The molecular biology of anthocyanin biosynthesis in grape berry skins

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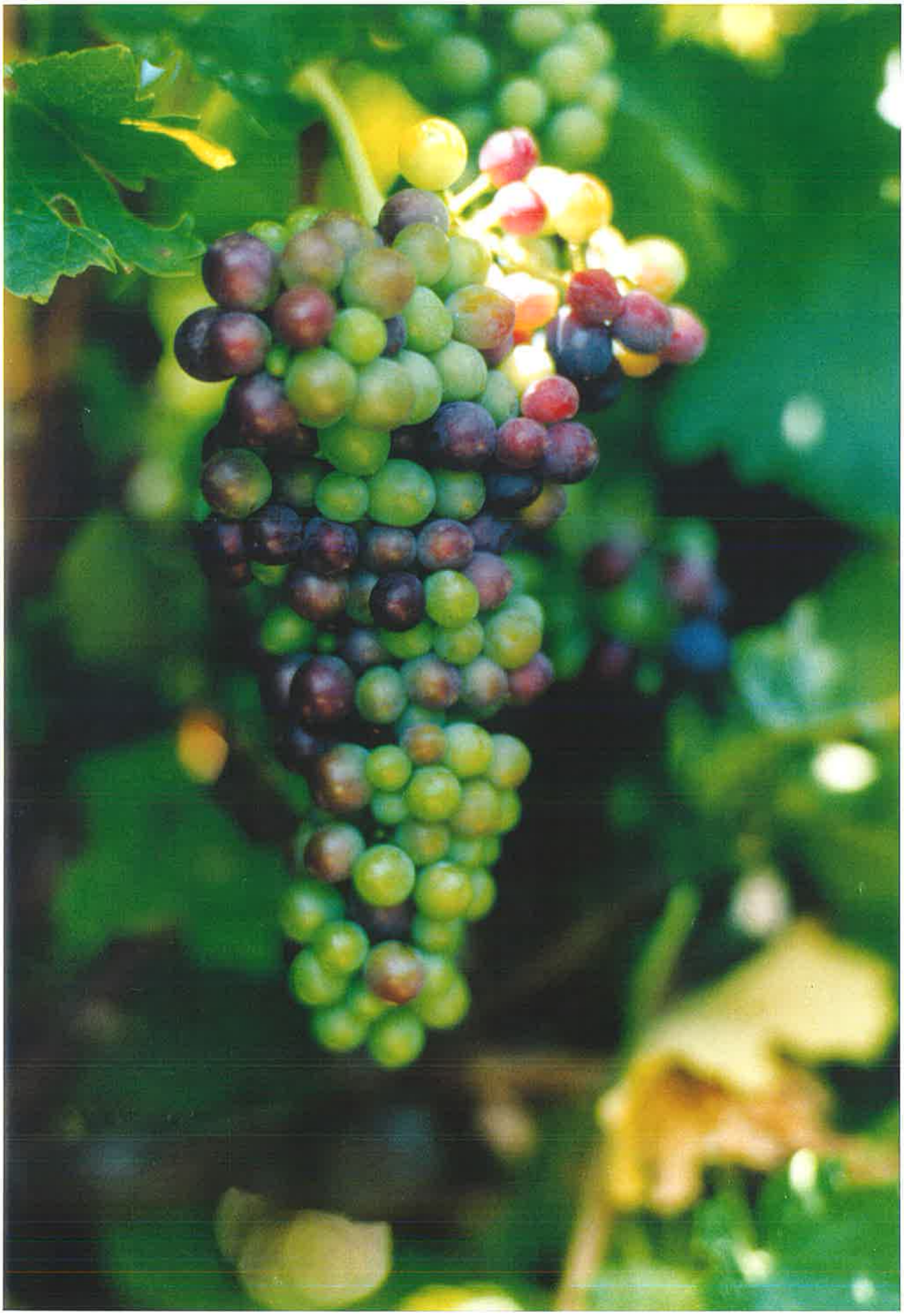

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Abstract

Anthocyanin synthesis in Shiraz grape berries began 10 weeks postflowering and continued throughout berry ripening. In flowers and grape berry skins, expression of all the anthocyanin biosynthesis pathway genes, except UDP-glucose: flavonoid 3-*o*-glucosyl transferase (UFGT), was detected up to 4 weeks postflowering followed by a reduction in expression 6 to 8 weeks postflowering. Expression of all the genes in the pathway, including UFGT, then increased at 10 weeks postflowering, coinciding with the onset of anthocyanin synthesis. Expression of all of the anthocyanin pathway genes except UFGT was detected in most unpigmented tissues, and UFGT was the only gene that showed an absolute differential expression pattern between white and coloured grape berry skins. These results suggest that UFGT is under a different regulatory regime compared to the other anthocyanin pathway genes in grapevine and that anthocyanin synthesis in grapes is controlled at a later stage than seen in other previously studied plant species. Expression of all of the anthocyanin genes except UFGT in the unpigmented tissues correlates with the production of condensed tannins and other flavonoids. Treatment of grape berries with a synthetic auxin-like compound caused a delay in the onset of ripening by approximately 2 weeks. This treatment also delayed, by 2 weeks, the increase in ABA level that normally accompanies ripening and altered the expression of a number of developmentally regulated genes. These observations suggest that auxins (perhaps in conjunction with ABA) have a role in the control of grape berry ripening by affecting the expression of genes involved in the ripening process. By analogy to other species, it is thought that the anthocyanin pathway in grapes is likely to be controlled by *myc*- and *myb*-like transcription factors. Attempts were made to clone these factors from grape berry skins. Although two *myb*-like cDNAs were cloned, their expression patterns suggest they are not involved in the regulation of anthocyanin accumulation in berries.

Declaration

This work contains no material which 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.

