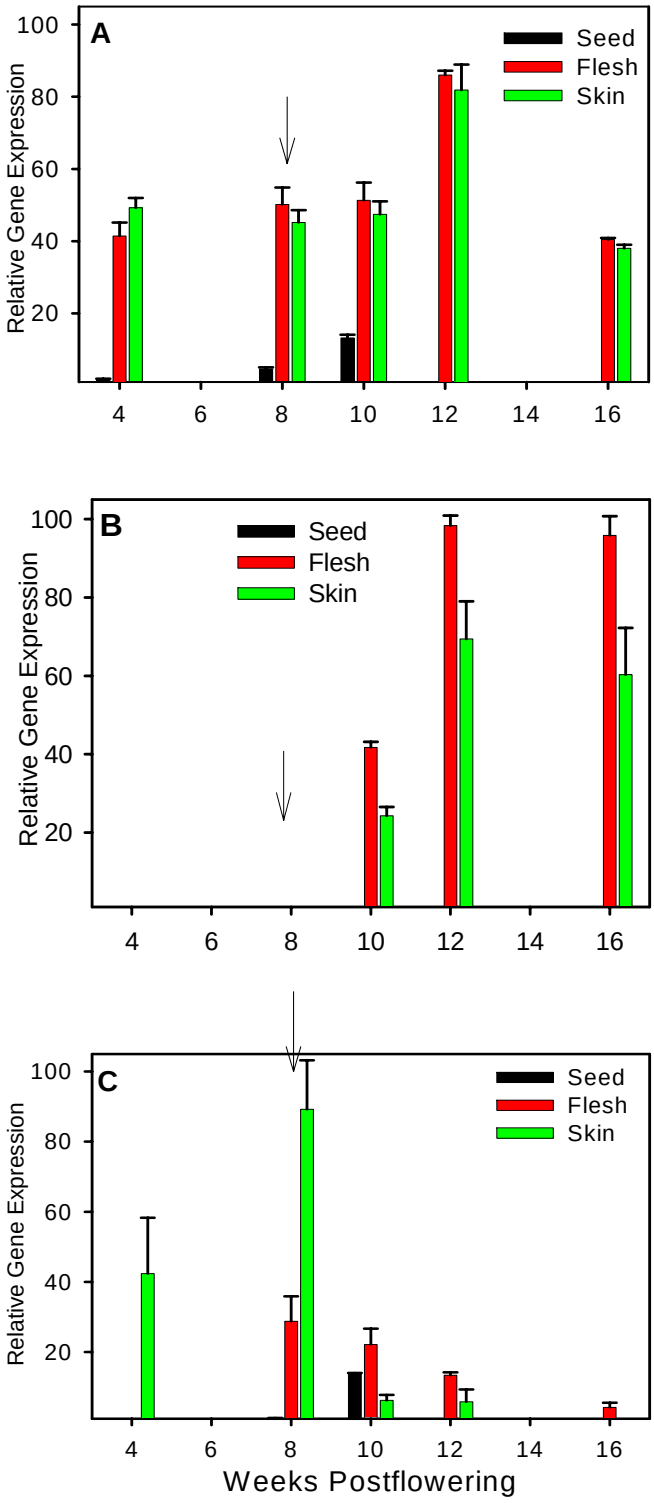


Figure 3.8: Relative gene expression of ripening associated genes measured during the development and ripening of cv. Cabernet Sauvignon grape berries skin, flesh and seed tissues during the 2003-04 Berry Series: A) *VvASR* expression; B) *VvGRIP4* expression; C) *VvACO* expression. The arrows denote veraison, which is the last sampling time point before an increase in soluble solids.

The *VvASR* mRNA profile was similar to the deseeded berry sample for the 2003-2004 season, with the highest level in the 12 weeks postflowering sample, not surprisingly as the levels of *Vv*ASR transcripts in the skin and flesh were highly similar and there was little expression in the seed. The other peak which was observed in the deseeded berries was at six weeks postflowering, however, this was not one of the time points selected for the skin, flesh and seed analysis.

The *VvGRIP4* transcript was only detected in the postveraison samples of the skin and flesh for the selected 2003-2004 time points examined, which is in agreement with the whole deseeded berry samples.

The *VvACO* transcript result is similar to the whole deseeded berry series for 2003-2004 with higher levels in the preveraison samples than the postveraison. There was *VvACO* transcript detected in the skin samples only at four weeks postflowering, and not in the flesh or seed samples from this same time point. The only seed sample that had detectable levels of *VvACO* transcript was the ten weeks postflowering. The preveraison skin samples had the highest levels of *VvACO* transcript.

3.5 Discussion

In order to study the levels of ABA biosynthesis pathway gene transcripts during grape berry development a system was developed which utilised qReal-Time PCR. First, experiments were undertaken to ensure that all the members of the important gene families had been cloned and sequenced. Second, good quality RNA and cDNA was produced for the required samples and tested. Third, a suitable gene to act as the internal standard was identified. Forth, primers to detect the ABA biosynthesis pathway genes of interest and also to detect other genes of interest and those whose developmental pattern of gene expression had previously been described were designed and tested. These genes were used as further controls to ensure that the expected patterns were identified using the system developed.

3.5.1 Degenerate oligo PCR

The cleavage of 9-cis-xanthophylls to xanthoxin which is catalysed by NCED has been shown to be a key regulatory step in ABA biosynthesis in plants (Qin and Zeevaart, 1999). In other plant species, such as *Arabidopsis* and Avocado, multiple genes encoding the NCED enzyme have been identified (Tan *et al*., 2003; Chernys and Zeevaart, 2000). In

Arabidopsis, nine carotenoid cleavage dioxygenase genes have been identified in the complete genome sequence (Tan *et al*., 2003) and of these carotenoid cleavage dioxygenase genes, at least five encode NCEDs involved in ABA synthesis (Iuchi *et al*., 2001). Tan *et al*. (2003) demonstrated the differential expression and localization of the NCED genes through qReal-Time PCR analysis and *AtNCED* promoter:GUS expression studies. The differential regulation indicates that overlapping programs exist that regulate ABA biosynthesis in plants. In avocado, three genes encoding NCED cleavage-like enzymes were cloned (Chernys and Zeevaart, 2000). Two of these genes were shown through northern blot analysis to be strongly induced in parallel during fruit ripening. In avocado fruit ABA biosynthesis appears to be redundant, in the sense that two genes appear to encode proteins with identical functions (Chernys and Zeevaart, 2000). Chernys and Zeevaart (2000) suggest that as well as synthesis, transport and degradation are also important in regulating ABA levels. They suggested that the accessibility of the enzyme to the substrate and the isomerisation of violaxanthin to neoxanthin may also explain why two *NCED* genes exist.

Previous work by Soar *et al*. (2006) identified two NCED genes in *V. vinifera* (cv. Shiraz) using degenerate oligo PCR on leaf RNA. Tan *et al*. (2003) and Chernys and Zeevaart (2000) showed that the *NCED* genes in *Arabidopsis* and avocado are expressed in a tissue specific manner. Therefore, not all grapevine *NCED* genes may have been isolated using leaf RNA as the template for the degenerate oligo PCR. Using degenerate oligo PCR primers and cDNA prepared from RNA extracted from berries collected throughout the berry developmental series for 2002-2003, PCR products were obtained and sequenced. Sequences that were homologous to both the previously identified *VvNCED1* and *VvNCED2* genes were obtained, but no novel NCED sequences were identified. Further investigation by Dr Jim Speirs (pers. comm.) showed there were only two grape *NCED* genes detectable by Southern blot analysis. The grapevine cDNAs *VvNCED1* and *VvNCED2* both have high homology to the *NCED* genes from *Arabidopsis* and other *NCED* genes from other species whose functions have been verified through experimental means (Iuchi *et al*., 2001). This indicates that the two putative grape NCEDs most likely act in grapevine to cleave 9-cis-xanthophylls to form xanthoxin.

The conversion of zeaxanthin to violaxanthin is catalysed by ZEP, and it has been shown by Buridge *et al*. (1999) in tomato that this gene is not up-regulated in response to water

stress. This suggests that ZEP is not a key regulatory enzyme in the ABA biosynthesis pathway. Dr Jim Speirs (pers. comm.) had previously identified a full-length *V. vinifera* cv. Shiraz *ZEP* gene and through Southern blot analysis confirmed that only one copy of this gene was likely to be present in the grapevine genome. The grapevine *VvZEP* has a high level of sequence homology with the *Arabidopsis* ZEP gene and those from other species indicating that it most likely encodes ZEP in grapevine and converts zeaxanthin to violaxanthin *in planta*.

Other genes identified in the ABA biosynthesis pathway include the *AtABA2* gene from *Arabidopsis*, which is a short-chain alcohol dehydrogenase (Gonzalez-Guzman *et al*., 2002) and catalyses the conversion of xanthoxin to abscisic aldehyde, and the abscisic acid oxidase gene *AtAAO3* (Seo *et al*., 2000) which converts abscisic aldehyde to abscisic acid. The grape homologues to these genes have not yet been identified and so were not included in the qReal-Time PCR analysis on the berry developmental series that is detailed in this chapter.

3.5.2 qReal-time PCR, justification of technique

The technique of gene expression analysis known as qReal-Time PCR technology has removed many of the difficulties associated with quantitative gene expression studies, and qRT-PCR is rapidly being adopted as a standard method for in-depth expression studies (Brunner *et al*., 2004). qRT-PCR differs from classical PCR by measurement of the amplified PCR product at each cycle throughout the PCR reaction. Thus qRT-PCR PCR allows the amplification to be followed in qReal-Time during the exponential phase of the process (Gachon *et al*., 2004). Closely related genes that are very similar at the sequence level may cross-hybridize during northern blot procedures and therefore, it may be difficult to determine the RNA level of a specific member of a gene family. This problem is resolved by the high specificity of qRT-PCR which uses two gene specific oligonucleotides.

qRT-PCR provides a high sensitivity for the detection of RNA due to a combination of the amplification performed by the PCR step and the system of detection (Gachon *et al*., 2004). It is therefore, a convenient technique when the amount of starting material to be studied is limited, such as the skin, flesh and seed samples of the berry. The methodology of the qReal-Time PCR used in the gene expression analysis detailed in the following experiments is based on the measurement of the fluorescence emitted by SYBRgreen

molecules intercalated into the double strand DNA. As intercalating agents bind regardless of the nucleotide sequence, they can be used to detect any DNA fragment (Gachon *et al*., 2004).

To enable comparison of levels of gene expression between tissues, the qReal-Time PCR analysis must be normalised. The purpose of normalization is to correct for non-specific variation, such as differences in RNA quantity and quality, which can affect efficiencies of the reverse transcription and PCR reactions. The most commonly used method is relative quantitation, whereby gene expression level is normalized to that of an internal reference gene. While this avoids the problems and limitations of absolute quantitation, selection of an internal control gene expressed at a nearly constant level in all tissue samples being investigated is required. Previous experiments by Downey *et al*. (2003) identified *VvUbiquitin1* (TC32075, TIGR database), a putative ubiquitin extension protein as having relative constant expression throughout grape berry development. This gene was used as the internal control gene for the qReal-Time PCR experiments described in this chapter.

3.5.3 Changes in the expression of ABA biosynthesis genes, and genes involved in berry development, in Cabernet Sauvignon berries and the implications for transcriptional control

Cheng *et al*. (2002) suggested that in *Arabidopsis* it was clear that during developmental (fruit ripening) and physiological changes (wilting), ABA biosynthesis is regulated at the level of cleavage of C_{40}-carotenoid precursors into xanthoxin. They showed that two *NCED*-like genes are up-regulated during fruit ripening, but only one is induced in dehydrated leaves. The situation in grape berries is somewhat different. The expression of *VvNCED1* in all three berry developmental series was consistently higher in the preveraison tissues than in the veraison and postveraison tissues. The highest concentration of free ABA was measured in every season in the ten weeks postflowering sample, however, in none of the seasons, was the highest amount of either *VvNCED1* or *VvNCED2* relative gene expression calculated to be in this sample. The peak in *NCED* relative gene expression occurred prior to the corresponding increase in free ABA in the developing berries. This is consistent with *VvNECD1* exerting transcriptional control over ABA synthesis in the berry. However, increased mRNA abundance is not always linked with increased activity of the gene product (Thompson *et al.,* 2000). Multi-gene families often encode genes with related functions, but in cases where the function is the same,

differential regulation ensures that distinct genes are activated in response to different environmental and other stimuli. Tan *et al*. (2003) developed a specific transcript quantification assay based on fluorescent qRT-PCR in order to quantify mRNAs of each member of the *AtNCED* gene in organs of developing *Arabidopsis* plants. They stated that the differential regulation of the five *AtNCED* genes indicated the presence of overlapping programs of developmental and environmental signals that regulate ABA biosynthesis in plants. Through these experiments it was demonstrated that *AtNCED3* was the most highly expressed gene in both stress and non-stressed leaves. However, stress increased mRNA levels of all *NCEDs*. The fact that there are two *NCED* genes expressed in grape berry tissues may be an indication that sensitive mechanisms are needed to regulate the amount and location of the ABA synthesized. Xiong and Zhu (2003) proposed that gene family members may be regulated differentially by different stresses or may have different thresholds of stress induction.

While ZEP catalyses an essential step in ABA synthesis, ZEP transcript levels do not appear to be the rate limiting step in leaf tissue as they remained constant while leaf ABA concentration increased in the leaves of water stressed tomato plants (Thompson *et al.,* 2000). *VvZEP* does not appear to be expressed with a consistent pattern during grape berry development. In the 2000-2001 berry series, the highest expression was in the ten weeks postflowering sample. However, this up-regulation at 10 weeks postflowering was not reproduced in the other berry developmental series. The expression profile of *VvZEP* during the 2002-2003 berry series was fairly uniform for all time points, and the 2003-2004 weekly developmental series had higher *VvZEP* expression detected in the preveraison berry tissues than in the postveraison berries. One potential explanation for the differing mRNA profiles for the three developmental series is that it has been shown in tobacco and tomato plants that transcript levels of *ZEP* genes in leaves were regulated diurnally with high transcript levels during daylight hours, which may reflect regulation by circadian rhythm (Audran *et al.,* 1998) rather than the periods of diurnal water potential changes in plants. However, despite the diurnal variation in transcript levels, no changes in the level of ZEP protein were found. The potential circadian regulation could be related to the involvement of ZEP products in the light-harvesting complex but not to the role of ZEP proteins in ABA biosynthesis (Audran *et al.,* 1998). Although the berries for the three developmental series were harvested at a fairly similar time every day (approximately 11am), the time they were harvested after sunrise would change throughout the season due

to the change in day length. Therefore, some differences may result in the *VvZEP* mRNA expression being influenced by the circadian or diurnal rhythm of the grapevine. A 24 hour time course sampling of the grape berries at a particular developmental stage, for example, ten weeks postflowering, may indicate whether *VvZEP* mRNA levels vary as a result of circadian or diurnal rhythms. There are other potential reasons for the variations, such as weather conditions on the sampling day (including sunlight levels, temperature, soil water availability and humidity).

Cheng *et al*. (2002) used an *ABA2/GIN1* reporter gene to show that *AtABA2* expression is highly restricted to specific vascular tissues (predominantly in the roots and the stems) in *Arabidopsis*. This is distinct from the tissue expression patterns of other genes, such as *ZEP*, *NCED3*, *AAO3*, and *ABA3*, that are important for ABA biosynthesis (Audran *et al*., 1998; Qin and Zeevaart, 1999; Iuchi *et al*., 2000, 2001; Thompson *et al*., 2000; Xiong *et al*., 2001, 2002) which are expressed in a greater variety of tissues. The product of the *ABA2* gene is a cytosolic short-chain dehydrogenase reductase which catalyses the conversion of the plastid-derived xanthoxin to abscisic aldehyde. These results indicate that inter-organ, inter-cellular, and inter-organelle transport of ABA precursors and/or ABA may be required for the completion of ABA synthesis and for ABA to reach target sites. As the grape homologue to *AtABA2* has not yet been identified, its expression pattern during the grape berry developmental has not been determined.

As hormones are such powerful coordinators of gene expression their levels are usually thought to be tightly controlled. The correct, physiologically active ABA level is controlled through fine-tuning the balance between *de novo* synthesis and catabolism. The hydroxylation at the 8'-position of ABA is known to be the key step of ABA catabolism, and this reaction is catalyzed by ABA 8'-hydroxylase, a cytochrome P450 (Saito *et al*., 2004). In grape, the ABA 8'-hydroxylase has not been identified, so its mRNA expression during the grape developmental series is yet to be determined. Once the remaining genes in the ABA biosynthesis and catabolism pathways have been identified, their mRNA expression profiles will give a more complete understanding of the degree of influence the control of transcript levels in the developing grape has on free ABA levels.

As well as the mRNA expressions of the ABA biosynthesis genes that had been identified in grape, the qRT-PCR expression profiles of several other genes of interest were determined. The first was *VvACO*, which catalyzes the conversion of 1-

aminocyclopropane-1-carboxylic acid (ACC) to ethylene. In climacteric fruit it has been shown that ethylene plays an important role in ripening. A burst of ethylene production occurs at around the time of the commencement of ripening and exogenously applied ethylene induces ripening and endogenous ethylene production (Giovannoni, 2001). Liu *et al*. (1999) showed that the expression of the banana *ACO1* gene was detectable at the preclimacteric stage and increased when ripening commenced and then remained high throughout the later ripening stage despite of a rapid reduction in the ACO activity. They explained that the discrepancy between enzyme activity and gene expression of ACO could be, at least in part due to reduced contents of ascorbate and iron, cofactors for the enzyme during ripening. Addition of these cofactors to the incubation medium greatly stimulated the *in vivo* ACO activity during late ripening stages. The results suggest that ethylene production in banana fruit is regulated by reduction of ACO activity possibly through limited *in situ* availability of its cofactors once ripening has commenced. Chervin *et al*. (2004) measured the mRNA expression of *VvACO* in Cabernet Sauvignon berries, up until ten weeks postflowering using northern blot analysis. *VvACO* mRNA was detected in the preveraison berries, with the maximal level at seven weeks post flowering, after which it decreased below the level of detection at ten weeks post flowering. This is a very similar result to that obtained for two of the berry developmental series, 2000-2001 and 2002-2003, with the highest level of *VvACO* mRNA detected in the eight weeks postflowering sample, after which the level decreased to almost below detection at ten weeks postflowering. The comparison with the results of Chervin *et al*. (2004) produced by an alternative method for mRNA expression determination validates to some extend the qRT-PCR approach. During the 2003-2004 season earlier samples were assayed for ACO gene expression than in the previous two series discussed above. High levels were detected in the closed flower and one week postflowering samples, and much lower mRNA levels detected in the other preveraison berry tissue. The inclusion of these data points on the graph (Fig. 3.8C) gives the impression that the levels of *VvACO* expression at around veraison in this season are unusually low. However, when the Ct values are compared, the levels in the later preveraison berry samples for 2004-2005 are comparable with the other two seasons. Chervin *et al*. (2004) did not record the early levels of *VvACO* gene expression in their developmental series.

The ASR proteins, which are induced by ABA, stress, and ripening, were first described in tomato (Iusem *et al*., 1993; Amitai-Zeigerson *et al*., 1994; Rossi and Iusem, 1994). *ASR*

genes are expressed in various organs, such as the fruit of tomato (Iusem *et al*., 1993). ASR genes seem to be involved in processes of plant development, such as senescence and fruit development, and in response to abiotic stresses, such as water deficit, salt, cold, and limited light (Schneider *et al*., 1997; de Vienne *et al*., 1999; Maskin *et al*., 2001; Jeanneau *et al*., 2002). Cakir *et al*. (2003) published results showing that a grape homologue, *VvMSA*, had upregulated gene expression during the early stages of fruit development and during the later stages of grape ripening with lower levels at veraison. The expression data by Cakir *et al.* (2004) was obtained using northern blot analysis on the grape variety Ugni blanc. No details on the developmental stage the berries were harvested are given, or whether the berries were whole or deseeded. The 2003-2004 *VvASR* qRT-PCR results match closely with the data from Cakir *et al*. (2004). The data from the other seasons however, are not consistent with this. Possible explanations for the discrepancy between the various years is that the *VvASR* may also be under circadian/diurnal rhythmic control like the ABA biosynthesis gene *ZEP*, and the samples were not taken at the same time every sampling time point. Also there may be gene expression differences between the variety Cakir *et al*. (2004) used in their published work, Ugni blanc, and that which was used in this study, Cabernet Sauvignon.

As qRealTime-PCR is a relatively new approach for determining mRNA levels in plant tissues, the expression profile of several genes was determined in the grape developmental series from the three seasons and compared with published data. One gene, Grape ripening-induced 4 (*VvGRIP4*), had previously been identified by Davies and Robinson (1996) through differential screening of a post-veraison grape berry cDNA library. Northern blot analysis of a developmental series of Shiraz berries showed that GRIP4 mRNA expression was highly upregulated in post veraison berry tissues. The qRT-PCR data obtained for all three developmental series of the Cabernet Sauvignon berries sampled was verified by this previously published work, with *VvGRIP4* expression detectable at high levels in the postveraison berries. This result goes some way to validate the qRT-PCR approach as a valuable method in determining gene transcript levels in grape tissues. The patterns of *VvNCED1, VvNCED2* and *VvZEP* are such that the berry ABA may be produced *de novo* in the berry, or could be imported into the berry, or in another case, could be a combination of both.

Chapter 4: Grape Tissue *In vitro* Experiments

4.1 Introduction

4.1.1 Hormones in fruit ripening

Fruit ripening can be influenced by both internal, developmental cues, and external cues such as light, temperature, water and cultural practices. Hormones play an important role in the plants response to these stimuli and fruit ripening is one process in which hormones are crucial. Ethylene is widely recognised as being essential to ripening in climacteric fruit (Adams-Phillips *et al*., 2004). However, the hormonal control of ripening in other, non-climacteric fruit is still not well defined and three hormones (including ABA) have now been identified as having some role in this process (see Chapter 1 for a full discussion of this).

4.1.2 Sugar signalling

Other compounds apart from the classical hormones can have roles in signalling. Sugars have signalling roles in a wide variety of developmental processes in plants and they may interact with other hormone signalling pathways (Arroyo *et al*., 2003). Sugar signal transduction in plants has been shown to be tightly associated with some processes affected by ABA, such as the response to osmotic stress and ethylene signalling (Eckardt, 2002). ABA biosynthesis and signalling can be induced by osmotic stress (Arroyo *et al*., 2003). This effect can be due to an increased osmotic potential caused by the addition of solute to the medium, rather than a specific effect of the sugar. Cheng *et al*., (2002) showed that the application of mannitol to *Arabidopsis* seedlings caused the induction of a number of ABA biosynthesis and signalling genes. However, under some circumstances there does seem to be a sugar- specific effect that is not related to osmotic level alone; i.e. the sugar in question appears to act as a signalling molecule in its own right. Cheng *et al*. (2002) showed that in the *Arabidopsis gin1/aba2* mutant, 6% glucose resulted in elevated expression of *AtABA1, AtABA2 and AtAAO3*, genes which are involved in ABA biosynthesis, whereas 6% mannitol diminished the expression of these genes suggesting that in this case the response was glucose-specific. The gene which catalyses the conversion of xanthoxin to abscisic aldehyde, *ABSCISIC ACID DEFICIENT2* (*ABA2*), has been shown to function as a molecular link between nutrient signalling and plant hormone biosynthesis in *Arabidopsis* (Cheng *et al.,* 2002). Cheng *et al.* (2002) also demonstrated that in *Arabidopsis AtABA2* expression is induced significantly in the presence of 2 and

6% glucose; however, this gene is not activated by ABA alone, and its glucose activation requires endogenous ABA. These extensive studies in *Arabidopsis* ABA biosynthesis mutant plants provide direct molecular evidence for the intimate involvement of glucose in the control of genes involved in ABA biosynthesis and signalling. Although sucrose may be the sugar compound used for transport in the grapevine, it is cleaved either in the apoplast, the cytoplasm of the berry mesocarp cells, or in the vacuole by invertases where glucose and fructose are stored in roughly equal amounts. Therefore, in most cellular compartments of the berry mesocarp tissues there is likely to be high concentrations of glucose (Ageorges *et al*., 2000).

ABA accumulation in response to developmental and environmental cues has been extensively studied in a range of species. It has been shown that the expression of *NCED* is highly activated by stress conditions and encodes a rate-limiting enzyme for the stress-inducible accumulation of ABA in many plant species (Tan *et al.*, 2003 Qin and Zeevaart, 1999; Iuchi *et al.*, 2000, 2001). However, as Cheng *et al.* (2002) demonstrated *AtNCED3* expression was not induced by glucose application in *Arabidopsis*. This finding suggests that stress- and glucose-induced ABA accumulation are mediated by distinct mechanisms.

As found for *NCED*, the expression of *ZEP* also does not appear to be up-regulated by glucose. Arenas-Huertero *et al.* (2000) demonstrated in *Arabidopsis* that the ABA biosynthesis gene *AtZEP1* was not regulated by glucose as there was no differential expression of *AtZEP1* under high glucose conditions in either wild type *Arabidopsis* or the glucose insensitive mutant *gin5*.

The availability of, or lack of, sugars triggers many developmental responses, and sugars affect the expression of a large number of genes (Smeekens and Rook, 1997). As sugar sensing occurs at the individual cell level, the responses of such cells must be integrated at the tissue, organ and plant level. Koch (1996) showed that many plant metabolic and developmental processes were control by sugars, although the exact mechanisms by which plants sense sugar and initiate the signal transduction are still largely unknown.

In addition to interactions with sugars, the ABA signalling pathway can also be influenced by, and can in turn influence, other hormonal responses. Saniewski *et al*. (1986) showed that methyl jasmonate treatment in preclimacteric apples stimulated ethylene production,

however, in postclimacteric apples methyl jasmonate inhibited ethylene production. In tomatoes Riov *et al.* (1990) demonstrated that exogenous ABA application increased ethylene levels. There is considerable evidence that both antagonistic and synergistic interactions can occur between different hormones.

4.1.3 *In vitro* experimental techniques

Their woody nature and long generation time coupled with the lack of mutants with useful phenotypes make grapevines a challenging system in which to study hormone interactions and signalling at the detailed level. Isolated berries and suspension cell cultures offer the promise of a more easily manipulated model system in which various parameters can be readily manipulated. Excised whole *V. vinifera* berries and suspension cell cultures have been used by a number of researchers to investigate berry biology. Hiratsuka *et al,* (2001) used whole *V. labruscana* Bailey ('Olympia') berries harvested from field grown vines to investigate the anthocyanin formation in response to ABA and sugar. These researchers demonstrated that pigment synthesis was accelerated in the berries stimulated with ABA compared to the water control. This indicated that the berries were able to synthesize the pigments without nutrients from the vine. Çakir *et al*. (2003) used berry cell cultures to determine that *VvMSA*, the grape ASR (ABA-, stress-, and ripening-induced) gene expression was induced by sucrose, and that this induction was strongly enhanced by ABA. Atanassova *et al*. (2003) also used grape berry cell cultures to determine the induction of *VvHT1* (*Hexose Transporter 1*) gene by both sucrose and palatinose. Cell cultures are valuable model systems that allow the experimental investigation of gene expression in grape berries in a homologous system at any time of the year. They are also useful as the media can be rapidly changed and all the cells bathe in the same media under the same conditions. The drawback is, however, that cultured cells are dedifferentiated and the responses induced by different media *in vitro* may not be representative of what occurs *in planta.* The use of excised whole berries circumvents some of these limitations.

4.1.4 Experimental outline

In research described in this chapter, pre-veraison berries from Cabernet Sauvignon were used for *in vitro* inductions to investigate the effect of a variety of osmolites and ABA on ABA biosynthesis gene expression and the accumulation of ABA. The carotenoid biosynthesis inhibitor, norflurazon was also applied to berries to determine its effect on ABA accumulation and ABA biosynthesis pathway gene expression. As well as using

whole preveraison berries, grape cell cultures were used to allow experiments to be carried out year round, when berries were unavailable. The expression of other grape berry ripening-associated genes was also examined.

4.2 Materials and methods

4.2.1 Plant tissue

Whole berry induction experiments

Cabernet Sauvignon berries were harvested from *Vitis vinifera* L. cv Cabernet Sauvignon vines grown at the Slate Creek vineyard in Willunga, South Australia at selected times (see figure legends for dates). Whole bunches were harvested and stored on ice for transportation to the laboratory where they were sterilised by overnight immersion in 1L of 0.05% (v/v) Tween 20 (BDH) with a Milton sterilisation tablet (Milton). Bunches were then washed in autoclaved tap water and treated aseptically from this step. In 2003 the berries were removed from the bunch and sliced in half longitudinally. Between ten and fifteen berry halves were place in 6 cm diameter Petri dishes containing liquid Cormier media, which is Gamborgs B-5 Basal Medium, (PhytoTechnology) supplemented with 250 mg/L casein hydrolysate (Sigma), 0.93 μM kinetin (Sigma) and 0.54 μM NAA (Sigma). The osmolites sucrose (BDH) or mannitol (BDH), were added at a final concentration of 3 or 12% (w/v) sucrose and 12% (w/v) mannitol. The plates with the liquid media were placed in the dark for 48 hours after which the berry halves were washed three times with autoclaved tap water, blotted dry and immediately frozen in liquid nitrogen.

In 2004 10-12 berries were surface sterilized as described above and then sliced just adjacent to the pedicel through the top of the berry and the berry placed with the cut surface on the Petri dish with Cormier media as described above but without NAA. Osmolites were used at equivalent osmolalities by addition to final concentrations of 12% (w/v) sucrose, 7% (w/v) mannitol, 6.9% (w/v) D(+) glucose (Sigma) and 7% (w/v) D(-) fructose (Univar). Selected inductions were also supplemented with 50 μM (+) ABA (Valent Biosciences) and 50 μM norflurazon (a kind gift from Syngenta Crop Protection, Sydney, Australia). The Petri dishes containing the berries were placed in the dark for 24 hours. The berries were then washed with autoclaved tap water 3 times, blotted dry and immediately frozen in liquid nitrogen.

Grape suspension cell cultures

The initial Cabernet Sauvignon grape petiole suspension cultures were obtained from Dr C. Davies (CSIRO, Adelaide). The *Vitis vinifera* cv. Riesling cultures were initiated from callus culture from pre-veraison berries harvested from the Coombe vineyard, Waite Campus, The University of Adelaide. The cells were grown in grape Cormier media, supplemented with 30 g/L sucrose (Sigma).

The grape berry cell suspension was maintained at 25°C on an orbital shaker (100 rpm) by weekly subculture in Grape Cormier liquid media adjusted to pH 5.8 using 1.0 M potassium hydroxide or 1.0 M hydrochloric acid supplemented with 30 g/L sucrose. During subculture the grape suspension cells were allowed to settle, washed carefully, and suspended again in fresh medium. Suspension cultures were contained in 125 mL or 250 mL autoclaved Erlenmeyer flasks to the volume of 30 mL and 50 mL respectively. The inductions were conducted in Cormier media (described above). Various osmolites, i.e. sucrose, mannitol, D(+) glucose and D(-) fructose, were added as required to give the final concentrations as stated in 4.2.1a. All flasks were covered with aluminium foil to prevent light ingress, and the cultures were incubated at 25°C on an orbital shaker (100 rpm) for 24 hours. To harvest the grape suspension cells they were transferred to a funnel lined with blotting paper, washed carefully with autoclaved tap water 3 times, blotted dry and immediately frozen in liquid nitrogen.

4.2.2 ABA measurement by Phenomenex strata-X column purification

Protocol given previously in 2.2.2b.

4.2.3 Oligodeoxyribonucleotides

Details given previously in 3.2.2.

4.2.4 Preparation of grape RNA

Protocol given previously in 3.3.14.

4.2.5 RNeasy purification of grape RNA

Protocol given previously in 3.3.15.

4.2.6 cDNA synthesis

Protocol given previously in 3.3.16.

4.2.7 Real-time PCR amplification

Protocol given previously in 3.3.18.

4.3 Results

In the 2002-2003 whole berry *in vitro* induction experiments, the berries used to initiate the experiments were harvested on the 23-01-2003 and used on the 24-01-2003 after overnight sterilization. These berries were still hard, showing no signs of colour and were considered to have not entered the ripening phase. The concentration of the induction solutions was calculated on a percentage weight per volume basis. The free ABA analysis of the berries induced for 48 hours showed an increase in free ABA concentration of approximately four-fold for the 3% and 12% sucrose and 12% mannitol inductions compared to the level in the Cormier media alone which was 152 ng ABA/g berry fresh weight (Fig. 4.1). The berries harvested at this stage for the developmental series had an average ABA concentration of 180 ng/g fresh weight (Fig. 2.2B), which was very similar to the *in vitro* induction 0% control berries (163 ng/g fresh weight, Fig. 4.1).

The berry inductions conducted using fruit harvested on 30-01-2003 were nine weeks post-flowering. Only hard green (i.e. immature) berries were selected for the induction experiment as some berries seemed to be softening and showing signs of entering the ripening phase. The berries induced with sucrose and mannitol showed greatly increased levels of free ABA compared to the control inductions (Fig. 4.2) similar to those seen for the previous week's samples (Fig. 4.1). The free ABA level in the berries stimulated with 3% sucrose was approximately ten fold higher than the control, in the 12% sucrose treatment, free ABA was 14-fold higher and in the 12% mannitol treatment it was 16-fold higher (Fig. 4.2). A similar experiment was conducted five days later, on the 4th of February. In this case the berries selected appeared to be in the early stages of ripening as they were beginning to soften and degreen. The ABA levels of the control fruit were already elevated and there seemed to be little stimulation arising from the application of either sucrose or mannitol (Fig. 4.3).

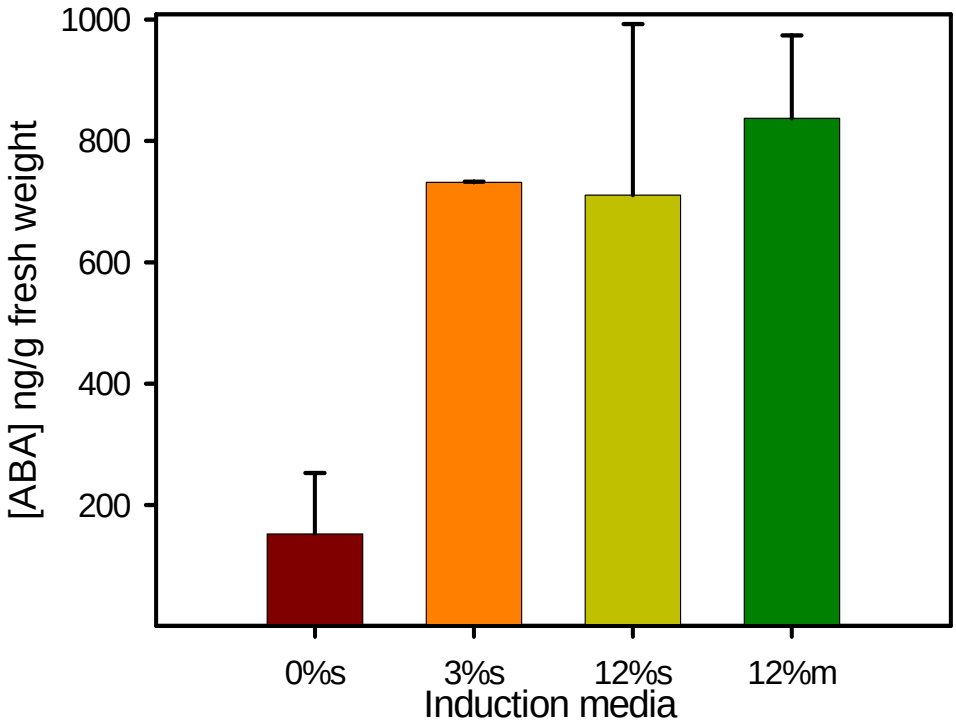


Figure 4.1: Comparison of free ABA levels measured during the 48 hour *in vitro* inductions of cv. Cabernet Sauvignon grape berries harvested on the 24-01-03, 0% is Cormier media alone, 3% is Cormier + 3% sucrose (w/v), 12%s is Cormier + 12% sucrose (w/v) and 12%m is Cormier + 12% mannitol (w/v).

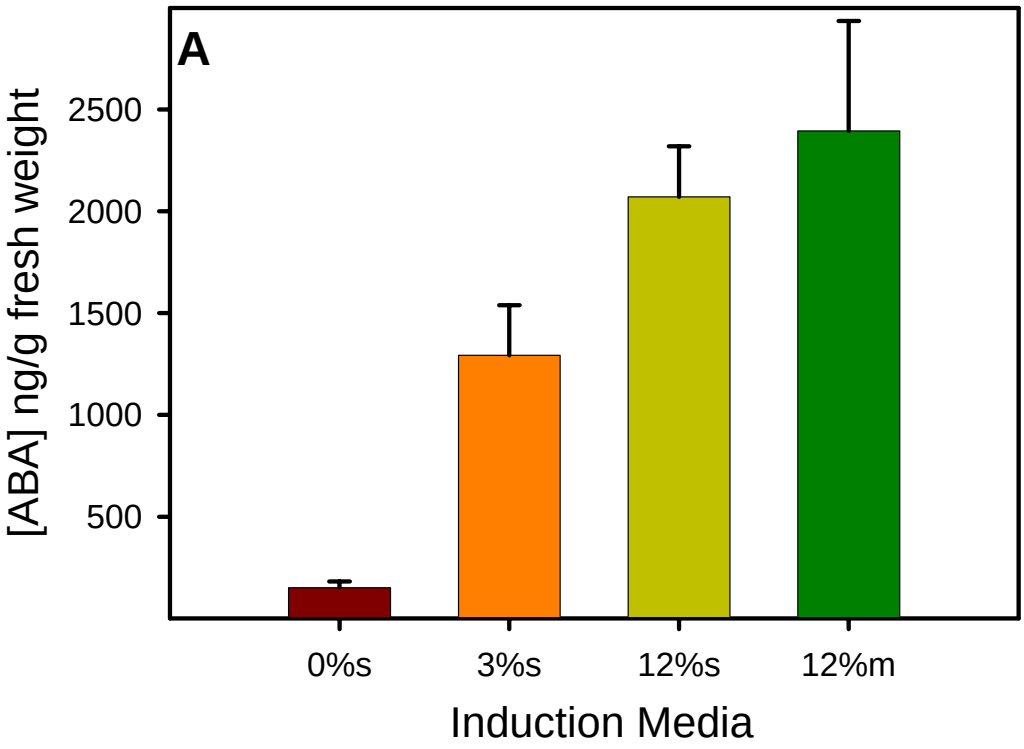


Figure 4.2: Comparison of free ABA levels measured during the 48 hour *in vitro* inductions of cv. Cabernet Sauvignon grape berries harvested on the 30-01-03, 0% is Cormier media alone, 3% is Cormier + 3% sucrose (w/v), 12%s is Cormier + 12% sucrose (w/v) and 12%m is Cormier + 12% mannitol (w/v).

The experiments above suggested that whole berries may be a useful system for studying ABA synthesis *in vitro*. To pursue this, an experiment was set up in 2004 in which the putative inducers of ABA synthesis sucrose, mannitol, glucose and fructose were tested for their effect on grapevine cell suspensions. These compounds were added to the media at equivalent osmolalities. The carotenoid biosynthesis pathway inhibitor norflurazon, and (+) ABA, at 50 μM, were also added in the presence of 12% (w/v) sucrose and the control solutions. For this series of experiments the berries were cut just below the pedicel and the cut surface was immersed in the liquid induction media to ensure maximum contact. The berries were harvested after 24 hours (as compared to 48 hours in the previous year's experiments) and the tissue analysed for free ABA. The berries were harvested on 09-01-2004 and were approximately eight weeks post-flowering, which was the last time point before the onset of veraison for this season. Selected samples from the various treatments i.e. the 0% and 12% sucrose inductions as well as the same inductions supplemented with norflurazon and ABA were also assayed for gene expression by qRT-PCR analysis (see below).