Paul K. Boss

January 1998

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Boss PK, Davies C, Robinson SP (1996a) Analysis of the expression of anthocyanin pathway genes in developing *Vitis vinifera* L. cv. Shiraz grape berries and the implications for pathway regulation. Plant Physiol **111:** 1059-1066

Boss PK, Davies C, Robinson SP (1996b) Expression of anthocyanin biosynthesis pathway genes in red and white grapes. Plant Mol Biol **32:** 565-569

Boss PK, Davies C, Robinson SP (1996c) Anthocyanin composition and anthocyanin pathway gene expression in grapevine sports differing in berry skin colour. Aust J Grape Wine Res **2:** 163-170

Davies C, Boss PK, Robinson SP (1997) Treatment of grape berries, a nonclimacteric fruit with a synthetic auxin, retards ripening and alters the expression of developmentally regulated genes. Plant Physiol **115:** 1155-1161

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Abbreviations

Abbreviations accepted by *Plant Physiology* to be used without definition are not included in this list. For definitions, consult the *Plant Physiology* 'instructions for contributors'.

3AT	3-aminotriazole
bHLH	basic helix-loop-helix
BTOA	benzothiazole-2-oxyacetic acid
4CL	4-coumarate CoA ligase
C4H	cinnamate 4-hydroxylase
CHI	chalcone isomerase
CHS	chalcone synthase
CSIRO	Commonwealth Science and Industrial Research Organisation
DFR	dihydroflavonol 4-reductase
F3H	flavanone-3-hydroxylase
F3'H	flavonoid 3'-hydroxylase
F3'5'H	flavonoid 3'5'-hydroxylase
FLS	flavonol synthase
GST	glutathione *S*-transferase
GUS	β-D-glucuronidase
LAR	leucoanthocyanidin reductase
LDOX	leucoanthocyanidin dioxygenase
MT	methyltransferase
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
pers. comm.	personal communication
ppm	parts per million
RT	UDP-rhamnose:anthocyanidin-3-glucoside rhamosyltransferase
U	unit(s)
UFGT	UDP-glucose: flavonoid 3-*o*-glucosyl transferase
vvm	volumes of air per volumes of culture per minute
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactoside

Chapter 1

Introduction

1.1 Grapes

Grape (*Vitis* spp.) is a temperate fruit crop with an ancient origin. There is evidence that grapevines were a source of food as long ago as 8000 BC. Carbonised grape seeds have been found in European prehistoric sites, and there is evidence of grape cultivation dating back to about 3000 BC in locations around the Mediterranean Sea (Zohary and Hopf 1988). The major cultivated species is *Vitis vinifera* L. which bears black, red or green grapes depending on the cultivar, and it is native to southern Europe and the Near East. There are also a number of *Vitis* species native to America or eastern Asia (Reisch and Pratt 1996). American species and interspecific hybrids are mainly used as phylloxera-resistant rootstocks for *V. vinifera*, although there are some interspecific crosses of *V. vinifera* with American species which are used for wine making.

In terms of production, grapes are only exceeded by oranges among all fruit crops world wide. However, grape product value far surpasses any other fruit crop. This is due to the multiple uses of grapes, including fresh fruit, juice, jelly, dried fruit and wine. The latter three products are also able to be stored for long periods which has lead to a complex system of trade for these commodities. Wine can represent the ultimate value-added product with single bottles being sold for thousands of dollars.

Grapes are the largest horticultural crop in Australia by production volume and gross value (Mackay *et al.* 1997) and are grown for three main purposes - wine production, dried fruit and fresh or table grape production. The wine industry is by far the largest earner of the three Australian grape industries with a total wine production value of approximately $1.8 billion in 1996-97, with $575 million of this coming from the export of wine (Source: Australian Wine and Brandy Council). The dried fruit and table grape production values are much less, being worth $68 million each in the 1994/95 season (Source: Australian Horticultural Corporation). The wine industry is also the only expanding industry of the three, wine export value alone has more than doubled since 1992, whereas dried fruit and table grape production figures have been static for the last ten years.

1.2 Grape breeding

The ancient origin of grapes is reflected in the age of the grape varieties used today for wine-making. Before the early nineteenth century, most cultivated varieties were naturally occurring vines and the new varieties arose as bud mutations or chance seedlings (Antcliff 1988). Controlled breeding began in France in the early 1800's and many of the varieties produced are still grown in France and other countries. Perhaps the most successful variety produced by hybridisation is Müller-Thurgau which was produced in 1882 and is a leading variety in Germany. Since then there have been many attempts to improve existing varieties through conventional breeding, but there are few cases of new varieties derived by cross hybridisation being grown widely. There are multiple barriers to the introduction and acceptance of new wine grape varieties and new cultivars need to be significantly better than existing ones to be adopted. As a result, most wine grape cultivar improvement programs

involve clonal selection, where spontaneous somatic variants of a cultivar are propagated and tested in a growing region and the best performing vines released as numbered clones.

There are many limitations to the use of conventional breeding for the improvement of grape cultivars. Grapevines are extremely heterozygous, show inbreeding depression (Alleweldt and Possingham 1988) and have a long life cycle. The long life cycle means the assessment of the fruit quality of crosses can not be carried out for several years. Any individual genotype can not be propagated by seed and must be vegetatively propagated (Gray and Meredith 1992). All of these problems make the task of introducing a trait by the typical strategy of crossing a desirable variety with a source of the desired trait and then repeated backcrossing a very difficult process. Generally, grape breeding has relied on the assessment of the F_1 generation for progeny which are improved cultivars. However, in general, the F_1 generation are intermediate to both parents and thus do not fully display the desired traits of both parents.

Genetic transformation presents an opportunity to improve existing grape cultivars through the introduction of selected genes. If the genetically modified grapes are still considered to be variants of the original cultivar, this will provide new improved material which maintains the identity of the traditional cultivar. The onus now lies on the molecular biologists to identify genes which upon introduction into the existing grape cultivars will thus improve the variety. One field of interest is fruit quality which is in turn a result of the processes that occur during fruit development and ripening. The understanding of the molecular changes that occur during the ripening process will allow the identification of processes that can be targeted and then altered for the benefit of the grape industries. Also, molecular techniques can be used as tools for analysis and for developing new viticultural practices.