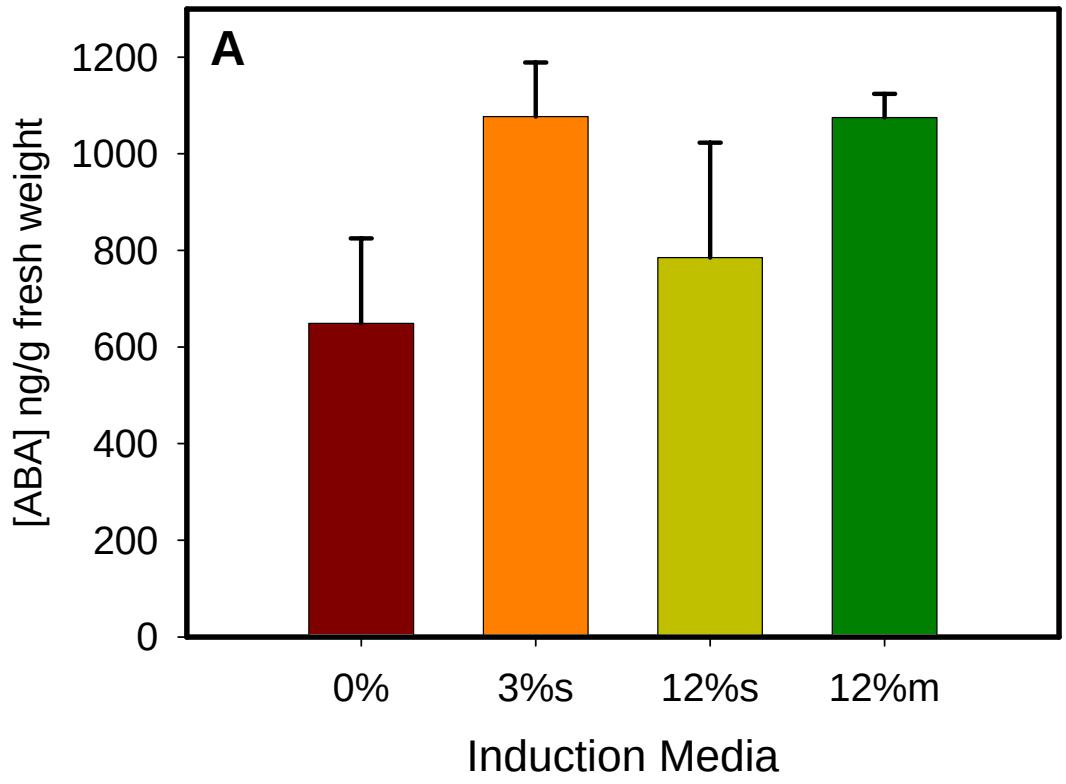


Figure 4.3: Comparison of free ABA levels measured during the 48 hour *in vitro* inductions of cv. Cabernet Sauvignon grape berries harvested on the 04-02-03, 0%s is Cormier media alone, 3%s is Cormier + 3% sucrose (w/v), 12%s is Cormier + 12% sucrose (w/v) and 12%m is Cormier + 12% mannitol (w/v).

The ABA analysis results show that in this experiment 12% sucrose caused an increase in the level of free ABA measured in the berries (Fig. 4.4). The level in the 12% sucrose treated fruit was approximately four-fold higher than in the control. The addition of norflurazon to the berries treated with 12% sucrose containing Cormier media decreased the measured level of free ABA to a level similar to that of the control berries (Fig. 4.4). None of the other treatments at similar osmolarity to the sucrose treatment resulted in the accumulation of elevated levels of ABA.

Gene expression analysis using qRT-PCR showed that the 12% sucrose + ABA treatment greatly increased the level of *VvNCED1* expression compared to all the other inductions (Fig 4.5A). *VvNCED2* expression however, was highest in the 12% sucrose treated sample, with the level reduced two-fold in the 12% sucrose + ABA treated fruit, and reduced even more, approximately ten fold (down to levels similar to the untreated control), in the 12% sucrose + norflurazon treated berries (Fig. 4.5B). The *VvZEP*, *VvASR* and *VvACO* expression patterns were similar to that shown for *VvNCED2*, with the highest relative expression in the 12% sucrose induction, with all other samples containing approximately ten fold lower levels (Figs. 4.5, 4.6).

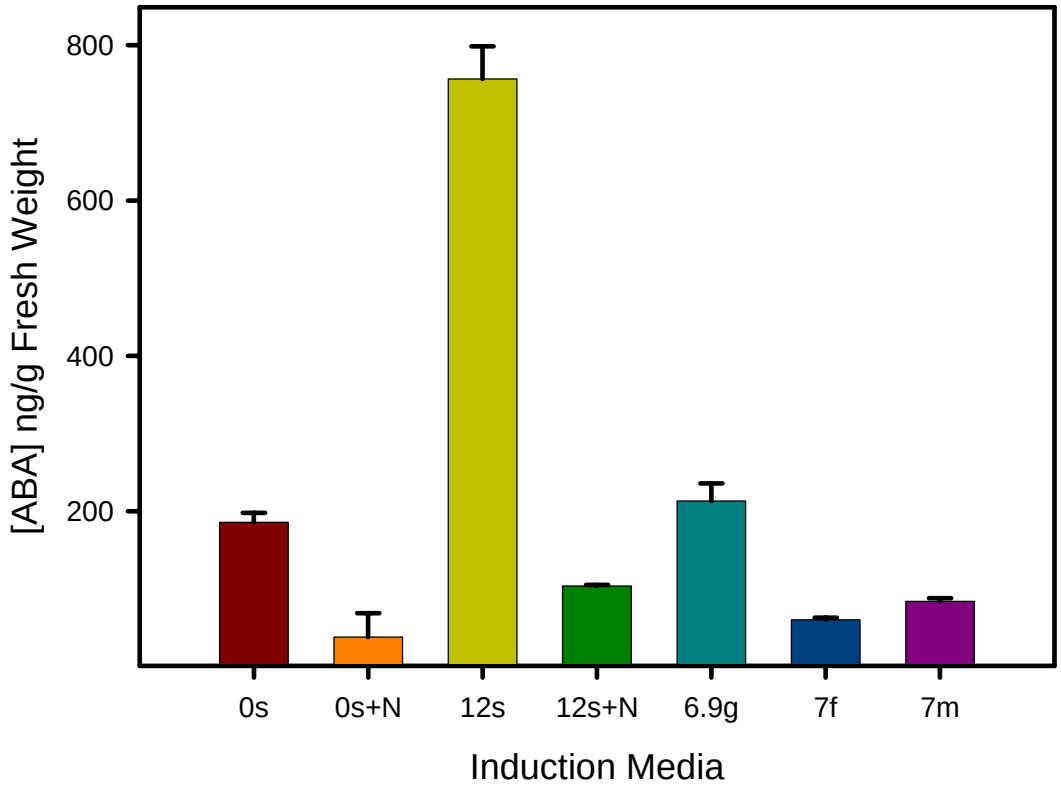


Figure 4.4: Comparison of free ABA levels measured during 24 hour *in vitro* inductions of cv. Cabernet Sauvignon grape berries harvested on the 09-01-04. 0% is Cormier media, +N is + Norflurazon (50 μM), 12s is Cormier + 12% sucrose (w/v), 12+N is Cormier + 12% sucrose (w/v) + Norflurazon, 6.9g is Cormier media + 6.9% glucose (w/v), 7f is Cormier media + 7% fructose, 7m is Cormier media + 7% mannitol.

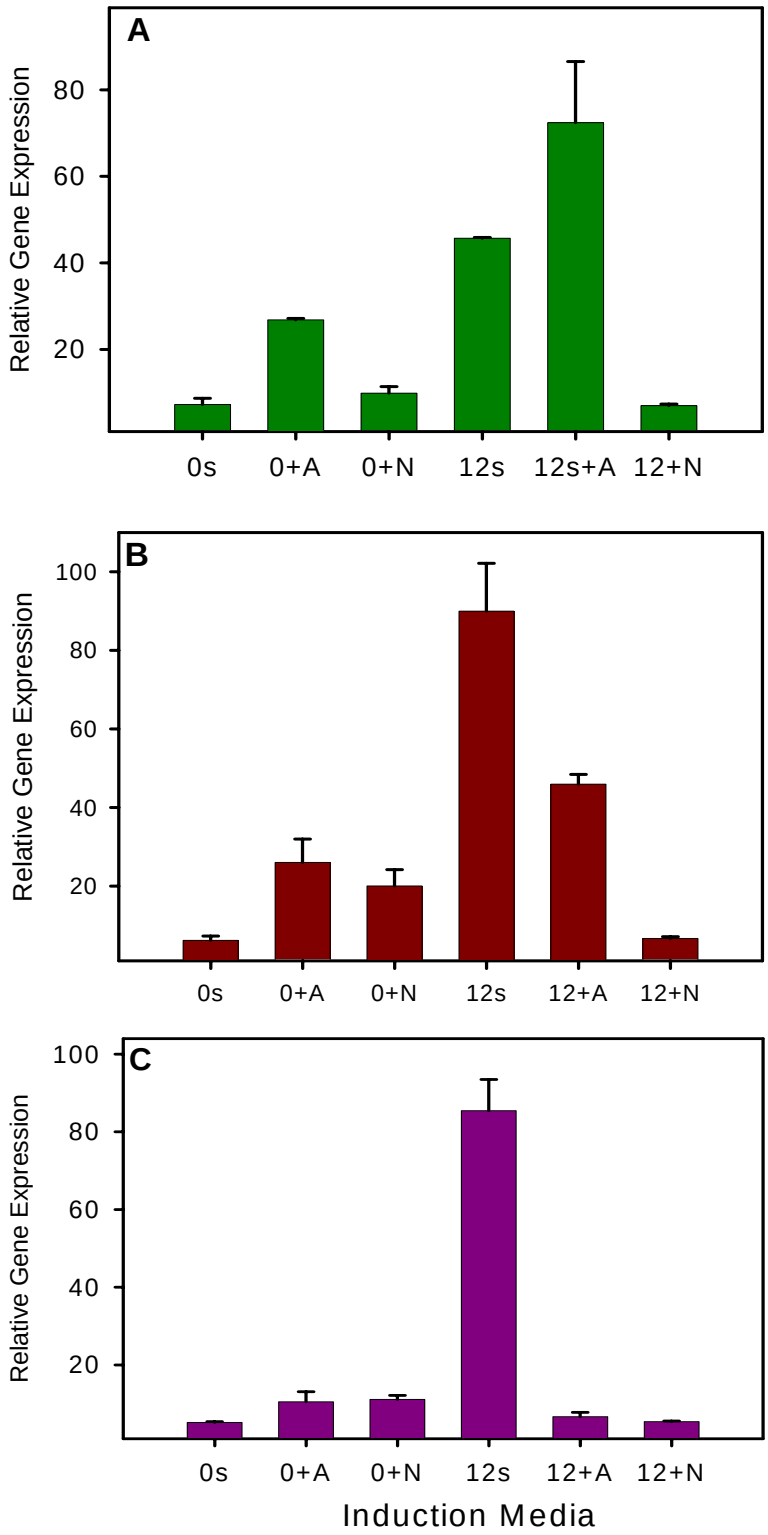


Figure 4.5: Relative gene expression of ripening associated genes measured during the 24 hour *in vitro* inductions of cv. Cabernet Sauvignon grape berries harvested on the 09-01-04: 0% is Cormier media, 0+A is Cormier + 50 μM ABA, 0+N is Cormier + 50 μM Norflurazon, 12s is Cormier + 12% sucrose (w/v), 12+A is Cormier + 12% sucrose (w/v) + 50 μM ABA 12+N is Cormier + 12% sucrose (w/v) + 50 μM Norflurazon. A) *VvNCED1* expression; B) *VvNCED2* expression; C) *VvZEP* expression.

The Cabernet Sauvignon suspension cell cultures used in the induction experiments with various osmolites were from two different sources of starting tissue, petioles and pre-veraison berries. The induction experiments were for 24 hours and a variety of osmolites, at the same osmolarity, were tested. The levels of free ABA in the petiole-derived suspension cells supplemented with sucrose, glucose and mannitol, but not fructose, were all increased by approximately four to five-fold compared to the 0% sucrose control (Fig. 4.7A). However, in the preveraison berry derived suspension cell inductions, only the 12% sucrose treatment caused an increase in free ABA levels of approximately eight-fold over the control inductions (Fig. 4.7B).

In addition to cells derived from Cabernet Sauvignon, Riesling preveraison berry derived suspension cells were also used in 24 hour induction experiments with various osmolites and the cells harvested for ABA analysis. The level of free ABA was increased approximately six-fold when the cells were induced with glucose and approximately 12-fold when induced with sucrose over the 0% sucrose control (Fig. 4.8).

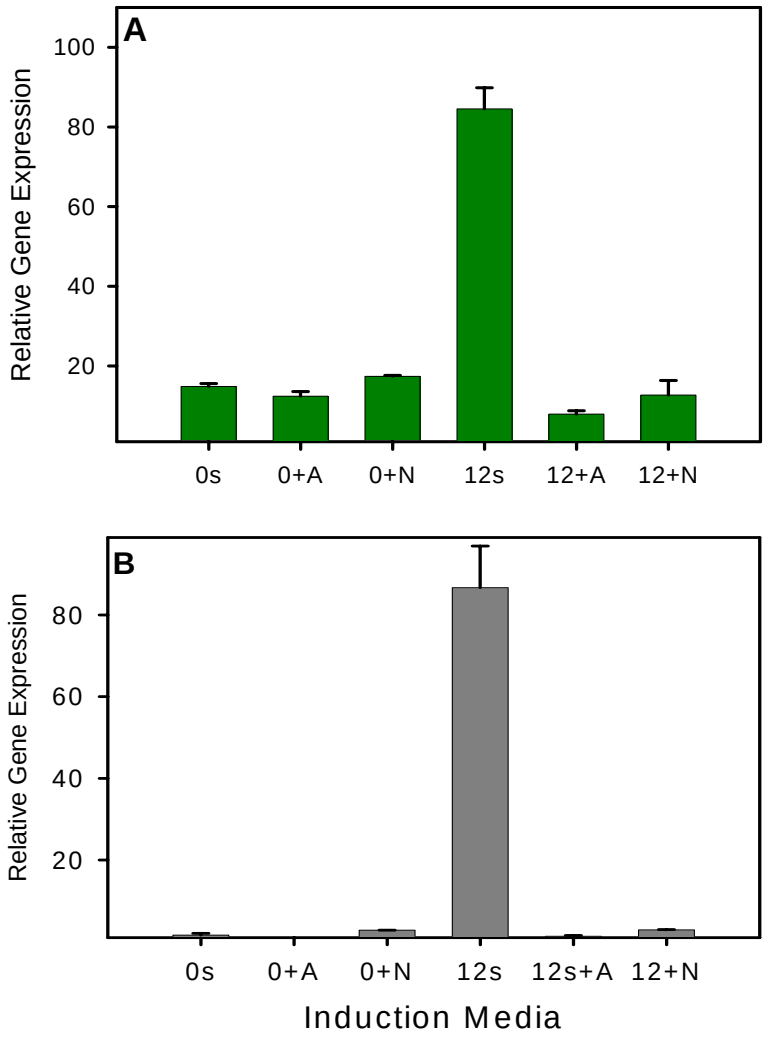


Figure 4.6: Relative gene expression of ripening associated genes measured during the 24 hour *in vitro* inductions of cv. Cabernet Sauvignon grape berries harvested on the 09-01-04; 0% is Cormier media, 0+A is Cormier + 50 μM ABA, 0+N is Cormier + 50 μM Norflurazon, 12s is Cormier + 12% sucrose (w/v), 12+A is Cormier + 12% sucrose (w/v) + 50 μM ABA 12+N is Cormier + 12% sucrose (w/v) + 50 μM Norflurazon. A) *VvASR* expression; B) *VvACO* expression

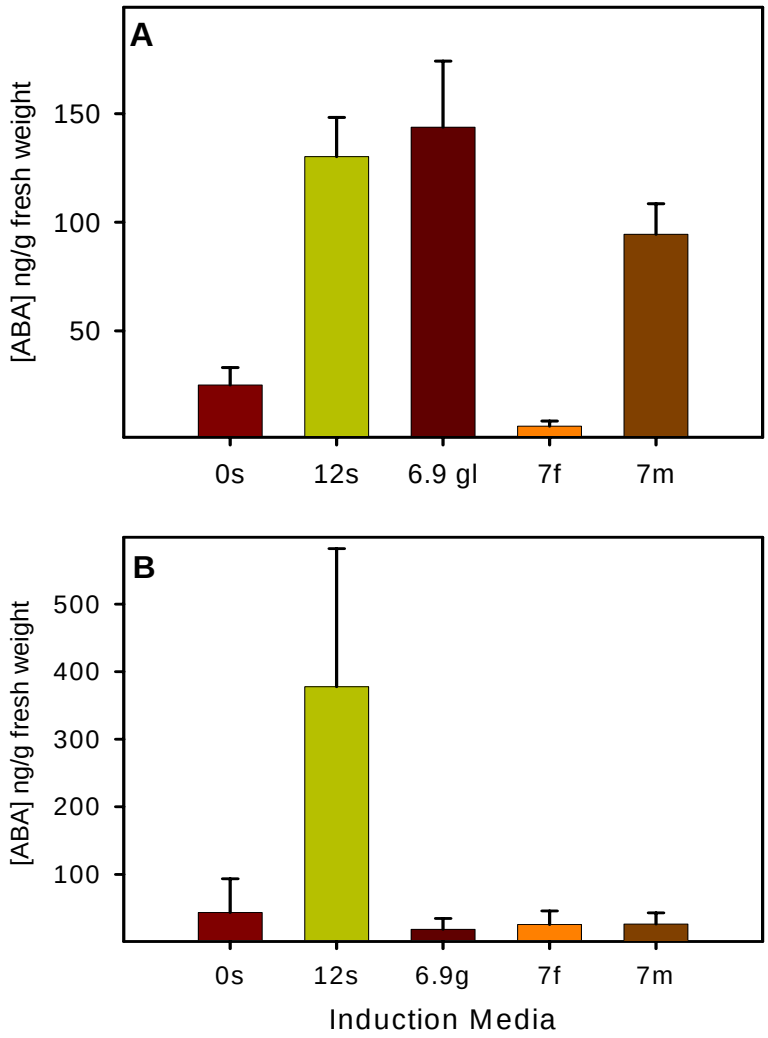


Figure 4.7: Comparison of free ABA levels in Cabernet Sauvignon suspension cell cultures induced with various osmolites for 24 hours; 0% is Cormier media, 12s is Cormier + 12% sucrose (w/v), 6.9g is Cormier media + 6.9% glucose (w/v), 7f is Cormier media + 7% fructose, 7m is Cormier media + 7% mannitol: Cabernet Sauvignon petiole suspension cell inductions; B) Cabernet Sauvignon berry suspension cell inductions.

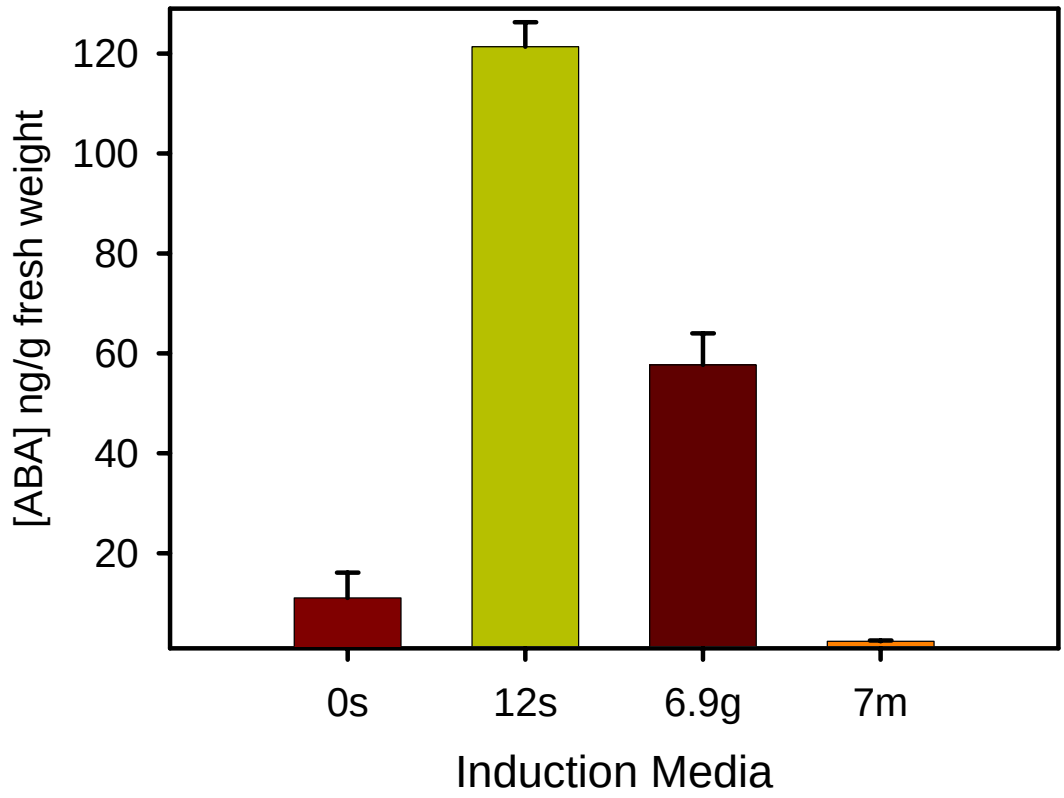


Figure 4.8: Comparison of free ABA levels in Riesling berry suspension cell cultures induced with various osmolites for 4 hours; 0s is Cormier media, 12s is Cormier + 12% sucrose (w/v), 6.9g is Cormier media + 6.9% glucose (w/v), 7m is Cormier media + 7% mannitol.

4.4 Discussion

The process of ripening in grapes is characterised by a coordinated change in transcriptional control of gene expression (Davies and Robinson, 2000; Waters *et al.,* 2005). As in other well studied fruit it seems likely that this change in gene expression is coordinated through the action of hormones. In grape berries a role for ABA in ripening seems likely due to the rapid increase in ABA levels which occurs at ripening initiation (Fig. 2.2, Davies *et al*., 1997). The timing of the increase in ABA levels at veraison correlates closely with the increase in sugar concentration and anthocyanins. It might be expected from this that ABA may either advance the timing of ripening or enhance the rate of ripening. Although ABA is likely to affect ripening its relative contribution to this process is further complicated by recent evidence suggesting that ethylene (Chervin *et al*., 2004) and brassinosteroids (Symons *et al.,* 2006) may also participate in the control of ripening. In contrast, the application of an auxin-like compound, benzothiazole-2-oxyacetic acid, to Shiraz grapes just prior to veraison resulted in both berry ripening and the increase in ABA levels being delayed by several weeks (Davies *et al*., 1997) which suggests that auxins can delay ripening. Studies using *Arabidopsis* ABA biosynthesis mutant plants provide direct molecular evidence for extensive glucose control of the genes involved in ABA biosynthesis and signalling (Cheng *et al.,* 2002; Eckardt, 2002). This suggests that the control of berry ABA levels, which influence ripening, might also be affected by sugar levels. The *in vitro* induction experiments described in this chapter were an attempt to further elucidate the relationship between sugars and ABA in the control of grape berry ripening.The experiments outlined in this chapter used *in vitro* techniques to study the synthesis of ABA and its role in grape berry ripening in more detail. This approach was attractive as such systems are more easily manipulated than whole grapevines. In many other species transgenic plants have been used to dissect the synthesis and action of hormones during development. This option is not currently feasible in grapevine within the time scale of a PhD project due to the laborious nature of transformation, the cost and the long time span for grapevine regeneration and fruit production.

Two model experimental systems were employed during this work. One of these, described above, involved the use of isolated, field grown berries *in vitro*. Grape suspension cell cultures were also used. It was thought that cell cultures may have the advantages of being available throughout the year, and allow more rapid and, because the

individual cells are bathed in the same solution, a more even, and instantaneous access of the solutes to the cells. The use of suspension cell cultures to investigate the alteration in berry physiology by the application of hormone or other chemical compounds is a common experimental technique. Tassoni *et al*. (2005) used *V. vinifera* Barbella suspension cells to determine the effect of jasmonic acid on resveratrol production. Curtain *et al*. (2003) also used cell cultures to determine the effect of jasmonic acid on the anthocyanin profile of *V. vinifera* cells.

The origin of the tissue used to establish suspension cell cultures appeared to have an influence on the physiological response in the induction experiments described here. Initially suspension cell cultures that had been established from *V. vinifera* Cabernet Sauvignon petioles were used. Analysis of ABA levels in the berries induced for 24 hours showed that the osmolites sucrose, glucose and mannitol (but not fructose) all caused an increase in free ABA levels compared to the control induction (Cormier media alone) (Fig. 4.7A). This was not the same as the induction experiment on the pre-veraison Cabernet Sauvignon berries in 2004, where it was observed that only 12% sucrose caused an increase in free ABA (Fig 4.4). When suspension cell cultures from Cabernet Sauvignon berries were used, the result was that only 12% sucrose potentiated an increase in free ABA, the other osmolites did not affect ABA levels (Fig. 4.7B). This result may indicate that although suspension cells appear to be dedifferentiated and totipotent, the original tissue used to initiate the callus bears some influence on the response of the suspension cells in experiments. It is also possible that cultures derived from the same tissues may reach different levels of dedifferentiation (C.M. Ford, pers. comm.). There may also be varietal differences as suspension cell cultures derived from Riesling berries responded to the various osmotica in a different manner than either of the Cabernet Sauvignon cultures (Fig. 4.8). As the whole berry induction experiments appeared to progress successfully and because of the limitations of the cell culture system this approach was not pursued further.

The whole berry inductions showed that the addition of either sucrose or mannitol (a non-metabolisable sugar alcohol) to the culture media could result in an increase in free ABA levels depending on the stage of berry development and the concentration of mannitol used. The 12% sucrose and 12% mannitol-treated berries shown in Fig. 4.1 showed a four to five fold increase in free ABA concentration compared to the control berries. The increase in free ABA concentration was even more pronounced in the berries sampled a

week later, the nine weeks post-flowering berries harvested on the 28-01-2003, which exhibited an increase in free ABA levels of approximately ten to 20-fold in the berries treated with 12% sucrose or 12% mannitol compared to the control berries (Fig. 4.2). In contrast to this, in the experiment conducted during the 2003-2004 season (Fig. 4.4), only 12% sucrose but not 7% mannitol induced a large increase in ABA levels. This difference in response may be due to the difference in mannitol concentration used in the two years experiments. For the 2002/2003 season's experiments 12% mannitol was used but for the 2003-2004 season the mannitol levels was adjusted (to 7%) to give solutions of equivalent osmolarity. The higher mannitol concentration in the first year's experiments may have induced a response merely because of the extreme solute level. The responses to the different solutes may be complex. It is possible that the response to sucrose and mannitol act through different pathways e.g. the response to sucrose is through a sucrose specific pathway (with or without an osmotic component) while the response to mannitol is activated by a pathway that responds to severe changes in osmotic conditions.

Apart from the adjustment in the mannitol levels used in the two season's experiments there were a number of other differences in experimental protocol which may have affected the results obtained, and which therefore, must be taken into account. These changes were instigated to try to improve the experimental system, building on the first year's experiments. During the 2003-2004 experiment the berries were at approximately the same stage of ripening as the berries used to produce the data presented in Fig. 4.1 but the inductions were only conducted for 24 hours rather than 48 hours. The shorter induction period was selected after noticing some degree of contamination from fungus and bacteria of the cultured berries during the previous years 48 hour inductions. The shorter duration of the 2003-2004 experiment may therefore, also contribute to the observed difference i.e. the induction of ABA accumulation by 12% mannitol in Fig. 4.1 (which occurred at a later stage of development also, Fig. 4.2, sampled at 48 hrs) that did not occur in the presence of 7% mannitol (Fig. 4.4, sampled at 24 hrs). The berries were also prepared in different ways. In the 2002-2003 season the berries were sliced lengthways which resulted in the seeds as well as the flesh being sliced through while in the second season (2003-2004), only a small portion of the berry near the brush was removed which may have been less stressful to the berry and less invasive. The other difference was that NAA was not used in the induction media during the second year's experiments. It has been shown previously auxins may retard ripening and ABA

accumulation in field experiments (Davies *et al.*, 1997, Inaba *et al*., 1976) and so it seemed prudent to delete NAA from the *in vitro* induction medium for fear of preventing any response to ABA.

There are a number of other considerations to be made when analysing the results of these experiments. The uptake and dispersal of the sugars and other substances of interest in the media is time dependent. In the fairly brief time span of these experiments the berries may not reach equilibrium with the induction media and the distribution of the solute within the berry may not be even. For example, the response to mannitol may be different to that for sucrose because of differential rates of movement of the solute into and through the berry tissue. It also means that because a berry has been placed on media containing 12% sucrose doesn't necessarily mean that the cells in the berry will be exposed to this concentration. The other point is that although mannitol is thought not to be metabolized in plants sucrose certainly is. It is highly likely that a considerable portion of the sucrose that is taken up is cleaved by invertases into its hexose components. Obviously, this would give rise to elevated fructose and glucose concentrations which might be at least partly responsible for the effects observed. The uptake of sucrose and subsequent cleavage may result in hexose being at different concentrations and in different locations which may explain why this treatment might act differently to treatment with hexoses. The cleavage of sucrose to its component monosaccharides also nearly doubles the osmolarity which is an additional effect to be taken into account.

The substantial increase in free ABA that was measured in the eight and nine weeks post-flowering *in vitro* berry inductions with the sucrose and mannitol treatment for 2003 was not replicated in the berries harvested at ten weeks post-flowering (Fig. 4.3). For these berries there was a less than two-fold increase in free ABA levels compared to the levels in 0% control inductions. The ABA levels detected in the 3%, 12% sucrose and 12% mannitol inductions were comparable to the field grown berries that were harvested on 04-02-2003 (the day the berries were harvested to initiate this induction experiment). This result indicates that while the pre-veraison berries (the eight and nine weeks post-flowering samples) are capable of increasing ABA levels in response to the osmolite induction, the berries at ten weeks post-flowering presumably already had sufficiently high basal levels of ABA and the addition of increasing amounts of osmolite to the Cormier media did not greatly affect the free ABA concentration.

An interaction between ABA and sugar uptake may also have been a factor in the experiments presented here. Kobashi *et al*. (2001) showed experimentally that the application of ABA to peach slices incubated *in vitro* in the presence of various osmolites caused an increased uptake of the osmolites sorbitol, glucose and fructose into the fruit discs. Other reports of ABA influencing the uptake of sugar uptake of apple, sugar beet and strawberry have been published (Beruter, 1983; Daie and Wyse, 1983; Ofosu-Anim and Yamaki, 1994). This raises the possibility that ABA may act during berry ripening to initiate the downloading and uptake of sugars. This could be a cyclic process as those solutes such as sucrose that can increase ABA accumulation may further enhance their own uptake.

The experiments described above where the addition of solutes to isolated berries induced ABA accumulation confirm that pre-veraison grape berries have the potential to produce free ABA, either by the *de novo* synthesis of ABA, or by the conversion of a bound form to free ABA. This suggests that the ABA detected in the harvested berries from the field grown vines presented in Chapter 2 may have been produced within the berries rather than being imported from the vegetative tissues, in particular from the roots of the grapevine.

In the 2004 whole berry *in vitro* induction experiment (Fig. 4.4), berries were incubated with and without sucrose with and without the carotenoid biosynthesis inhibitor norflurazon. Experiments using norflurazon to treat whole plants were not considered feasible due to norflurazon's action as a highly mobile herbicide. Norflurazon is used commercially as a broad-spectrum pyridazinone herbicide as it has been shown to inhibit phytoene desaturase, a key enzyme in the biosynthesis of carotenoids (Chamovitz *et al.,* 1991). As Figure 4.4 shows, only the berries cultured in the presence of 12% sucrose have an increased level of free ABA compared to the 0% control berries. When norflurazon was co-incubated with 12% sucrose, the level of free ABA was lower than the 0% sucrose control berries. This indicates that the observed increase in ABA levels was due to *de nova* synthesis rather than release of ABA from a bound form. The qReal-Time PCR data for this experiment showed that the 12% sucrose treated berries had high relative levels of *VvNCED2* and *VvZEP* expression in comparison to all the other inductions, and that this increase did not occur in the 12% suc + norflurazon treated berries (Fig. 4.5). These results when analysed in tandem suggest that the increase in free ABA measured in preveraison berries induced with 12% sucrose was due to the *de novo* synthesis of ABA, and this

synthesis required the induction of ABA biosynthesis gene expression i.e. an increase in the expression of *VvNCED2* and *VvZEP*.

The lack of induction of *VvNCED's* and *VvZEP* transcript accumulation in 12% sucrose + norflurazon-treated berries may be due to a lack of substrate being present as the ABA pathway is inhibited further upstream at the step catalysed by phytoene desaturase. It would seem that substrate levels for ZEP (and hence NCED) rapidly become limiting as if they existed in large amounts norflurazon would not have been effective in depressing the levels of ABA upon induction with sucrose.

Interestingly, in this experiment as well as the correlation between ABA biosynthesis gene expression (*VvNCED2* and *VvZEP*) and ABA levels, there was also a good correlation with the expression of two genes expressed during normal berry development, *VvACO* and *VvASR* (Fig. 4.6). The expression of these genes seems to be under quite sophisticated control. Both *VvACO* and *VvASR* expression was elevated by 12% sucrose only (Fig. 4.6). We know that ABA accumulation is increased by this treatment (Fig. 4.4). It also appears that ABA synthesis is required for this induction, as norflurazon inhibits the increase in *VvACO* and *VvASR* transcript levels even in the presence of sucrose (Fig. 4.6). In addition, exogenous ABA also inhibits this induction despite the presence of sucrose. One way of interpreting this is that while ABA (induced by sucrose treatment) stimulates the expression of these genes at lower levels, at higher levels (when added exogenously as in this case) it acts to inhibit gene expression. There is some support for ABA stimulating *ASR* gene expression as it is generally more highly expressed during normal berry development during the period when ABA (and sugar) levels are higher (compare Figs. 2.2; 2.3, 3.2A,C; 3.4A,C; 3.8A,C). Note that we might expect some of the sucrose presented to the berries via the media may be converted to hexoses via endogenous invertases. Also, ASRs are induced by ABA, stress, and ripening in tomato (Iusem *et al*., 1993; Amitai-Zeigerson *et al*., 1994; Rossi and Iusem, 1994). In contrast, *VvACO* is consistently expressed preveraison during normal berry development with a pattern that doesn't relate to the pattern of sugar (or ABA) accumulation (Figs. 3.2C; 3.4C; 3.8C). It could be that ethylene is being made as part of a stress response in the berries treated with 12% sucrose and that the increase in *VvACO* expression is coincident with, but not directly related to, the increase in ABA.

Although these results clearly need further investigation they do suggest a link between sugars, ABA and ethylene. Given that ABA (this work) and ethylene levels (Chervin *et al*., 2004) rise at about the time of ripening when sugar accumulation is also occurring it seems significant that an interaction between these three has been observed in the whole berry induction experiments.

These experiments do not identify the mechanism by which ABA levels increase during normal berry development, it may be that the increase in ABA levels at around veraison (Fig. 2.2) is developmentally controlled. However, these results raise the possibility that it could arise as a response to the presence of sucrose (or its metabolites) and that there may be an osmotic component to this response. These *in vitro* data are supported in part by the observation by Leon and Sheen (2003) that in the presence of high-glucose levels, *Arabidopsis* seedlings contain higher endogenous levels of ABA, and that an increase in osmolarity in the culture media causes an increase in the free ABA measured in plant tissue.

The interaction between sugar accumulation and ABA has been noted in other plant species. The mutant *gin1* identified by Zhou *et al*. (1998) was discovered in *Arabidopsis* germination screens where high levels of glucose did not arrest seedling development as observed in wild-type seedlings. The *gin1* mutant was determined to be allelic to the *Arabidopsis* mutant *aba2*, which is defective in ABA biosynthesis (Leon-Kloosterziel *et al*., 1996) and encodes a short-chain dehydrogenase (Gonzalez-Guzman *et al*., 2002). In *Arabidopsis* seedlings, the application of exogenous glucose caused an increase in the level of ABA (Arenas-Huertero *et al.,* 2000; Cheng *et al.,* 2002). The application of exogenous glucose to germinating *Arabidopsis* seeds retarded the rate at which endogenous ABA levels declined (Price *et al.,* 2003).

Many biosynthesis pathways are regulated by their end products. Cheng *et al*. (2002) showed that *ZEP* and *NCED* genes were induced by ABA in *Arabidopsis* of certain genetic backgrounds. For example, *AtNCED3* expression was upregulated by ABA in the Landsberg background. This observation strongly suggests a positive regulation of its own synthesis by ABA. ABA biosynthesis gene expression also appears to be regulated by substrate availability in grape berries. The rather complex pattern of ABA regulation may be a reflection of its importance during plant development.

Catalysis of ABA can also be induced by elevated ABA levels. Cutler and Krochko (1999) suggested that ABA can activate catabolic enzymes such as 8'-hydroxlase which result in ABA breakdown. In *Arabidopsis* the activity of ABA 8'-hydroxylase was also stimulated by exogenous ABA (Uknes and Ho, 1984).

It would appear that grape berries may be capable of regulating the level of ABA in response to changes in osmotic potential within the developing grape berry. Further investigation of the expression of other ABA biosynthesis and catabolism genes in grapes would provide a more thorough understanding of the ABA metabolism during the course of berry inductions. Although a study of changes in biosynthesis gene transcription can tell us much, the level of active enzyme is a key determinant in the accumulation of ABA. In tobacco and tomato plants the transcript levels of *ZEP* genes in leaves were not regulated by drought stress, but were found to be regulated diurnally with high transcript levels in the day, which may reflect regulation by circadian rhythm (Audran *et al*., 1998). However, despite the diurnal variation in transcript levels, no changes in the level of ZEP protein were found. The determination of the grape NCED1, NCED2 and ZEP protein levels would be useful in establishing whether the increase in mRNA expression also increases the level of the grape ABA biosynthesis enzymes as well.

These studies have shown that isolated berries and to a lesser extent cultured cells may be useful tools for further studies and a number of the experiments could be extended and investigated in more depth. It is obvious from the data presented in this chapter that the control of ABA metabolism in berries is a complex process. Berry response differs widely with developmental stage and the stimuli used. While this presents a complicated picture there are a number of interesting conclusions that can be drawn.

1. Isolated berries can clearly be induced to make their own ABA. This means that it is possible that the increase in ABA levels that occurs about the time of veraison in grape berries may be due, at least in part, to *in situ* synthesis rather than import from the rest of the plant.

2. Both low to moderate levels of sucrose and much higher levels of mannitol can induce the accumulation of ABA. Glucose, fructose and mannitol at levels osmotically equivalent to the level of sucrose did not induce ABA accumulation in isolated berries.

3. The sucrose-induced increase in ABA levels appears to be due to *de nova* synthesis as it is blocked by an inhibitor of carotenoid biosynthesis. This also indicates that for berries to be able to make their own ABA they need to be able to import or make carotenoids as otherwise the substrate levels for ZEP become limiting.

4. The increase in ABA that occurred during the berry induction experiments appears to be under transcriptional control as it is accompanied by increased levels of NCED and ZEP transcripts.

5. There is evidence of end-product negative feedback control of ABA biosynthesis gene transcription.

6. Cutured cells may respond differentially to exogenous sugars depending on the tissue and variety from which they were originally isolated.

7. There appears to be a link between sucrose, ABA and expression of the ethylene biosynthesis gene *VvACO* which may relate to berry ripening.

Chapter 5: ABA Field Experiments

5.1 Introduction

There have been many field studies investigating the effect of the application of exogenous plant growth regulators on developing grape berries. These studies have gone some way to describing the likely role of endogenous plant growth regulators during grape berry development. However, there are still many unanswered questions that need to be resolved. The application of some hormones can affect bunch and berry development without appearing to impact greatly on the timing or rate of ripening. For example, it is common commercial practice in the table grape industry to apply gibberellin to seedless cultivars to control both bunch size and berry size (Lynn *et al*., 1966). Gibberellin is thought to have a pivotal role in influencing berry and bunch size (Inaba *et al*., 1976) but has not been shown to influence the timing of veraison and therefore, berry ripeness. This is not surprising as the levels of active GAs are low and constant throughout all but the early stages of Cabernet Sauvignon berry development (Symons *et al*., 2006). Cytokinin application at full bloom can increase fruit size (Roubelakis and Kliewer, 1976), perhaps through an effect on cell division, and may also be important in fruit set but there is no evidence of any effect on ripening.