1.3 Grape berry development and ripening

Much of the research that has been conducted on fruit ripening has focused on climacteric fruit. Climacteric fruit are characterised by having a burst of respiration during ripening which is often accompanied by an increase in ethylene production by the fruit. This ethylene production is usually autocatalytic and may act as a coordinator of the ripening process (Tucker 1993). Although ethylene has been considered to be the 'ripening' hormone in climacteric fruit, the concept of ethylene as the only 'switch' for ripening in climacteric fruit is now being challenged. Studies on tomato show that there is a dramatic change in gene expression in the fruit before any increase in ethylene (Lincoln *et al.* 1987) and some ripening events can still occur when tomatoes fail to produce normal levels of ethylene (Goodenough *et al.* 1982). Cohen (1996) found elevated levels of auxin can delay the ripening of tomato fruit grown in culture. Thus, ripening in climacteric fruit may be the result of a complex interaction between ripening retarding growth regulators (e.g. auxins and cytokinins) and ripening enhancing growth regulators (e.g. ethylene). Nevertheless, ethylene perception is required throughout the normal ripening process in climacteric fruit and unripe fruit can be ripened off the plant by exposure to exogenous ethylene.

In non-climacteric fruit there is generally no increase in respiration during ripening. Respiration often decreases during the ripening period and there is also no autocatalytic production of ethylene. Exogenous ethylene increases the rate of respiration in non-climacteric fruit and can thus decrease the shelf life of the fruit. However, unlike climacteric fruit, unripe non-climacteric fruit are not induced to ripen by exogenous ethylene. The nature of the ripening trigger in non-climacteric fruit is not known, but is assumed to involve the interaction of certain plant growth regulators.

Grapes are non-climacteric fruit which are unable to ripen once removed from the vine (reviewed in Coombe 1992). Studies into the changes in the levels of endogenous growth hormones and the effects of the application of growth regulators on grape ripening are discussed in detail in Section 5.1. Grape berry growth follows a double sigmoid curve which is characteristic of most berry fruits (Coombe 1976). Generally, grape berries display two periods of volume increase separated by a lag phase where there is little increase in the berry volume. The duration of each of these phases depends on the grape variety and the environmental conditions in which the grapes are grown (Coombe 1973; Hale and Buttrose 1974). The volume increase associated with the first phase of berry development is characterised by an initial period of rapid cell division followed by a marked cell enlargement and a slower rate of cell division (Coombe 1976). As the berries approach the lag phase they possess chlorophyll as the dominant pigment and are still hard. At the end of this first growth stage the growth rate slows and in some cases may stop completely. The end of this lag phase is characterised by a sudden and dramatic change in the development of the berry. This point of development where these dramatic changes occur is called véraison which more specifically refers to the colour change of the berries (see Fig. 1.1). Coombe (1984) listed a host of events which take place at this time which include: further volume increase; accumulation of glucose and fructose; softening of the pericarp (increase in deformability); decrease in organic acid concentration; loss of chlorophyll; and an accumulation of flavour and colour compounds (in black and red grape varieties). These factors are major determinants of fruit quality. Colour in red wine is a very important quality parameter and relates to the accumulation of anthocyanin compounds in the skin of the grape berry. The value of colour in wine is recognised by the fact that three points out of a total of 20 are awarded specifically for colour in Australian wine show judging.

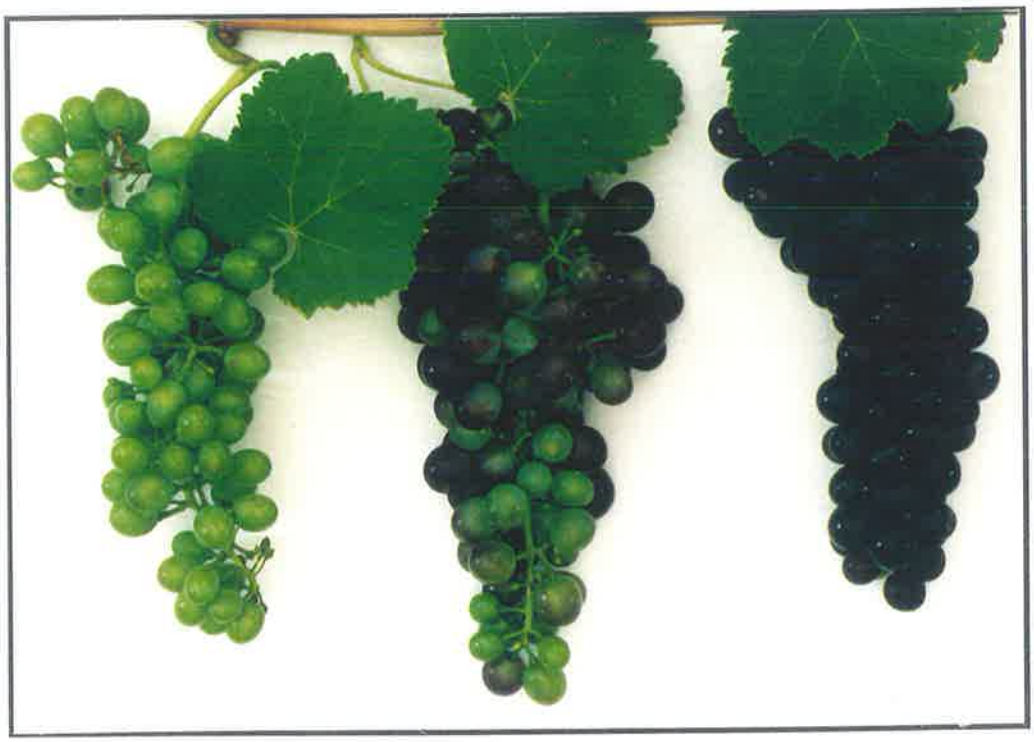


Figure 1.1 Bunches of Shiraz grapes at different stages of development. The bunch on the left represents a typical bunch just prior to véraison, the bunch in the middle is just going through véraison and the bunch on the right is almost fully ripe. Note the variation in the amount of colour on the individual berries on the middle bunch showing the difference in the timing of ripening in these berries.