It is known that sugar accumulation in many fruits is affected by plant hormones, such as ABA (Giovannoni 2001; Ofosu-Anim *et al.,* 1996). For example, the application of exogenous ABA application to developing peach fruit (a climacteric fruit) was shown to result in an increase in the total sugar content accompanied by changes in sorbitol metabolism (Kobashi *et al.,* 1999). ABA application to developing peach fruit significantly increased fruit weight and total soluble solids in fruit flesh (Kobashi *et al*., 2001).

Treatment with some naturally occurring hormones and their synthetic analogues can delay the initiation of grape berry ripening. For example, the delaying of ripening by auxins was discussed in Chapter 1. Three hormones have now been demonstrated to advance ripening. Ethylene has been known for some time to be involved in the control of climacteric ripening in a range of fruit (Adams-Phillips *et al*., 2004). In tomato Beaulieu and Saltviet (1997) showed that ethanol could trigger ethylene production and also advance ripening. As discussed in Chapter 1 ethylene may either advance or delay veraison depending on the timing of application (Hale *et al*., 1970; Chervin *et al*., 2004).

Recently, Symons *et al*. (2006) showed that veraison could be advanced by the application of a brassinosteroid and delayed by an inhibitor of BR synthesis.

Recent experimental data has implicated another group of plant growth regulators, BRs, in influencing grape berry ripening. BR levels increase significantly at veraison in Cabernet Sauvignon berries and the exogenous application of epi-brassinolide, (a synthetic epimer of the naturally occurring BR brassinolide) to preveraison berries significantly promoted veraison (as measured by the increase in berry colour and Brix) (Symons *et al*., 2006). In contrast the application of a BR synthesis inhibitor, brassinazole, significantly delayed veraison suggesting that the presence of BRs may advance berry ripening.

It has been proposed that ABA may induce ripening in some non-climacteric fruits, including grape (Coombe, 1976). As detailed in Chapter 2 of this thesis, the levels of free ABA were shown to increase at veraison in all three seasons that were examined. This was in agreement with previous published data (e.g. Davies *et al*., 1997). There is a limited number of published accounts of the effect of exogenous ABA application on grape berry development and these have been detailed in Chapter 1. The consensus of these studies is that ABA can hasten veraison, or at least promote some aspects of ripening such as colour accumulation, when applied at an appropriate before the event. However, some of the experiments yielded somewhat conflicting results and as a range of cultivars were used it is difficult to compare some of these results.

Studying the effects of the manipulation of field grown grape berries by exogenous plant growth regulator application is difficult for a variety of reasons. The non-synchronous ripening of grape berries on the same bunch and the variation between bunches on the same vine and between different vines are considerations when designing sampling and experiment regimes. This lack of synchrony suggests that individual berries have some autonomy over the rate of timing of their development and that a generalized, plant wide, ripening signal is not the trigger for individual berry ripening (Kanellis *et al.*, 1993).

The uptake of exogenous hormones, and inhibitors of their action, is also an important consideration. The berry has a waxy cuticle and skin cells with thick cell walls which act as a protective barrier against fungal pathogens, reduce water loss due to transpiration and contribute to the control of gaseous exchanges (Giovannoni, 2001). This cuticle could inhibit the transport of the exogenously applied hormone into the berry. Some

investigators have used invasive techniques such as feeding the compound of interest via a wick threaded through the peduncle of the berry (Hale and Coombe, 1974) or by injecting the compound directly into the berry using a syringe (Kraeva *et al.,* 1998). Another reason for using the invasive approach is the high cost of various hormones such as ABA which limits the amounts available and therefore, precludes the extensive use of spray-based delivery techniques. The invasive methods described above are not suitable for large numbers of samples, and both result in physical damage to the developing berry which may influence development. More recent berry ripening manipulation experiments with the application of exogenous compounds have been done through spraying bunches with the hormone dissolved in a carrier solution (Symons *et al*., 2006; Jeong *et al*., 2004), and this is the approach that was taken in the experiments described in this chapter. This has been possible due to the greater availability of significant quantities of (+) ABA.

In this chapter the results from two field experiments conducted in successive years are described. (+) ABA was sprayed on preveraison Cabernet Sauvignon and the berries were sampled frequently and assayed for various developmental parameters such as berry weight, anthocyanin concentration and total soluble solids (TSS), as well as the free and total ABA content. The implications of this work for the role of ABA during grape berry ripening are discussed.

5.2 Materials

5.2.1 2004 ABA field experiment

The berries used in this study were sampled from *Vitis vinifera* L. cv Cabernet Sauvignon vines grown at the Nepenthe vineyard in Lenswood, South Australia. Spray and sampling dates for the experiment are presented in Table 5.1. Six adjacent vines in the same row were selected for the experiment. Twenty bunches from each vine were tagged for use. Ten of these bunches were used for the control treatment and ten for the ABA treatment. The distribution of bunches used was randomised for each vine. (+) ABA (99% w/w) was provided for the spray experiment by Valent BioSciences Corporation (Box Hill, Australia) and a 400 mg/L (+) ABA solution in 0.5% (v/v) Tween 20 was prepared on the day of spray application. The control solution was 0.5% (v/v) Tween 20. Bunches were sprayed with a hand-held sprayer in the late afternoon on the days presented in Table 5.1. Three treatments were applied to each bunch a week apart. Samples were taken at seven day intervals, five berries were taken randomly from each of the control and ABA sprayed

bunches and immediately frozen whole in liquid nitrogen. All samples were stored at -80^{o}C awaiting further analysis.

Table 5.1: 2004 ABA spray and sampling regime Nepenthe Lenswood Vineyard

Date	Spray	Sampling	Days Post First Spray
22/01/2004	Yes		0
29/01/2004	Yes	Yes	7
5/02/2004	Yes	Yes	14
12/02/2004		Yes	21
19/02/2004		Yes	28
26/02/2004		Yes	35
18/03/2004		Yes	56
26/03/2004		Yes	64

5.2.2 2005 ABA field experiment

The berries used in this study were sampled from *Vitis vinifera* L. cv Cabernet Sauvignon vines grown at the Nepenthe vineyard in Charleston, South Australia. Spray and sampling dates for the experiment are presented in Table 5.2. During the 2004/2005 season the experiment was set up with six vines on the same row. The vines were pruned to 20 bunches on each vine, and the bunches on each vine were sprayed with same treatment (rather than as a mix of control and ABA-treated bunches as described for the previous years experiment). On each day of spray treatments 1L of both 5 mg/L and 400 mg/L (+) ABA solutions in 0.5% (v/v) Tween 20 were prepared. First, the (+) ABA powder was dissolved in 1mL 70% ethanol which was then diluted to a final volume of 1L with tap water containing 0.5% (v/v) Tween 20. The control solution was 0.5% (v/v) Tween 20. The treatments were duplicated on separate vines. Bunches were sprayed with a hand-held sprayer in the late afternoon on the days presented in Table 5.2. Bunches were sprayed for a total of three sprays, each a week apart. Samples were taken on the days listed in Table 5.2, five berries were taken randomly from each of the control and the ABA sprayed bunches (at the two concentrations described above), the berries were first washed in 70% ethanol then rinsed twice in 0.5% (v/v) Tween 20 solution and then frozen whole in liquid nitrogen. Berries for the skin, flesh and seed analysis were frozen whole and the separate

skin, flesh and seed samples were obtained by peeling the frozen fruit and separating the seeds and flesh prior to extraction. All samples were stored at -80^{o}C awaiting further analysis.

Table 5.2: 2005 ABA spray and sampling regime Nepenthe Charleston Vineyard

Date	Spray	Sampling	Days Post First Spray
21/01/2005	Yes		0
28/01/2005	Yes	Yes	7
4/02/2005	Yes	Yes	14
11/02/2005		Yes	21
18/02/2005		Yes	28
25/02/2005		Yes	35
04/03/2005		No	49
11/03/2005		Yes	56
22/03/2005		Yes	67
06/04/2005		Yes	75

5.2.3 Berry development analysis

The total berry weight of the sampled berries was measured and the average berry weight from each time point and treatment calculated. The frozen berries were then ground using a coffee grinder, and this powered tissue was used in all future experiments. Sugar accumulation measured as total soluble solids (^{o}Brix), in the berry was used to determine progress of berry ripening and was determined with a refractometer (model 10430: Reichert, Vienna, Austria).

5.2.4 Abscisic acid analysis

Methods previously described in 2.2.2.b for free ABA analysis and 2.2.3 for bound ABA analysis.

5.2.5 Anthocyanin extraction

Methods previously described in 2.2.4.

5.3 Results

5.3.1 Treatment of Cabernet Sauvignon fruit with exogenous ABA: 2003/04 experiment

Visual observation of the untreated and ABA-treated fruit gave a general impression of the effects of ABA application. In the 2004 spray experiment bunches were photographed every three to four days and five berries were harvested at random from each bunch every week. The 400 mg/L (+) ABA-sprayed bunches showed some softening (data not shown) and anthocyanin development at 05-02-2004 which was 14 days post first spray (dpfs) (refer to Figure 5.1). The berries on the control bunches did not begin to colour until 10-02-2004 (19dpfs, Table 1) when approximately one to three berries on several of bunches showed some softening and anthocyanin accumulation (refer to Figure 5.2). At 28dpfs (19-02-2004, Table 1) the control bunches are approximately 50% coloured compared to the (+) ABA-sprayed bunches which are almost 100% coloured (Fig. 5.3).

The total soluble solids (^{o}Brix) and berry weight data for the 2004 spray experiment are presented in Figure 5.4A. Due to the limited sample size available, no replicates were done and so statistical analysis was not possible. The ABA-sprayed berries had begun accumulating soluble solids by 14 days post the first spray.

Figure 5.1: Nepenthe Lenswood Spray Experiment 05-02-2004 A) and B) Control bunches, C) and D) 400 mg/ml (+) ABA.



Figure 5.2: Nepenthe Lenswood Spray Experiment 10-02-2004 A) and B) Control bunches, C) and D) 400 mg/ml (+) ABA.



Figure 5.3: Nepenthe Lenswood Spray Experiment 19-02-2004 A) and B) Control bunches, C) and D) 400 mg/ml (+) ABA.



The levels of total soluble solids increased rapidly to 19.6 ^{o}Brix at 35dpfs after the first application of ABA and the (+) ABA-sprayed bunches reached a final Brix at harvest of 20.5. The control berries however, did not increase in soluble solids until after 21dpfs from the first spray. The control fruit reached a Brix of 19.4 at harvest. Also shown in Figure 5.4A are the average berry weights of the harvested berries. The (+) ABA-sprayed berries rapidly increased in size up until 56 days post the first spray when the berries had an average berry weight of 876 mg. The berries were lower in weight at the final harvest time point (64dpfs), which may indicate that they were dehydrated or may simply be due to sampling effects. The control berries had a delayed increase in average berry size and did not start increasing in weight until after 21dpfs. The final average weight for the control berries was 738 mg, less than that of the ABA-treated fruit. The A_{520} levels which are a measure of anthocyanins in the berry skins are shown in Figure 5.4B. In the (+) ABA-treated berries the A_{520} levels began to increase between 11 and 14 days after the first spray, which were the first sampling dates when the berries were observed to be beginning to colour, compared to the control berries which did not have a measurable increase in anthocyanins until between 18 days and 26 days after the first spray time point. The A_{520} of the harvested berries for both the ABA and control berries were very similar, 82.0/g fresh weight for the control berries and 81.6/g fresh weight.

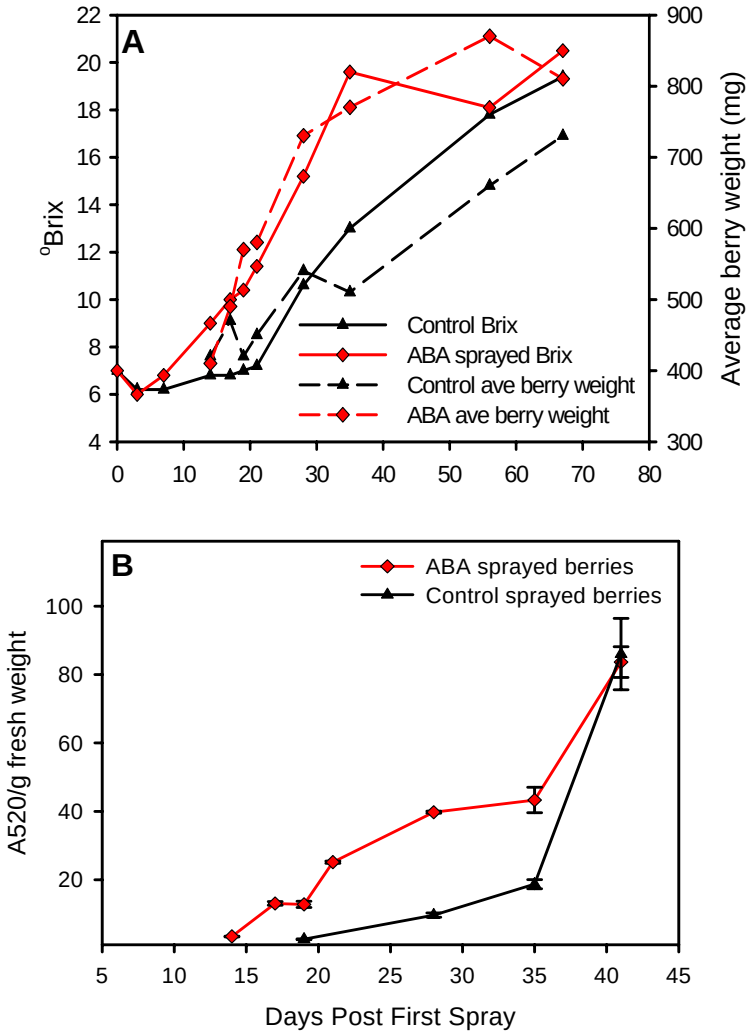


Figure 5.4: 2004 Nepenthe Lenswood Cabernet Sauvignon ABA Spray experiment; A) Berry Weight and Brix Data; B) Changes in absorbance at $A_{520.}$ Bars represent standard errors.

Figure 5.5A shows the free and total ABA levels for the ABA-sprayed berries compared to the control berries. ABA application caused a rise in free and total ABA levels soon after treatment. The increases measured one week after sprays at 0 and 7 days were approximately the same. The highest levels of both free and total ABA were at 10 days post the first spray i.e. three days after the second spray. At 10 days post the first spray the ABA-sprayed berries were found to have a total (hydrolysed) ABA concentration of 16.5 μg/g fresh weight at ten days post the first spray, compared to 13.0 μg/g fresh weight for free ABA sample in the same sample. The bound ABA for this sample was calculated to be 3.5 μg/g fresh weight, which is shown in Figure 5.5B. The control berries had ABA levels much lower than the ABA-sprayed berries and due to the very high levels measured in the ABA-sprayed berries, the increase in free ABA in the control berries after veraison is not obvious on the graph. The third ABA spray did not increase levels as much as the previous two and ABA levels had declined to near controls levels by the end of sampling.

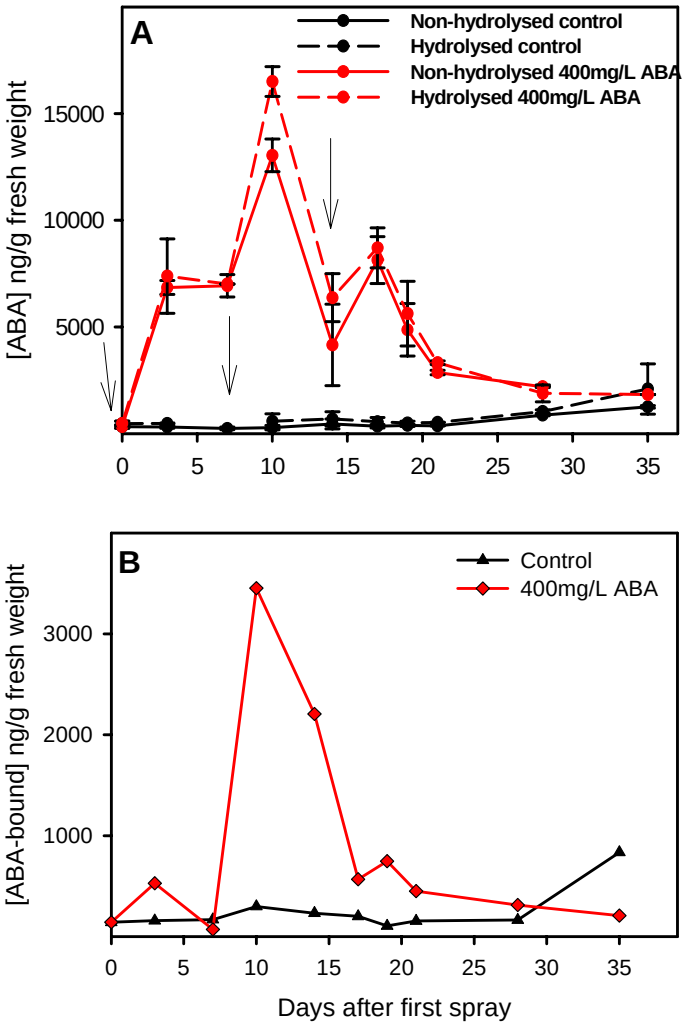


Figure 5.5: 2004 Nepenthe Lenswood Cabernet Sauvignon ABA Spray experiment; A) Free ABA and Hydrolysed ABA analysis; B) Bound ABA Bars represent standard errors. Arrows represent spray applications.

The amount of bound ABA was calculated by deducting the amount of free ABA from the amount of total (hydrolysed) ABA (Fig. 5.5B). As a result of calculating the bound ABA in this way is plotted it appears that there is a large increase in bound ABA in the 10 days post first spray sample. However, this is probably a misconception arising from the method of calculation where a large number is deducted from another large number to give a small residual value.

5.3.2 Treatment of Cabernet Sauvignon fruit with exogenous ABA: 2004/05 experiment

The 2005 (+) ABA spray experiment was carried out on replicate vines and the photographs shown in Figures 5.6 to 5.8 are representative for each of the treatments. The photographs in Figure 5.6 were taken two weeks post the first spray, and the 400 mg/L (+) ABA-sprayed bunches had approximately 40% coloured berries. The control and 5 mg/L (+) ABA-sprayed bunches had only one or two coloured berries per bunch at the same stage. The photographs in Figure 5.7 taken 11/02/2005 show that the 400 mg/L (+) ABA-sprayed bunches were almost completely coloured compared to the control and 5 mg/L (+) ABA-sprayed bunches which had mostly hard, green berries. By 28 days post the first spray nearly all the berries on the control and 5 mg/L (+) ABA-treated bunches were coloured (Fig. 5.8).

At each sampling time point, the number of coloured berries was counted and then berries were randomly harvested and removed for further analysis. At harvest, the number of berries remaining on each bunch was counted, and the percentage of coloured berries was calculated for each time point.

This data is shown in Figure 5.9A. Berries on the 400 mg/L (+) ABA-treated bunches were beginning to colour at seven days post the first spray and at 14 days 32% of the berries had some colour. By 21 days post the first spray the percentage of coloured berries was 96%. The 5 mg/L (+) ABA-sprayed and control berries did not begin colouring until between 14 and 21 days post the first spray but by 28 days post the first spray both treatments were almost 100% coloured (Fig. 5.9A).

Figure 5.6: Nepenthe Charleston Spray Experiment 04-02-2005 A) and B) Control bunches, C) and D) 5 mg/ml (+) ABA; E) and F) 400 mg/ml (+) ABA.