However, the influence of anthocyanins to the quality of wine extends beyond colour because they are able to interact with other phenolics, proteins and polysaccharides to contribute to the organoleptic and chemical qualities of the wine (Ribéreau-Gayon 1982; Haslam and Lilley 1988). The colour of table grapes is also important in attracting the customer to the product in the marketplace. This thesis describes studies into the accumulation of anthocyanins in grape berry skin and the control of the genes involved in this process.

1.4 Anthocyanin chemistry

There have been many different types of anthocyanins described in plants. The anthocyanidin precursors from which the anthocyanins derive possess a basic three-ring structure, which is shown in Figure 1.2. The differences in these substances depends upon (i) the number and position of hydroxyl groups attached to the rings, (ii) the degree and position of methylation of the hydroxy groups, (iii) the nature and the number of sugars attached, and the position of their attachment, and (iv) the nature and the number of aliphatic or aromatic acids attached to these sugars (Mazza and Miniati 1993). The names given to the chemical 'backbones' which have no sugars attached are anthocyanidins or aglycones. There are six common anthocyanidins in plants; pelargonidin, cyanidin, peonidin, delphinidin, petunidin and malvidin (see Fig. 1.3).

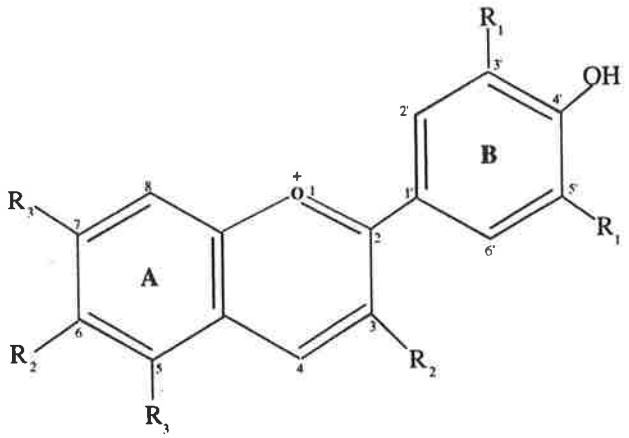


Figure 1.2 The basic structure of naturally occurring anthocyanidins. R_1 = H, OH or OMe, R_2 = H or OH, R_3 = OH or OMe.

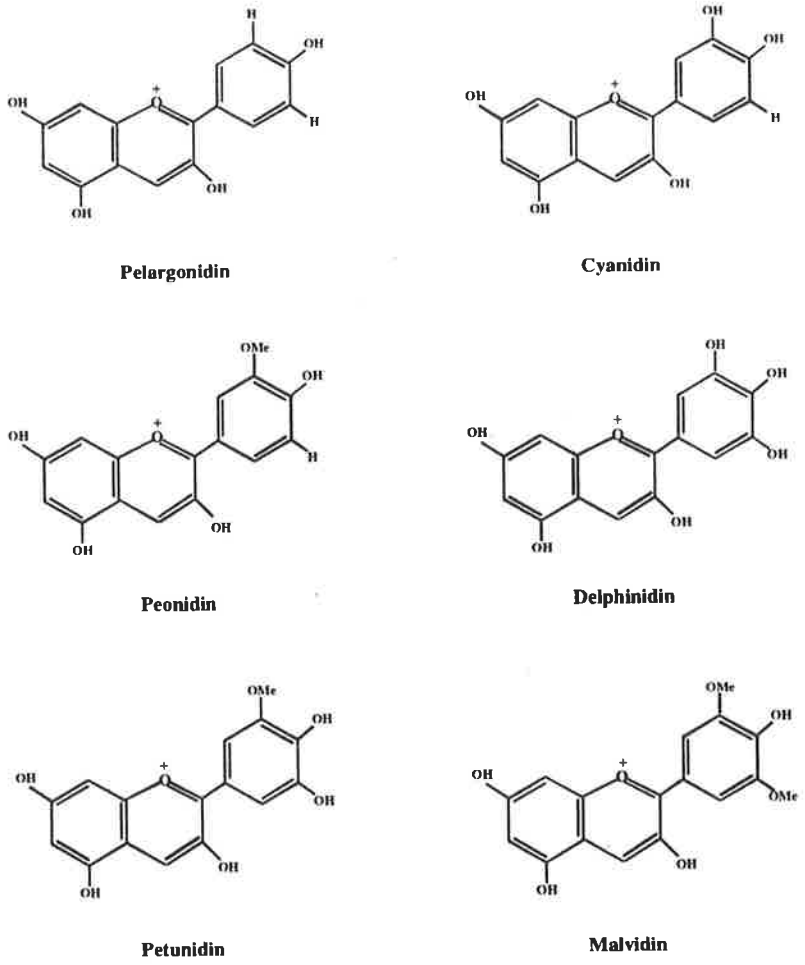


Figure 1.3 The six most common anthocyanidins found in plants.

Each of these anthocyanidins can be glycosylated and acylated at different sites and with different sugars and acyl groups, and so there are many more anthocyanins than anthocyanidins. The common sugars attached are glucose, galactose, rhamnose and arabinose. In many cases, the sugars are acylated by a number of acids, for example, *p*-coumaric acid, acetic acid or caffeic acid. *In situ* colours can not be generated unless there is a free hydroxyl group at either 5 or 7 of the A ring or 4' of the B ring (Brouillard 1982), and in aqueous media anthocyanins act very much as pH indicators, being red in low pH, bluish at intermediate pH and colourless at high pH. This is due to the changes in the equilibrium among four major anthocyanin structures that can exist (Brouillard 1982). For

malvidin 3-monoglucoside. the colour is lost as the pH of the solution approaches 7 as the equilibrium between the two major structures, the coloured flavylium cation and the colourless carbinol, is dominated by the carbinol (Brouillard 1982). However, in a complex mixture of anthocyanins and other compounds, there are several types of interactions that can affect the equilibrium between the anthocyanin species and thus the colour of the solution.

The *in situ* colour stabilisation and intensification of anthocyanins depends upon a number of factors. First, as has already been mentioned, the structure of anthocyanins varies considerably, and this has an effect on colour stability and intensity. This includes the number and nature of added hydroxyls, methoxyls, sugars and acylated sugars. For example, as the number of hydroxyl groups in the B ring increases, the colour spectrum moves from reds to blues. The replacement of these hydroxyls with methoxyl groups reverses this trend slightly (Harborne 1967). Second, increased concentration of anthocyanins intensifies colour and stability may be enhanced as well through self-association or intramolecular co-pigmentation. Intramolecular co-pigmentation occurs in anthocyanins with two or more aromatic acyl groups and results in increased colour stability. This increased stability is due to the reduction in hydration at C-2 and C-4 as a result of the sandwich-like stacking of aromatic residues (Brouillard 1982). The intramolecular interactions are influenced by the structure of the aglycone, the structure and location of the sugars as well as the number and nature of the acyl groups attached to these sugars. It has been observed that the colour intensity of an anthocyanin can increase more than linearly with an increase in concentration (Asen *et al.* 1972). This phenomenon is called self-association and is thought to occur by the helical stacking of the anthocyanin molecules (Hoshino 1991). There also exists intermolecular co-pigmentation, which

increases colour intensity and shifts the wavelength of maximum absorbance resulting in the colours being more blue or purple. There are many compounds which are able to co-pigment, for example flavonoids, alkaloids, amino acids, tannins and polyphenols. The interactions change the equilibrium of the hydration reaction between the coloured flavylium cations and the colourless carbinols. The intensity of the co-pigmentation depends upon the type and nature of both the anthocyanin and the co-pigment, and the pH and temperature of the medium (Mazza and Brouillard 1990). Metal-complexing also affects colour, perhaps by promoting self-association or by removing water of hydration (Goto *et al.* 1976), and a host of other factors including temperature, light, oxygen, acetaldehyde, ascorbic acid, sulphur dioxide, and sugars and their degradation products can affect the stability and intensity of the colour of anthocyanins (reviewed in Francis 1989).

Anthocyanins and their precursors contribute towards floral and fruit pigmentation, and the spectrum of colours produced by these substances ranges from yellows, through reds, to blues. The wavelength of maximum absorbance depends on the type of anthocyanidin backbone and the chemical nature of the species attached to the basic three ring structure common to the anthocyanins. The *in situ* colour can be further altered by intra- and intermolecular interactions and other factors briefly discussed above.

1.5 Grape anthocyanins

1.5.1 Introduction

Generally, grape varieties possess no pelargonidin, and each species/variety has a unique set of anthocyanins. *Vitis vinifera* varieties contain predominantly malvidin 3-monoglucoside

(or malvidin derivatives), although this is not necessarily the case in other *Vitis* species (Mazza and Miniati 1993). The total anthocyanin content of grapes also varies considerably. For example, in a review published by Mazza and Miniati (1993), Pinot Noir was recorded as containing 33 mg of anthocyanins/100 g whereas Vincent contained 439 mg/100 g. However, these results do not take into account some of the variables that may influence the amount of anthocyanins which accumulate in the fruit (see Section 1.5.2).

The anthocyanin composition of a grape berry is generally studied by the use of reversed-phase high performance liquid chromatography (HPLC). This technique was originally developed for use with grapes by Williams *et al.* (1978) and Wulf and Nagel (1978) and allows the rapid and almost complete separation of the different anthocyanins from the complex mixtures often seen in grape extracts. The peaks seen on an HPLC trace of grape anthocyanins have been identified by degradation analyses (Wulf and Nagel 1978), HPLC-diode array spectroscopy (Hebrero *et al.* 1988), and HPLC-mass spectroscopy (Baldi *et al.* 1995). The order of elution of the anthocyanins is related to their hydrophobic properties and is thus the same for all mixtures allowing the identification of anthocyanins in grape samples by relating the peaks and elution order back to these original studies. This allows for a rapid and simultaneous separation of each of the different anthocyanins in a grape extract.

1.5.2 Changes in anthocyanins during the ripening of grapes

The anthocyanin content of grapes increases during ripening, and the accumulation begins at véraison (Fig. 1.1). Different patterns of anthocyanin accumulation have been reported depending on the cultivar and environmental conditions. It has been reported that there are

three phases of development of anthocyanins in grapes from véraison: (i) a slow increase. (ii) a rapid increase, ending in a stabilisation phase, and (iii) a decrease at the end of ripening (Hrazdina *et al.* 1984). Somers (1976) found that the maximum concentration in South Australian grapes was measured 20-30 days after véraison when the sugar content of the berries was 20-24%. In another study, Roggero *et al.* (1986) found that there was a successive accumulation of anthocyanins, or their derivatives, depending on their position in the biochemical pathway. Thus, as the fruit ripened, the substances that accumulated were from later parts of the biosynthetic pathway.

The development of anthocyanins in grapes is influenced by several variables. These include differences in cultivars, seasons, production site and cultural practices. For example, it has been shown that a 15% reduction in solar radiation reduces anthocyanin content by 60%. This reduction was apparently due to changes in the activity of the phenylalanine ammonia-lyase (PAL) enzyme (Roubelakis-Angelakis and Kliewer 1986). Growth temperatures also influence fruit coloration. A high fruit coloration was recorded in fruit which had been grown in 15-30°C day temperatures and 10-20°C night temperatures (Crippen and Morrison 1986). Conversely, in sensitive cultivars, anthocyanin synthesis is inhibited at high temperatures (Kliewer 1970; Kliewer and Torres 1972). An excess of nitrogen fertilisers has been shown to reduce anthocyanins (Kliewer 1977), and if water supply is limited, the grapes are smaller and richer in anthocyanins. Anthocyanins can be oxidised by an extracellular laccase produced by *Botrytis cinerea*, a common pathogen of ripe grape berries (Dubernet *et al.* 1977). Finally, Matsushima *et al.* (1989) found that the application of abscisic acid (ABA) to grapes 3-4 days after véraison results in an increase in anthocyanins 2-3 weeks later.