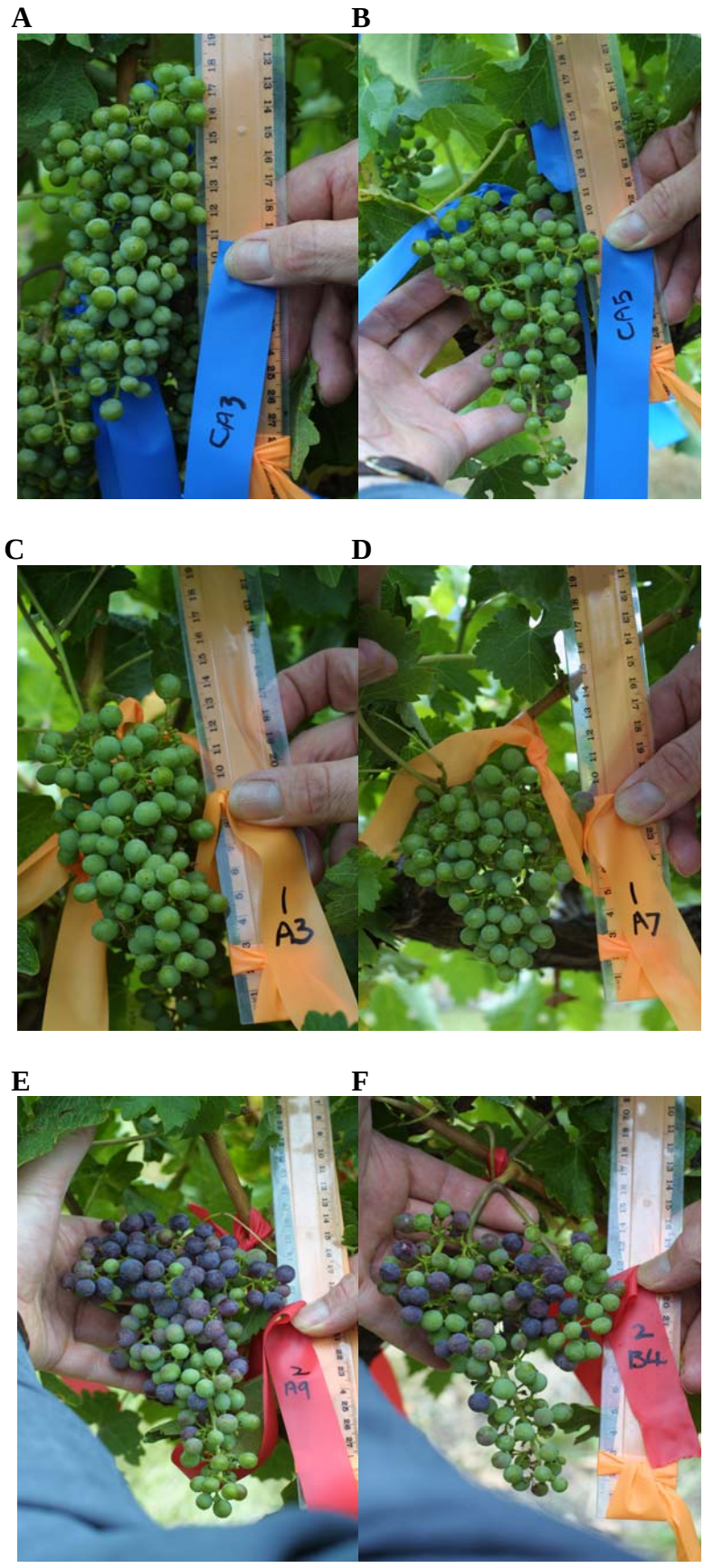


Figure 5.7 Nepenthe Spray Experiment 11-02-2005 A) and B) Control bunches, C) and D) 5mg/ml (+) ABA; E) and F) 400mg/ml (+) ABA.

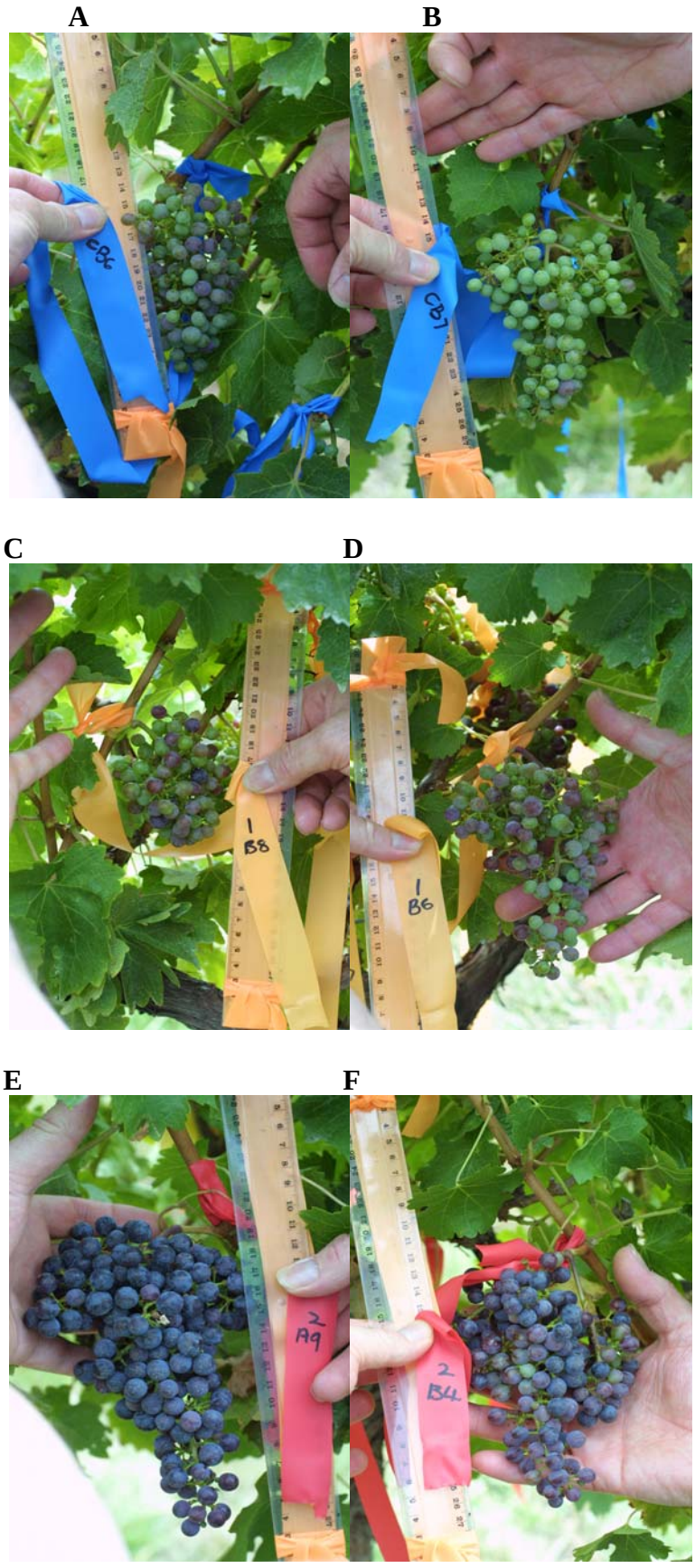
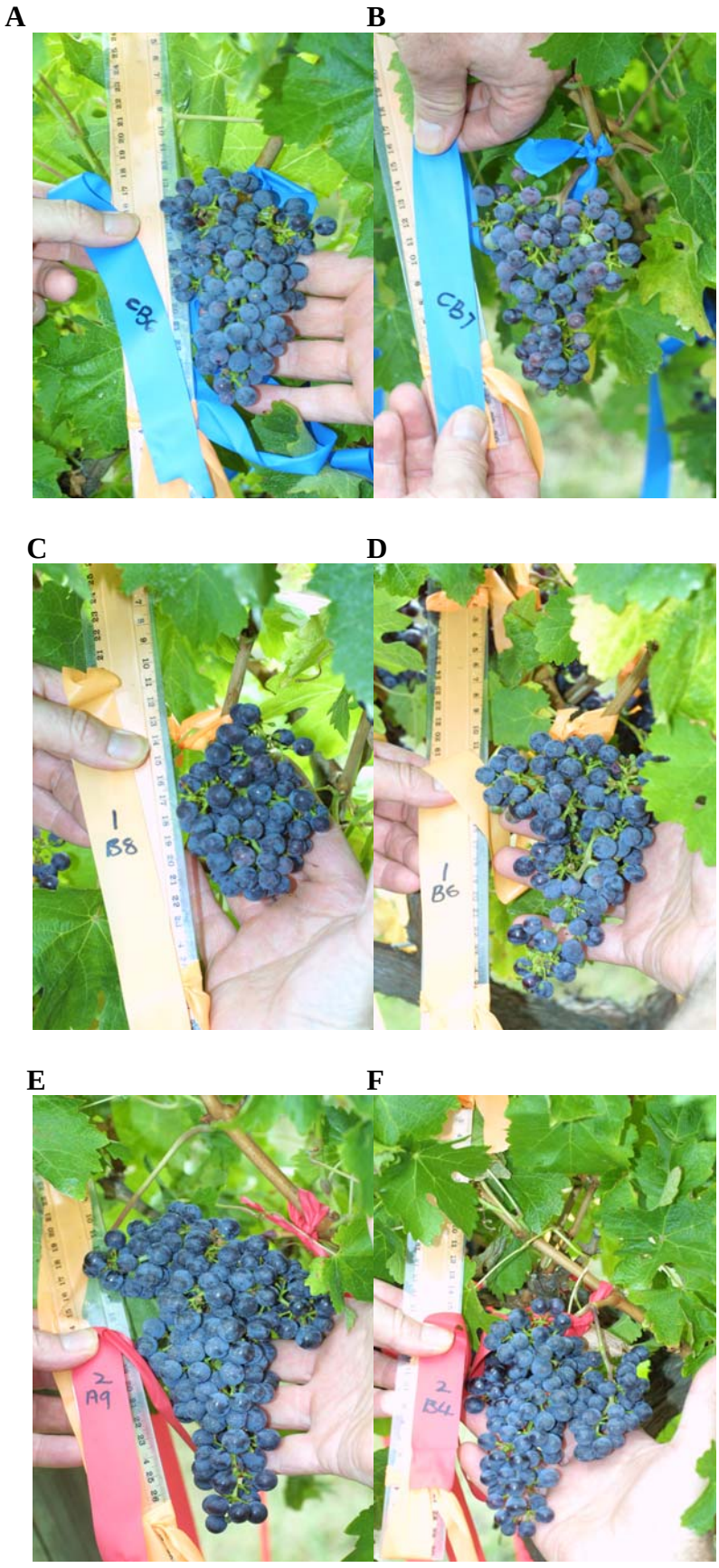


Figure 5.8 Nepenthe Spray Experiment 18-02-2005 A) and B) Control bunches, C) and D) 5mg/ml (+) ABA; E) and F) 400mg/ml (+) ABA.



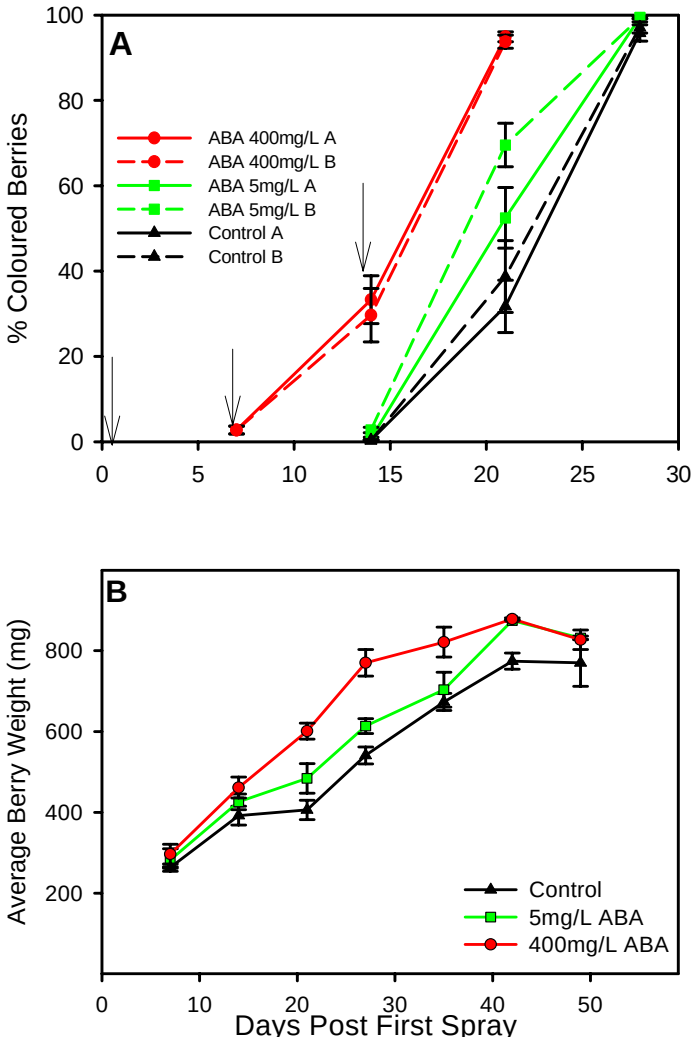


Figure 5.7: 2005 Nepenthe Charleston Cabernet Sauvignon ABA Spray experiment A), % Coloured berries; B) Berry Weight Data. Bars represent standard errors. Arrows represent spray applications.

The average berry weights for the harvested berries from each sampling time point are presented in Figure 5.9B. The 400 mg/L (+) ABA-sprayed berries had higher average berry weights for samples taken at 21, 28, 35 and 42 days post first spray than the control berries. The 5 mg/L (+) ABA-treated fruit had berry weights intermediate between these treatments (Fig. 5.9B). By harvest time the average berry weights for the three treatments were almost the same, approximately 800 mg.

Total soluble solids were measured (as degrees Brix) for the sampled berries (Fig. 5.10A). The 400 mg/L (+) ABA-treated berries had slightly higher Brix measurements for the 14 days post first spray time point through to the 36 days post first spray compared to the other two treatments. There seemed to be little if any difference in Brix levels between the control fruit and that treated with 5 mg/L ABA. The final three sampling time points had very similar Brix measurements for each of the three treatments and at harvest the Brix was approximately 25 degrees.

The A_{520} measurements of the fruit from the 2004/05 experiment are presented in Figure 5.10B. The 400 mg/L (+) ABA-treated berries had higher A_{520} values and hence higher anthocyanin levels than the control samples for much of the time course. The increase in anthocyanins seemed delayed in the 5 mg/L (+) ABA treated berries compared to those treated with the higher concentration of ABA and contained similar levels to those in the control fruit, except at the 49 days post spray sampling time point where the A_{520} absorbance was $16.04A_{520}$/g fresh weight for the 5 mg/L (+) ABA sample, compared to $10.9A_{520}$/g fresh weight for the control sample. The A_{520} levels in the final samples were similar for all treatments.

ABA levels were analysed for the 2005 ABA spray experiment. Figure 5.11A shows the free and hydrolysed (total) ABA analysis for the 400 mg/L (+) ABA-sprayed berries compared to the control berries for the early sampling time points, from seven to 28 days post the first spray application. The amount of bound ABA deduced from the difference between total and free ABA levels is not shown due to the difficulties in accurately determining it by this method (see also Fig. 5.5B). Similar to the data presented for the 2004 spray experiment (Figs. 5.5A,B) the amount of free and hydrolysed ABA is much higher in the 400 mg/L (+) ABA-sprayed berries than the control berries sampled at equivalent time points.

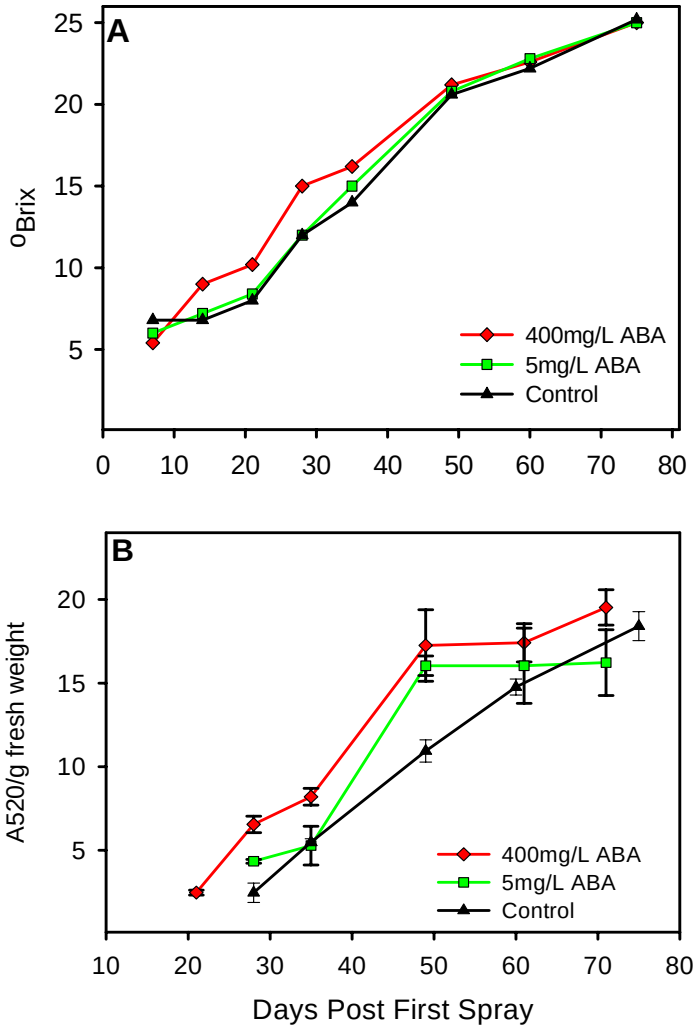


Figure 5.8: 2005 Nepenthe Charleston Cabernet Sauvignon ABA Spray experiment; A) Total soluble solids (^{o}Brix); B) Changes in absorbance at A_{520}. Bars represent standard errors.

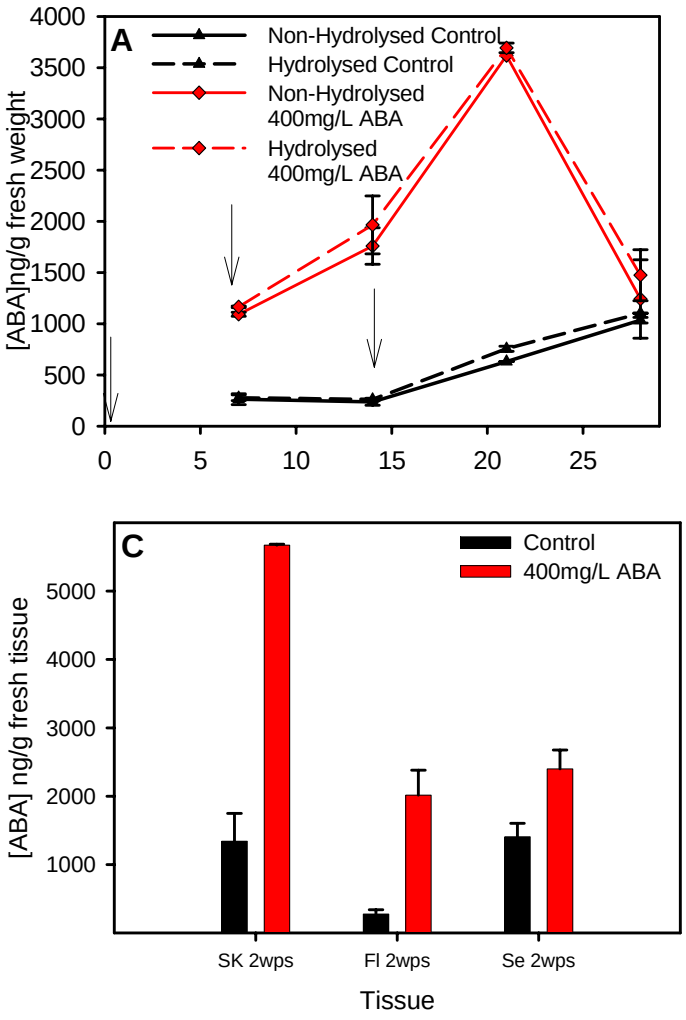


Figure 5.9: 2005 Nepenthe Charleston Cabernet Sauvignon ABA Spray experiment; A) Free ABA and Hydrolysed ABA analysis; B) ABA in skin, flesh and seed tissues at two week post first spray. Bars represent standard errors. Arrows represent spray applications.

The peak ABA concentration in the sprayed berries was calculated to be 3.62 μg/g fresh weight in the berries sampled 21 days post the first spray. The much lower levels of ABA detected in this experiment compared to the previous years work are probably due to the washing of residual ABA from berries during sampling (see Materials and Methods). Much of the ABA is likely to be located on the surface of the berry but not absorbed into the berry and may be washed off by the ethanol and detergent treatment used. The control berries by comparison had a peak ABA concentration of 1034 ng/g fresh weight in the 28 days post first spray sampling time point. As for the previous year the levels of both total and free ABA were similar in the ABA-treated and control fruit in the last sample taken (Fig. 5.11A).