1.5.3 Anthocyanins in cultured grape cells

Grape cell cultures are used commercially as sources of anthocyanins. This has become important to the food industry as a means of harvesting anthocyanins to be used as food dyes, to replace synthetic dyes. The types and concentrations of anthocyanins produced depends upon the source of the culture material and the composition of the medium in which they are cultured. Yamakawa *et al.* (1983) found that cell cultures derived from anthers had higher amounts of anthocyanins than those derived from "vines" (sic; presumably vegetative tissue). Anthocyanin production and cell growth were both affected by the relative concentrations of growth hormones and the source of nitrogen. Phosphate stimulated cell growth, but had an adverse effect on anthocyanin formation. Light stimulated anthocyanin production, as did an aeration rate of 0.4 vvm. High sucrose was also shown to enhance the production of anthocyanins by Cormier *et al.* (1989). They found that when the sucrose concentrations became stressful, the accumulation of anthocyanins was increased. Do and Cormier (1991) later showed that osmotic stress, caused by high sugar concentrations in the media, resulted in the production of more peonidin 3-monoglucoside in grape cell cultures. In general, media conditions which are high in sucrose, low in nitrate and which increase the length of the initial lag phase induce high levels of anthocyanin accumulation (Hirose *et al.* 1990; Hirasuna *et al.* 1991).

1.5.4 Anthocyanins and colour in red wine

Anthocyanins are the main compounds involved in red wine colour and, along with tannins, influence the organoleptic quality of red wine. The processes of wine making and the subsequent storage and aging of the wine have large impacts on the colour of red wine.

Therefore the fate of anthocyanins during these processes and their influence on wine colour have been the subject of much research which has been extensively reviewed (Ribéreau-Gayon 1982; Mazza and Miniati 1993; Mazza 1995). It is important to realise that the colour of red wine does not depend solely on free anthocyanin content. In young wines, free anthocyanins account for approximately 40% of the total colour but this decreases rapidly during aging (Ribéreau-Gayon 1982). Condensed tannins and tannin-anthocyanin combinations become the major participants in wine colour after several years of aging (Ribéreau-Gayon 1982). Therefore, the colour of wine depends not only the amount of anthocyanins in the grapes, but also, the type of anthocyanins produced, the levels of other phenolic species which may interact with anthocyanins to enhance or reduce colour and the conditions of winemaking, aging and storage which will influence these interactions.

The extraction of anthocyanins (and other phenolics) from the grape skins into the resultant wine begins with crushing and extends through maceration, fermentation and pressing. The conditions employed for each of these processes influences the colour composition of the wine. Extensive crushing will enhance the extraction of anthocyanins, but often results in bitter astringent wines as more phenolics are also extracted and the oxidation of these compounds is enhanced. Prior to fermentation, whole or crushed grapes may be heated to 50-80°C to increase the extraction of anthocyanins and flavour compounds in a process known as thermovinification. Peynaud (1984) points out that the gain in colour extraction during thermovinification is often lost after a few years and so it is probably only useful in improving the colour and flavour of a young wine when the grapes have deficiencies. Two fermentation techniques are generally used for wine making and are known as either classic vinification, when the grapes are crushed, or carbonic maceration when whole grapes are used for fermentation. The duration of the fermentation will determine the amount of

anthocyanin in the resultant wine, with 5 to 6 days giving maximum levels (Ribéreau-Gayon 1982). However, if this maceration period is extended further the anthocyanin levels and colour intensity of the wine will decrease (Ribéreau-Gayon 1982). Following fermentation of the grape must, the marc is pressed and this again results in the release of more anthocyanin and phenolic compounds from the grape tissue.

Two interesting studies which compared different winemaking techniques also demonstrate the fact that anthocyanin levels alone do not determine the amount of colour in the wine. Timberlake and Brindle (1976) found that thermovinification resulted in a more coloured wine than traditional vinification, but it contained less anthocyanin and more polymeric pigment. Wine made by carbonic maceration was the least coloured, but had anthocyanin levels similar to the wine made by thermovinification. Timberlake and Brindle (1976) suggested that the difference in colour is due to variations in the physicochemical state of the anthocyanins. Somers and Evans (1979) looked at the effect of pH on the changes in anthocyanins, total phenolics and colour density during the thermovinification and traditional fermentation of Shiraz grape juice. At pH 3.83 the colour density decreased fivefold during fermentation, but anthocyanins and total phenolics only decreased by approximately 30%, and at pH 3.4 a threefold decrease in colour density coincided with only a 20% decrease in anthocyanins and phenolics. Somers and Evans (1979) suggested that ethanol has a destructive effect on pigment aggregates that are present in the juice or formed early in the fermentation which would account for the loss in colour density.

The changes in levels of the individual anthocyanin species during winemaking has been the subject of few studies, probably due to the difficulty in detecting individual anthocyanins on HPLC traces in wines as polymeric pigments are produced and absorb in the same range.

Leone *et al.* (1984) followed the changes in 15 anthocyanins during the production of wine from three Italian grape cultivars. In all cases the major pigments present in these grapes at maturation were malvidin 3-monoglucoside and the acetylated and coumaroylated derivatives of malvidin. The data suggested that malvidin 3-monoglucoside was more stable than the acylated anthocyanins and that malvidin 3-acetylglucoside was more stable than malvidin 3-*p*-coumaroylglucoside. This confirmed the findings of McCloskey and Yengoyan (1981) who found that acylated monoglucosides disappeared faster than the other monoglucosides in Cabernet Sauvignon and Zinfandel wines. Similarly, in Tinta Roriz wines the rate of loss of the acylated malvidin derivatives was reported to be quicker than the loss of malvidin 3-monoglucoside (Dallas *et al.* 1995). However, Nagel and Wulf (1979) found that there was no significant difference in the rate of loss of acylated and non-acylated anthocyanins during the fermentation and aging of Merlot and Cabernet Sauvignon wines. The data from all these studies is limited as it only accounts for the loss of the anthocyanin monomers. It is possible that the acylated anthocyanins are more reactive and thus contribute more to pigmented polymers or are perhaps degraded hydrolytically to malvidin 3-monoglucoside. Thus, the real contribution of the individual anthocyanin species to colour, both as free anthocyanins and as contributors to pigmented polymers has yet to be determined.

The storage and aging of wine following vinification are periods where there are further changes in the colour characteristics of the wine. During this time the grape anthocyanins are replaced by more stable polymeric pigments (Somers 1971). These polymeric pigments are thought to be formed by a number of reactions including acetaldehyde-mediated condensation, co-pigmentation and self association (Mazza 1995). The stability and reactivity of these polymeric pigments are affected by several factors which include

temperature, oxygen, acetaldehyde, SO_2, pH and the availability of copigments (Mazza 1995). Bakker and Timberlake (1997) have recently identified four new anthocyanin pigments believed to be produced during the maturation of wine called vitisin A and B and acetylvitisin A and B. They are thought to be formed from malvidin 3-monoglucoside and malvidin 3-acetylglucoside, and unlike other anthocyanins are wholly or partially resistant to SO_2 bleaching and express more colour at higher pH values than malvidin 3-monoglucoside (Bakker and Timberlake 1997). Dallas *et al.* (1996) have used model wine solutions to investigate the interactions between anthocyanins, procyanidin B_2 and acetylaldehyde and have shown that different anthocyanins have different degradation rates, but all are able to form new compounds in the model solution. Nevertheless, the structure of the coloured polymers in wine is yet to be elucidated and so the role total anthocyanin levels and individual anthocyanin species play in this colour development is unknown.

1.6 The biochemistry of anthocyanin production

The biosynthesis pathway of flavonoid production has been well studied in plants, and especially in flowers where colour depends on the substances produced by this pathway. The pathway has a branching nature, with the early steps resulting in products which act as precursors for many types of related compounds (see Fig. 1.4). However, the enzymes involved in the later biosynthetic steps act specifically for the production of anthocyanins. The structural genes of the pathway appear to be under the control of regulatory genes which encode *myc*- and *myb*-like transcription factors, although in the cases studied to date these regulatory genes appear to control the pathway at different steps in different species. The following is a brief overview of the steps involved in anthocyanin biosynthesis and the pathway is depicted in Figure 1.5.

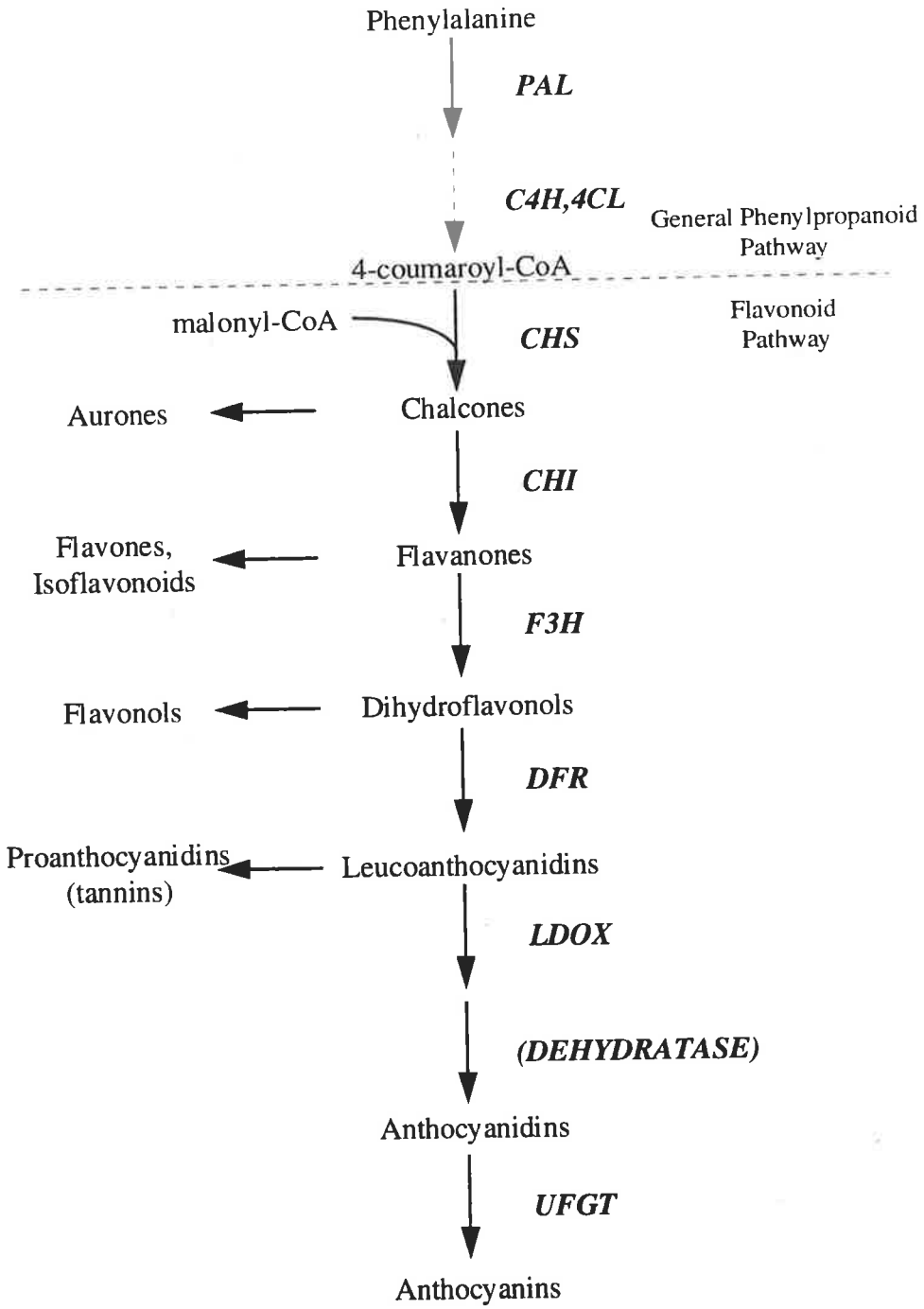


Figure 1.4 A schematic diagram of the general phenylpropanoid and flavonoid biosynthesis pathways. The dotted line indicates the start of the flavonoid pathway. The enzymes catalysing each reaction are indicated down the right of the pathway and the dehydratase is putative. Arrows indicate that the intermediates are precursors of other biosynthesis pathways with final products indicated.