Berries sampled two weeks after the first spray application were separated into the skin, flesh and seed components for the control berry and 400 mg/L (+) ABA-sprayed bunches. These samples were analysed for free ABA concentration (Fig. 5.11B). In control berries the highest ABA levels were in the skin with the lowest in the flesh. In ABA treated fruit the ABA concentration was again highest in the skin but the levels in the flesh and seed were roughly equal. All ABA-treated samples had higher levels of ABA than did the corresponding control samples. Not only was there considerably more ABA present in the skins of ABA-treated fruit (ABA, 5672 ng/g fresh wt.; Control, 1342 ng/g fresh wt.) but the levels in the flesh, and to a lesser degree in the seed, were also elevated.

5.4 Discussion

The application of (+) ABA to field grown preveraison Cabernet Sauvignon berries at 400 mg/L hastened the onset of veraison as measured by an increase in berry colour, weight and TSS. The fact that ABA treatment enhances the progress of a number of ripening-associated processes (colour and sugar accumulation, berry weight increase) indicates that its effect appears to be on ripening in general not just on one aspect. The results regarding the effects of ABA application to berries were similar for both field experiments described in this chapter despite being conducted in different vineyards in successive years and using slightly different experimental procedures. The application of (+) ABA at a concentration of 400 mg/L to preveraison bunches weekly for three weeks resulted in the hastening of anthocyanin accumulation of the berries compared to the control bunches. In tandem with the colouration of the ABA treated berries, there was also an increase in berry weight and

total soluble solids in the early sampling time points, when compared to the control bunches, and in 2005 compared to the 5 mg/L ABA sprayed bunches. The berry weight for the ABA-treated berries was higher than the control berries up to the final berry harvest time point when the ABA treated berries decreased in weight, possibly due to experimental variation as there were no visible signs of berry shrivelling.

The sprays commenced nearly three weeks preveraison for the 2004 and two weeks preveraison for the 2005 experiments, and as there were three spray applications in both years, this meant that the last application in both cases was one-two weeks postveraison. The sprays in both these experiments were applied well before harvest. The Brix, berry weight and anthocyanin levels in the treated berries were similar to those of the control fruit by harvest, possibly due to the sink strength of the ABA treated berries becoming less than the control berries as the soluble solid concentration of the ABA treated berries increased. This meant the control berries were stronger sinks and this may have increased the soluble solid accumulation in these less ripe berries, allowing them to reach the same Brix as the berry weight at the harvest date. The effect of ABA sprays later in berry development would be interesting to study, and whether the hastening of ripening could be maintained if further sprays were applied after the berries had begun to develop colour and soften. Spraying bunches after veraison had occurred, or indeed applying more frequent sprays, may have a different effect on berry development than those experiments described in this chapter.

The high levels of ABA measured in the ABA-sprayed berries from the 2004 experiment lead to the modification of the experimental protocol for the 2005 experiment, where after harvesting the sampled berries from each time point, the berries were washed in a 70% (v/v) ethanol solution, followed by a 0.5% (v/v) Tween + water solution to remove any ABA that was adhering to the outside of the berry prior to freezing. This berry washing removed much of the ABA which was apparently adhered to the outside of the berry, as can be noted from the differences in the peak levels of ABA measured in the two years (compare Figs. 5.5A and 5.11A). In 2004 the highest ABA concentration was in the ten days post first spray berries, and was 16.4 ng/g for the hydrolysed, compared to 3.6 ng/g fresh weight in the 21 days post first spray 400 mg/L ABA sampled in 2005. This substantial discrepancy indicates that little ABA is probably absorbed into the berry and also shows that ABA seems to be stable for a considerable period on the berry surface and

is not broken down quickly by light exposure. It suggests that absorption is a limiting factor effecting efficiency and that further experiments investigating different solutions for applying the ABA to the grape berry may be warranted.

Counting the number of coloured berries on each bunch is a useful, non-destructive method to determine the progression of berry ripening. This allowed us to count larger numbers of berries than we could sample from each bunch due to limitations on the number of bunches. It was important to ensure that we did not remove too many berries from each bunch throughout the time course of the experiment, as this could have altered the source sink relationship of the bunch and impacted on the progression of bunch ripening.

In the first years experiment each vine that was used for the spraying experiment had a combination of ABA sprayed bunches, control sprayed bunches and some untreated bunches. As the control sprayed and untreated bunches ripened later than the ABA treated fruit it might have been that they competed for photosynthate when they started to ripen and therefore slowed down the progress of the ABA-treated fruit. Initially, the ABA-treated fruit would have a competitive edge in regard to sugar accumulation as they commence accumulating sugar earlier than the other fruit. However, later in ripening the ABA-treated fruit have more stored sugar than the control fruit and therefore, may be less strong sinks. Under these circumstances one might expect the non-treated fruit to act as a stronger sink than the ABA-treated fruit and this may be the reason for the observed 'equalisation'. However, in the next years (2004/2005) experiment all bunches on the experimental vines were treated in the same manner, that is, on one vine all bunches were sprayed with the same treatment. The results for this year were similar with regard to the ABA- treated bunches and the control bunches all reaching similar ^{o}Brix, berry weight and anthocyanin accumulation as determined by A_{520} concentration by harvest time. To reduce crop load and to ensure that photosynthate wasn't a limiting factor in the 2004/2005 experiment the vines were pruned to 20 bunches. However, despite limiting bunch numbers per vine and treating all 20 bunches on each vine with the same reagent the ABA-treated fruit was similar, at harvest, to the other fruit in the physical and biochemical parameters measured. This may simply mean that there are physiological limits to the levels of various constituents and that although ABA-treated fruit may initiate

accumulation earlier they do not go beyond these limits. In the case of sugar storage, for example, this makes sense as eventually osmotic considerations must limit accumulation.

These results confirm experimental data published by Hale and Coombe (1974) where 1 mM (+/-) ABA was applied to Shiraz berries beginning one, or two to three weeks prior to veraison. The ABA was applied through a wick that was threaded through the peduncle of the bunch. The ABA was fed continuously for a week, and the berries were harvested 18 days after veraison of the water-treated control berries. The researchers showed, by counting the number of coloured berries on each bunch, that the effect of ABA application was more pronounced when applied approximately one week prior to veraison, compared to two to three weeks. The total soluble solids at the harvest time point also showed that the Brix was highest in bunches treated with ABA starting at one week preveraison compared to the control and other ABA treatment. They hypothesized that the berry is insensitive to applied ABA early in the lag phase, and this may be due to the concentration of ABA being required to remain low for a certain period before elevated concentrations can have an effect on the ripening process. It may also be that some inhibitory factor is present in pre-veraison fruit that needs to decrease in concentration before ripening can initiate. It is possible that the machinery required for ripening is not available earlier in development.

The results from the two years experiments described in this chapter show a similar trend, although the ABA application in the 2004 ABA experiment began approximately three weeks preveraison which Hale and Coombe suggested may be too early. Despite the differences in the experimental designs between the spray experiments described in this chapter and the ABA treatment done by Hale and Coombe (1974) both experiments indicate that the application of exogenous ABA to preveraison grape berries can induce ripening. Hale and Coombe applied ABA constantly for a week whereas in the experiments described here bunches were sprayed three times at weekly intervals. The ABA analysis data for the skin, flesh and seed from the 2005 experiment indicated that ABA was still present in the berries one week after the spray application at levels higher than those measured in the control berries, which potentially shows the ABA is not fully metabolized, or broken down by light and heat, after seven days. This indicates that the continuous application to the berry such as in the Hale and Coombe (1974) experiment may not be crucial. The spray application makes it difficult to calculate the total amount of

ABA applied to each berry and therefore the amount that is transported across the cuticle to the skin and flesh tissues, however, it is much less invasive to the grape berry than by application via a wick, and many more bunches were able to be treated thus providing a greater number of experimental data points.

The experimental results obtained in both years experiments indicated that the 400 mg/L (+) ABA sprayed berries had a higher concentration of anthocyanins in the early sampling time points when compared to the control berries, however, at harvest the two treatments had similar levels. This was different to the observations made by Kataoka *et al.* (1982). These researchers applied 1000ppm (+/-) ABA to *V. vinifera* cv. Kyoto in a 70% ethanol solution at veraison or two weeks post-veraison. The vines had been pruned to one bunch of 30 berries per shoot. The anthocyanin content of the treated berries at harvest (six weeks post veraison) was threefold higher in the ABA-treated berries than the controls. They did not find the ABA treatment had an effect on the soluble solids or the total acid content.

This result indicates that there maybe a synergistic relationship between the ABA and the ethanol that was used to dissolve the ABA. Chervin *et al*. (2004) showed that spraying ethanol at mid-veraison enhanced internal ethylene production which effected parameters related to Cabernet Sauvignon maturity, including levels of the polyphenol and organic acid pools. There also maybe a cultivar difference as Kyoto is a table grape variety and considerable variation may occur in response between cultivars. Chervin *et al.* (2005) published data showing that the spraying of 10% ethanol to Cabernet Sauvignon berries starting at four weeks prior to harvest resulted in increased anthocyanin accumulation, however, 20% ethanol decreased the accumulation. Kataoka *et al*. (1982) applied the ABA in 70% ethanol only twice, at veraison and two weeks after, compared to Chervin *et al*. (2005) who applied a single spray much later in berry development, at four weeks prior to harvest. The possible synergistic relationship between ABA and ethanol warrants further investigation and also demonstrates how factors such as the vehicle for applying the ABA to the grape berry is of critical importance. In the experiments described here the ABA was taken up in a small volume of ethanol which was then diluted in a large volume of Tween 20 solution. The final concentration of ethanol applied was 0.07 %. For this reason any putative synergistic interaction between ABA and ethanol would not have been noticed.

A more recent report by Jeong *et al*. (2004), showed the enhancement of various ripening-related processes through ABA application. Cabernet Sauvignon berries were sprayed at

veraison with 1000 mg/L ABA. The juice of the ABA-treated grapes at four weeks after veraison showed a higher concentration of soluble solids and lower titratable acidity than the control grapes. Early accumulation of anthocyanins was also enhanced by ABA treatment. The concentration of anthocyanins in the ABA-treated berries decreased at six weeks after veraison, indicating the berries were overripe at that time. These results suggested that, as for this work, ABA-treated berries ripened earlier than the control berries; however, there was no significant difference in the maximum concentration of anthocyanins. Jeong *et al.* (2004) used 1000 mg/L (±) ABA in their experiments. This racemic mix has equimolar amounts of both, the naturally occurring (+) form of ABA, and the (+) form of ABA, which is a synthetic enantiomer. Balsevich *et al*. (1994) described experimental work investigating the response of cultured maize cells to (+) and (-) ABA and found that the maize cells were able to metabolize the (-) ABA but at a much slower rate than the (+) ABA. The (-) ABA also inhibited growth of the maize cells but again this was much less than the inhibition by (+) ABA. This and other work suggests that both forms of ABA are active in physiological assays but the (+) form appears to have more potent effects in all cases studied thus far. The concentration of active ABA that was applied to the experimental bunches by Jeong *et al.* (2004) was therefore approximately 500 mg/L. In the field experiments described in this chapter, a pure preparation of (+) ABA was applied at concentrations 400 mg/L or 5 mg/L. The use of the pure (+) ABA is unique compared to the published papers described in this chapter which all used the racemic mixture of ABA, and thus the interpretation of the effect on the grape berry development is complicated by this fact.

Although the anthocyanin content of the ABA treated berries were determined and found to be of a similar level to the controls at harvest, there are many other parameters that may have been affected by the ABA application that could have implications for the flavour and composition of the grape berries. For example there are secondary metabolites, such as tannins whose level or composition may have been altered by the ABA sprays, and this would have an impact on the resulting wine made from the berries. In the design of future experiments it would be useful to treat sufficiently large numbers of bunches so as to allow wine to be made from harvested and control berries and then in-depth analysis by HPLC and other techniques to investigate whether the ABA treatment has affected the secondary metabolite profiles.

Symons *et al*. (2006) have recently published data implicating another plant hormone in grape berry development. It was shown that the application of exogenous epi-BR prior to veraison significantly promoted veraison, and conversely the application of a BR synthesis inhibitor, brassinazole, significantly delayed veraison. These results are consistent with the observed increase in endogenous BR at the onset of ripening these researchers measured in grape berries. They suggested that changes in BR levels in grape berries influence the ripening process as has been proposed for ABA. This raises the question of interaction between the different endogenous hormones. There now appears to be three hormones that may influence at least some aspects of grape berry ripening i.e. ABA, BRs and ethylene. Further experimentation is required to determine the role of each during ripening and how they may interact with each other. There are a number of ways in which they could interact, there may be a sequential signalling process for example or they may act through redundant pathways. There are many examples where interactions, both synergistic and antagonistic, between different hormones have been documented. Tanaka *et al*. (2006) have recently published reports on studies in *Arabidopsis* that demonstrate cytokinin and auxin inhibit ABA-induced stomatal closure through the modulation of ethylene biosynthesis, and that ethylene inhibits the ABA-induced reduction of osmotic pressure in the guard cells. Iglesias *et al*. (2001) showed that the exogenous application of sucrose to mandarin (*Citrus unshiu* (Mak.) Marc., cv. Okitsu) citrus fruit-peel promoted 'colour break', a process characterized by the conversion of chloroplast to chromoplast. The process involves the loss of chlorophylls and the gain of carotenoids, changing peel colour from green to orange. The rate of colour break was correlated positively with sucrose content. In both *in vivo* and *in vitro* experiments, colour change promoted by sucrose was unaffected by ethylene but delayed by GA_3. Ethylene inhibitors were shown to counteract the sucrose effects on colour change. The sugar regulation appears to operate via ethylene, whereas GA may act as a repressor of the sucrose-ethylene stimulation.

The results described in this chapter, indicate that berry development can be influenced by the exogenous application of ABA, although the effect is only easily detected at a concentration of 400 mg/L and not at 5 mg/L. The amount of ABA that enters the berry appears to be greatly inhibited by the berry cuticle and the actual effective concentrations are certainly much lower. The timing of the application may be crucial, and further experiments varying both the frequency and number of ABA applications would be

desirable. However, other researchers have published work during the course of this thesis showing that application of other substances, such as epi-BR and ethanol, also influence the progression of berry development and there is some evidence of interactions between the various signalling pathways. The precise mechanisms by which ABA controls development and the identity of the genes that it controls and their functions remain to be elaborated. More extensive experiments are needed to further elucidate the role exogenous application of ABA plays in berry development and the synergies between this hormone and others.

Chapter 6: General discussion

6.1 Introduction

The aim of this project (Section 1.3) was to investigate the role of ABA in grape berry development, and ripening in particular. There are two questions concerning the role of ABA in ripening grapes which have particular significance to the grape and wine industries. First, does ABA control the initiation of ripening? Second, are the levels of ABA related to berry quality, i.e. does ABA influence the accumulation of such compounds as anthocyanins, organic acids, flavour and aroma compounds and sugars? The answers to these questions will be found by understanding the role of ABA during berry development and the mechanisms through which ABA acts. This requires detailed knowledge of ABA accumulation, perception and action.

Some of the significant findings from this work, which have added to our understanding include:

- ABA concentration increased reproducibly at veraison at roughly same time as sugar, colour and berry weight change
- Net accumulation of ABA doesn't occur until veraison
- A substantial amount of bound ABA is present at all stages but there is no evidence of conversion to the free form within the berry
- Total and free ABA concentration decreased 2-3 weeks after veraison during each season, due to both breakdown and sequestration of free ABA into the bound form
- Free ABA levels in skin and flesh were fairly similar throughout development, levels in seeds were often a little higher
- The ABA biosynthetic pathway genes *VvNCED1*, *VvNECD2* and *VvZEP* were expressed throughout berry development, the *NCED* genes were normally expressed more highly before veraison but no clear pattern of transcriptional activity was relatable to changes in ABA level
- ABA synthesis can be induced in isolated berries through *de nova* synthesis by exposure to sucrose but not other osmotica, there is a concomitant increase in the levels of NCED and ZEP mRNAs

- Sucrose treatments that induce ABA accumulation also induce the levels of ACC oxidase mRNA
- ABA applied to pre-veraison berries in the vineyard can promote ripening as measured by an increase in sugar levels, skin colour and berry size

6.2 ABA accumulation in Cabernet Sauvignon berries

Most of the research described in this thesis was carried out using a single *V. vinifera* cultivar, Cabernet Sauvignon. It is not yet known whether the pattern of ABA accumulation observed in the Cabernet Sauvignon berry series from the three years sampled are representative of all of *Vitis vinifera* or indeed all members of the Vitaceae family. The changes in ABA levels reported here (Chapter 2) are consistent with patterns observed by other workers. The only other available data from *V. vinifera* cv. Cabernet Sauvignon berry series shows a similar trend to that determined in this work (Scienza *et al*., 1978) although there are discrepancies between the two studies with regard to the ratio of free ABA at the two weeks postflowering sample and the ten week postflowering sample, this may well be explained by differences in climate or viticultural practice between the two sites or in the definition of flowering time as the curve is very steep at this point and a small difference in timing would make a big difference to levels.

Some reports have shown different profiles of ABA accumulation (Coombe and Hale, 1973, Inaba *et al*., 1976). In general the data presented here agrees with previously published data for grape berries; however, comparisons are difficult for a variety of reasons. First, the techniques used to measure ABA in plant tissues have altered greatly over the years. The method that was used in determining the ABA concentrations presented in this work was modified from published methods which employ HPLC and GC-MS techniques (Soar *et al*., 2006) and an internal standard to correct for any variability during extraction. These measurements should be the most accurate available.

Another reason that complicates direct comparison of these results to other published ABA grape data is that few researchers have published ABA concentrations using *Vitis vinifera* cv. Cabernet Sauvignon. *V. vinifera* cv. Therefore differences observed in ABA profiles may be due to varietal variation. The only available data from *V. vinifera* cv. Cabernet Sauvignon berry series shows a similar trend to that determined in this work (Scienza *et al*., 1978).

As discussed in Chapter 2 although the pattern ABA accumulation appears stable over three years in Cabernet Sauvignon grapes from one site it may not fully represent the patterns of ABA accumulation across a range of varieties grown at sites with various climates. Future experiments that would give valuable additional information could include the sampling of Cabernet Sauvignon berries from other climatic regions. The McLaren Vale climate is regarded as a Mediterranean climate, warm sunny days with fresh sea breezes from the nearby Gulf of St Vincent to temper high summer heats. The area has long periods of dry weather from December through to late autumn, with a mean January temperature of 21.7ºC and 1920 heat degree days (source: www.bom.gov.au). The ABA profile of the same variety grown in a different climate may show differing results. Therefore measuring the ABA levels throughout the development of Cabernet Sauvignon fruit from divergent growing regions such as the hot dry Riverland in the central east of South Australia and a cool climate region such as Tasmania may provide some interesting insights. A comparison in the levels of ABA in fruit grown under different soil moisture conditions may also be of interest as ABA is thought to be made in the roots in response to water stress and can be transported to the shoots.

The comparison of ABA profiles between different varieties of *V. vinifera* grown within the same region would provide an indication of the range of variation within the *vinifera* genus which may be useful information to consider in the planning of future experiments and may improve our understanding of the role of ABA. Within the same geographical area (i.e. McLaren Vale) several different *V. vinifera* cultivars could be sampled to give a comparison of ABA levels at the same berry developmental stages.

6.3 ABA biosynthesis gene expression in Cabernet Sauvignon berries and the role of ABA in berry development

Obviously, knowing the site of ABA synthesis and how it is controlled is important if we wish to modify berry development through the manipulation of ABA levels. This study has not defined the site of synthesis of the ABA which accumulates in berries during ripening. Of course it may be that the ABA which accumulates is made at a remote site and is merely transported into the berry during ripening along with sugars. The results from Chapter 4 show that isolated berries can make ABA in response to the presence of sucrose. In this case the increase appears to be under control of *VvNCED2* transcription as it is induced by this treatment and the norflurazon treatment prevents ABA accumulation

indicating that the accumulation results from an enzymic synthesis rather than a release from the bound form.

Further evidence suggests that the berries are at least capable of ABA synthesis during development. Grape berries were found to have detectable transcripts for both *VvNCED* genes as well as *VvZEP*. The gene expression patterns don't suggest a clear cut transcriptional control of ABA accumulation within the berry. However, gene transcript levels were only measured every one or two weeks and there maybe short term oscillations in transcript levels that disguise any overall trend in transcriptional control. The variation in *VvNCED* (and to a lesser extent *VvZEP1*) mRNA accumulation between seasons may be due to differing environmental factors. One indication that there may be some level of transcriptional control is that although the levels of *VvZEP* transcripts were reasonably constant throughout development but the transcript levels of both *NCED*s are considerably reduced in all seasons later in development when the ABA levels begin to decline (Figs 3.1, 3.3, 3.5). The fact that there are two *NCED* genes expressed in grape berry tissues may also indicate that flexible transcriptional control is important in regulating the amount and location of ABA synthesis.

A number of other mechanisms other than transcriptional regulation suggest themselves for the control of ABA accumulation in berries. The most obvious one is that ABA synthesis in berries may be under post-transcriptional control e.g. through the control of mRNA splicing, translational control, or via the control of enzyme activity. The rate of breakdown may also vary and therefore affect the accumulated levels (Balsevich *et al*., 1994, Cutler and Krochko, 1999). Further experiments could investigate NECD and ZEP protein levels and enzyme activity.

This work has shown that an increase in free ABA levels correlates with the initiation of ripening and that ABA promotes ripening when applied to berries during the preveraison period. These results suggest that ABA may well be involved in the initiation of berry ripening. However, the situation in 'normally' grown fruit may not be this simple. Both BRs (Symons *et al*., 2006) and ethylene (Chervin et al., 2004) have also been implicated in the control of berry ripening. Therefore, there seems to be considerable scope for interaction between the signalling pathways of these three hormones. A number of hormone pathways have been found to interact (Eckardt 2002) and there is also the possibility of some redundancy within the control of grape ripening. Interestingly, the

application of ABA has been shown to induce a burst of ethylene production and so there may be some very obvious linkages between the hormones implicated in the control of grape ripening. The nature of the initial signal that triggers ripening is also unknown. The fact that ABA synthesis can be induced in single berries by the addition of sucrose to the media suggests that the influx of sugars at veraison may be the trigger for ABA accumulation (either by synthesis in the berry or import into the berry). If this is so the question the next question is what is the signal that triggers the unloading of sugars from the phloem. Curiously, it also seems from field experiments that the application of ABA to preveraison berries can result in the unloading and storage of sugars.

Grapevines mutated in the ABA biosynthesis and perception pathways would be valuable tools in unravelling the role that ABA plays in grape berry development. Unfortunately, there appear to be no such mutants currently available. Another way of manipulating the system is to use inhibitors of ABA biosynthesis and perception. In this work the carotenoid biosynthesis inhibitor norflurazon has been used to inhibit ABA biosynthesis inhibitor (Chapter 4). This compound causes lethal damage during plant growth because carotenoids play an important role in protecting photosynthetic organisms against damage by photo oxidation. Therefore, the use of this inhibitor in the investigation of ABA functions is limited to narrow physiological aspects. Recent work published by Kitahata *et al.* (2006) identified a compound known as abamineSG, which is a novel inhibitor of ABA biosynthesis that targets NCED and does not cause lethal damage to the plant. Thus, abamineSG could be used to examine a broad range of physiological aspects involved in the functions of ABA. If this compound was available, it potentially could be applied to isolated berries for *in vitro* experiments to verify that sucrose does induce an increase in ABA levels by *de nova* synthesis. The application of a specific inhibitor of the ABA biosynthesis pathway such as abamineSG to developing grape berries on field grown vines may also be possible as it may not have such a detrimental effect on the whole grapevine. The limitation of such a methodology is that the mobility of the reagent will limit the ability of the experimenter to specifically locate the site of ABA synthesis.

Towards the end of this research a paper was published by Gagne *et al.* (2006) where (+/-) ABA at a concentration of 2×10^{-4} mol/L was applied once to Cabernet Sauvignon berries at approximately three weeks postflowering. The developmental stage of the grapes used in the ABA experiment described by Gagne *et al*. (2006) was significantly earlier than

those used in the experiments described in Chapter 5. As in the work described here the ABA treatment stimulated the accumulation of sugars, however, untreated and treated berries had almost identical sugar levels at maturity. This work suggests that a single ABA application early during berry development may influence the progression of berry ripening. However, there are notable differences between the work reported here and the results of Gange *et al.* (2006). In Chapter 2 evidence was presented that despite differences in climatic conditions between the three years sampled the ABA levels were similar. Gagne *et al.* (2006) reported that the ABA levels they observed in two different seasons were very different, with the peak ABA concentration of the control berries in 2003 reaching approximately 0.3 nmol/g fresh weight, compared to 2004 where the control berries reached a peak concentration of approximately 1.6 nmol/g fresh weight. This difference of more than 5-fold was greater than any variation measured between the three years of ABA analysis detailed in Chapter 2. The researchers suggest that environmental differences between the two seasons could explain these discrepancies, with the 2003 vintage being characterized by long periods of high temperatures and rare rain events. Gange *et al*. (2006) applied a racemic mixture of equal amounts of (+)-S-ABA (the natural hormone) and (-)-R-ABA (the synthetic enantiomer) which also complicates the interpretation of their results as there is evidence that the two compounds exhibit different biochemical and physiological effects on different systems (Balsevich *et al*., 1994). The experiments described in Chapter 5 used the pure form of (+)-S-ABA which distinguishes this work from other published reports on the effect of ABA on grape berry development.

6.4 Implications for grape growing and winemaking

While the work here is of considerable interest from a physiological perspective, the greater immediate importance is how this new knowledge might impact upon grape production and ultimately on wine style. The outcomes of this research indicate that the application of exogenous ABA to developing Cabernet Sauvignon berries, although it hastened the onset of veraison, did not have a noticeable influence on the final total soluble solid or anthocyanin content at harvest time, compared to the control berries. There are a number of possible reasons why this may be so. Fruit did, however, begin to ripen earlier which may have advantages in controlling harvest time and promoting ripening in cool climates. There may be other berry metabolites which are influenced by the ABA application but were not measured within the relatively narrow constraints of this project, such as flavonols, tannins and flavour and aroma compounds. The experiments described

here did not include making winemaking and metabolic and/or sensory analysis which would determine the effect of ABA on changes in metabolite levels. Although the total anthocyanin content was similar between the ABA-treated and the control berries, the relative extractability and stability of different classes of anthocyanins during winemaking was not determined. There may have been a shift in the anthocyanin profile of the ABA treated berry which may influence the wine colour. Applications of ABA post veraison may resolve some of these issues. ABA applied towards the time of harvest may result in increased colour, sugar and flavour over the fruit produced with the preveraison treatments that promote the initiation of ripening. Altered final berry composition may then translate into altered wine characteristics.

There may be ways of altering ABA levels, and hence ripening that do not involve the direct application of ABA. Some viticultural practices, for example, reduced water applications may increase berry ABA levels with consequent changes in berry development. Finally, as the timing, extent and nature of the ripening process is under strong genetic control and as there is considerable variation in these parameters within *vinifera* it should be possible to develop vines with a range of desirable ripening characteristics, some of these might be expected to have altered hormone levels around the time of veraison.

6.5 Summary

During the course of this research three plant hormones have now been implicated in the control of berry ripening i.e. ethylene, the brassinosteroid castasterone and ABA (Symons *et al*., 2006, Chervin et *al*., 2004, Gagne *et al*., 2006). However, the role of ABA in grape berry development, and ripening in particular, is not well understood. The research described in this thesis goes some way in answering the questions that were raised at the beginning of this project as described in Chapter 1. 4. With the development of new ABA biosynthesis inhibitors such as abamineGS, and the availability of (+)-S-ABA more extensive field work can be undertaken to investigate the effect ABA plays in grape berry development, and ultimately the impact this may have on the subsequent wine produced from these berries.

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Appendix 1: Primer sequences

Primer	Genebank Accession Number	Sequence	Comment
NCEDden1F		5'TTCTTCGATGGAGATGGAATGRTNCAYGC3'	5' outer primer for degenerate oligo PCR
NCEDden2F		5'TGCTGGCTATGTXXGAGGAYGAYYTNCC3'	5'inner primer for degenerate oligo PCR
NCEDden3R		5'TGTTTCTGGCTGGTGGGANCRTTCCA3'	3' outer primer for degenerate oligo PCR
NCEDden4R		5'GGTTGGTTCAGCAAGTGGAATYTCNACRTC3'	3'inner primer for degenerate oligo PCR
VvNCED1F	AY337613	5'TGCAGAGGACGAGAGTGTAA3'	NCED1 Forward Real-Time PCR primer
VvNCED1R		5'AGCTACACCAAAAGCTACGA3'	NCED1 Reverse Real-Time PCR primer
VvNCED21F	AY337614	5'GAAGACTTGGCAAATCAAGCTTAGA3'	JS NCED2 Forward Real-Time PCR primer
VvNCED2R2		5'GCTGAGCTCCAATTCTACCG3'	NCED2 Reverse Real-Time PCR primer
VvZEPF	AY337615	5'TACCGGGTATTTTTGGGACA3'	ZEP Forward Real-Time PCR primer
VvZEPR		5'CTTCTTCATCCGTGGCAAGT3'	ZEP Reverse Real-Time PCR primer
VvUbiqF	CF406001	5'AACCTCCAATCCAGTCATCTACT3'	Ubiquitin Forward Real-Time PCR primer
VvUbiqR		5'GTGGTATTATTGAGCCATCCTT3'	Ubiquitin Reverse Real-Time PCR primer
VvGRIP4F	AJ237982	5'ATCCATGCCAATTGAACCAT3'	GRIP4 Forward Real-Time PCR primer
VvGRIP4R		5'TGGAGTAAGTGATGAAATGAGCA3'	GRIP4 Reverse Real-Time PCR primer
VvASRF	AF281656	5'GGTCTTTCTCCGACTTGTGC3'	ASR Forward Real-Time PCR primer
VvASRR		5'ACCAGCTACTCCGACACCAG3'	ASR Reverse Real-Time PCR primer
VvACOF		5'GGTGGAGAAAGAAAAGGAGACA3'	ACO Forward Real-Time PCR primer
VvACOR		5'TCTGTTGGCAAAGGACTCAA3'	ACO Reverse Real-Time PCR primer
B26		GACTCGAGTCGACATCGATTT(16)3'	Modified oligo dT primer from Frohman et al., 1988

Appendix 2: Sequence Alignment for Degenerate Oligo PCR

```
VvNCED1          MASPAAAAASTPNISACVKPKLCYSSS-SSSSSSSSALDLGSFSFHRPTIRPN--INCS-  56
VvNCED2          MASSTAPTSNTTVTWAGAHHHKLRSSS-SSLLDLG--FPSKSIPFRKPNSRRGNAIHCAA 57
ArabNCED2        MASFTATAAVSGRWLGGNHTQPPLSSSQSSDLSYCSSLPMASRVTRKLN-------VSSA 53
ArabNCED1        MACSYILTPNPTKLNLSFAPSDLDAPSPSSSVSFTNTKPRR----RKLS-------ANSV 49
ArabNCED3        MQHSLRSDLLPTKTSPRSHLLPQPKNANISRRILIN----------------------P 37
                 *         .               :  *

VvNCED1          LHSPSVLHFPKHTSSSTGVATVKTTLPKEKDDFSSSVSQQWNFLQSAAAMALDAVETALV 116
VvNCED2          LQSHSVLHYPRQPFHPKEFS--KDDFPENQ----ITQPPQWNFLQRAAALALDRAESALV 111
ArabNCED2        LHTPPALHFPKQSSNSPAIV----VKPKAK----ESNTKQMNLFQRAAAAALDAAEGFLV 105
ArabNCED1        SDTPNLLNFPNYPSPNPIIP--------------EKDTSRWNPLQRAASAALDFAETALL 95
ArabNCED3        FKIPTLPDLTSPVPSPVKLK---------------PTYPNLNLLQKLAATMLDKIESSIV 82
                  .     . .         .                    . * :*  *:  **  *  ::

VvNCED1          A-RETQHPLPKTADPRVQIAGNFAPVPEQPVQHSLPVDGRIPDCIQGVYTRNGANPLHKP 175
VvNCED2          S-HERQHPLPKSADPRVQIAGNFAPVPEQPVHHSLPVSGTIPDCMNGVYLRNGANPLFEP 170
ArabNCED2        S-HEKLHPLPKTADPSVQIAGNFAPVNEQPVRRNLPVVGKLPDSIKGVYVRNGANPLHEP 164
ArabNCED1        R-RERSKPLPKTVDPRHQISGNYAPVPEQSVKSSLSVDGKIPDCIDGVYLRNGANPLFEP 154
ArabNCED3        IPMEQNRPLPKPTDPAVQLSGNFAPVNECPVQNGLEVVGQIPSCLKGVYIRNGANPMFPP 142
                    *  :****..**  *::**:*** * .*: .* * * :*..:.*** ******:. *

VvNCED1          VAGHHLFDGDGMVHAVQFK-DGDASYACRFTETQRLIQERDFGRPVFPKAIGELHGHSGI 234
VvNCED2          VAGHHFFDGDGMVHAVTLN-GGSASYACRFTETQRLVQERKLGRPVFPKAIGELHGHSGI 229
ArabNCED2        VTGHHFFDGDGMVHAVKFE-HGSASYACRFTQTNRFVQERQLGRPVFPKAIGELHGHTGI 223
ArabNCED1        VSGHHLFDGDGMVHAVKIT-NGDASYSCRFTETERLVQEKQLGSPIFPKAIGELHGHSGI 213
ArabNCED3        LAGHHLFDGDGMIHAVSIGFDNQVSYSCRYTKTNRLVQETALGRSVFPKPIGELHGHSGL 202
                 ::***:******:*** :   ...**:**:*:*:*::**  :* .:***.*******:*:

VvNCED1          ARLLLFYARGLFGLVDHSHGTGVANAGLVYFNRRLLAMSEDDLPYQIRVTPSGDLETVGR 294
VvNCED2          ARLLLFYARGLFGLVDHNHGTGVANAGLVYFNQRLLAMSEDDLPYQVRIKPSGDLETVGR 289
ArabNCED2        ARLMLFYARAAAGIVDPAHGTGVANAGLVYFNGRLLAMSEDDLPYQVQITPNGDLKTVGR 283
ArabNCED1        ARLMLFYARGLFGLLNHKNGTGVANAGLVYFHDRLLAMSEDDLPYQVRVTDNGDLETIGR 273
ArabNCED3        ARLALFTARAGIGLVDGTRGMGVANAGVVFFNGRLLAMSEDDLPYQVKIDGQGDLETIGR 262
                 *** ** **.  *:::  .* ******:*:*: **************:::  .***:*:**

VvNCED1          YDFEGQLRSTMIAHPKVDPVSGEMFALSYDVVQKPYLKYFRFSPKGDKSPDVEIPLAEPT 354
VvNCED2          YDFDKQLRSTMIAHPKVDPVSGELFALSYDVVQKPYLKYFHFSPEGHKSPDVEIPIAGPT 349
ArabNCED2        FDFDGQLESTMIAHPKVDPESGELFALSYDVVSKPYLKYFRFSPDGTKSPDVEIQLDQPT 343
ArabNCED1        FDFDGQLSSAMIAHPKIDPVTKELFALSYDVVKKPYLKYFKFSPEGEKSPDVEIPLASPT 333
ArabNCED3        FGFDDQIDSSVIAHPKVDATTGDLHTLSYNVLKKPHLRYLKFNTCGKKTRDVEITLPEPT 322
                 :.*: *: *::*****:*. : ::.:***:*:.**:*:*::*.. * *: **** :  **

VvNCED1          MMHDFAITERFVVVPNQQVVFKLQEMISGGSPVVYDKNKMSRFGVLDKNATDASGMLWIE 414
VvNCED2          MMHDFAITENYVVIPDQQVVLKLQEMITGGSPVIYHKNKKSRFGILAKNAQTASDIIWVE 409
ArabNCED2        MMHDFAITENFVVVPDQQVVFKLPEMIRGGSPVVYDKNKVARFGILDKYAEDSSNIKWID 403
ArabNCED1        MMHDFAITENFVVIPDQQVVFKLSDMFLGKSPVKYDGEKISRFGILPRNAKDASEMVWVE 393
ArabNCED3        MIHDFAITENFVVIPDQQMVFKLSEMIRGGSPVIYVKEKMARFGVLSKQDLTGSDINWVD 382
                 *:*******.:**:*:**:*:** :*: * *** *  :* :***:* :    .* : *::

VvNCED1          APDCFCFHLWNAWEEPETD---EVVVIGSCMTPPDSIFNECDEGLKSVLSEIRLNLKTGK 471
VvNCED2          SPDTFCFHLWNAWEEPETN---EVVVIGSCMTPADSIFNECEENLQSVLSEIRLNLKTGE 466
ArabNCED2        APDCFCFHLWNAWEEPETD---EVVVIGSCMTPPDSIFNESDENLKSVLSEIRLNLKTGE 460
ArabNCED1        SPETFCFHLWNAWESPETD---EVVVIGSCMTPADSIFNECDEQLNSVLSEIRLNLKTGK 450
ArabNCED3        VPDCFCFHLWNAWEERTEEGDPVIVVIGSCMSPPDTIFSESGEPTRVELSEIRLNMRTKE 442
                  *: **********.   :    :*******:*.*:**.*. *  .  *******::* :

VvNCED1          STRRPILP-ESEQVNLEAGMVNRNRLGRKTQFAYLAVAEPWPKVSGFAKVDLSTGQVSKY 530
VvNCED2          STRRPIVP-ASEQVNLEAGMVDRTRLGRKTQYAYLAIAEPWPKVSGFAKVDLSTGDVQKY 525
ArabNCED2        STRRPIISNEDQQVNLEAGMVNRNMLGRKTKFAYLALAEPWPKVSGFAKVDLTTGEVKKH 520
ArabNCED1        STRRTIIP-GSVQMNLEAGMVNRNLLGRKTRYAYLAIAEPWPKVSGFAKVDLSTGEVKNH 509
ArabNCED3        SNRKVIVT----GVNLEAGHINRSYVGRKSQFVYIAIADPWPKCSGIAKVDIQNGTVSEF 498
                 *.*: *:.     :***** ::*. :***::::.*:*:*:**** **:****: .* *.:.




VvNCED1          IYGEQRYGGEPLFLPRDPNSGREDDGYILAFVHDEKTWKSELQIVNATNLQLEASVKLPS 590
VvNCED2          QYGDQRYGGEPYFVHKDPTSEREDDGYILAFVHDEKTWKSELHIVNAMNLQLEATVKLPS 585
ArabNCED2        LYGDNRYGGEPLFLPGE--GGEEDEGYILCFVHDEKTWKSELQIVNAVSLEVEATVKLPS 578
ArabNCED1        FYGGKKYGGEPFFLPRGLESDGEDDGYIMSFVHDEESWESELHIVNAVTLELEATVKLPS 569
ArabNCED3        NYGPSRFGGEPCFVPEG--EGEEDKGYVMGFVRDEEKDESEFVVVDATDMKQVAAVRLPE 556
                  ** .::**** *:        **.**:: **:**:. :**: :*:*  ::  *:*:**.

VvNCED1          RVPYGFHGTFISSKELEKQA- 610
```

```
VvNCED2          RVPYGFHGTFVNSEDLANQA- 605
ArabNCED2        RVPYGFHGTFIGADDLAKQVV 599
ArabNCED1        RVPYGFHGTFVNSADMLNQA- 589
ArabNCED3        RVPYGFHGTFVSENQLKEQVF 577
                 **********:.  :: :*.
```

Forward primers

NCEDden1F-
TTCTTCGATGGAGATGGA atgrtncaygc-3' Core: degen=16 len=11 Clamp: score=79, len=18 temp= 60.0

NCEDden2F
TGCTGGCTATGTCCGAGgaygayytncc -3' Core: degen=32 len=11 Clamp: score=77, len=17 temp= 62.9

Reverse primers

NCEDden4R
ctrcanctytaAGGTGAACGACTTGGTTGG -5' Core: degen=16 len=11 Clamp: score=63, len=19 temp= 61.3
NB The primer is written 5' to 3' so the ordered primer is actually-
ggttggttcagcaagtggaatytcnacrtc
NCEDden3R
accttrcgnacCCTCCTCGGTCTTTGT -5' Core: degen=8 len=11 Clamp: score=76, len=16 temp= 60.3
NB The primer is written 5' to 3' so the ordered primer is actually-
Tgtttctggctggtgggancrttcca