Phenylalanine ammonia lyase (PAL) is the first enzyme involved in anthocyanin production. It catalyses the production of cinnamic acid from phenylalanine (Hanson and Havir 1981). The cinnamic acid is then converted to *p*-coumaric acid by cinnamate 4-hydroxylase (C4H) (Nair and Vining 1965). The enzyme 4-coumarate CoA ligase (4CL) then ligates CoA to *p*-coumaric acid to produce a *p*-coumaroyl-CoA ester (see Heller and Forkmann 1988). The first flavonoid produced is a chalcone, and the enzyme involved is chalcone synthase (CHS). This is produced by the condensation of *p*-coumaroyl-CoA with three molecules of malonyl CoA (Kreuzaler and Hahlbrock 1972). The chalcone is then converted to a flavanone by chalcone isomerase (CHI) which catalyses a stereo-specific ring closure (Moustafa and Wong 1967). This can also occur spontaneously, although at a slower rate (Kuhn *et al.* 1978). Flavanone 3-hydroxylase (F3H) then hydroxylates flavanones to form dihydroflavonols (Forkmann *et al.* 1980).

The dihydroflavonols are the precursors for anthocyanin synthesis. There are several enzymatic steps required for this process and also the transport of the compounds from the cytoplasm to the vacuole. Dihydroflavonol 4-reductase (DFR) catalyses the first step in the conversion from dihydroflavonol to anthocyanin. It causes a reduction at the 4 position of the C ring to give leucoanthocyanidin (Stafford and Lester 1982). The next steps in the production of anthocyanins from leucoanthocyanidin are not well characterised. They are believed to involve a hydroxylase and a dehydratase (Heller and Forkmann 1988). The *Candica* (*Candi*) locus in *Antirrhinum majus* (Martin *et al.* 1991) and the *A2* locus in maize (Menssen *et al.* 1990) have been putatively identified as coding for leucoanthocyanidin dioxygenase (LDOX). The aglycones can then be stabilised through the addition of a

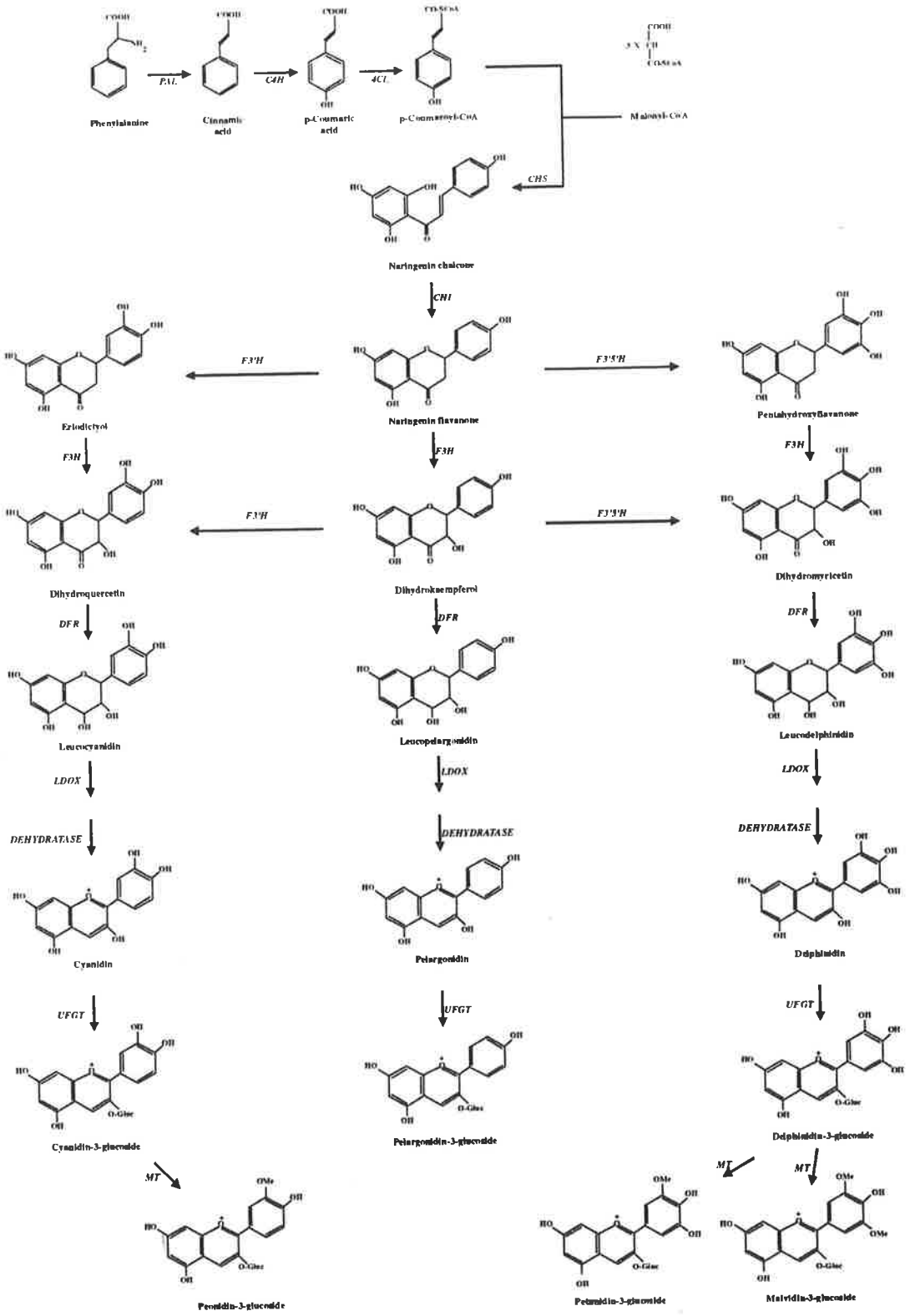


Figure 1.5 The anthocyanin biosynthesis pathway.

glucose residue at the 3 position of the C ring. This reaction is catalysed by UDP-glucose: flavonoid 3-o-glucosyltransferase (UFGT; Larson and Coe 1977), and the product is believed to be able to be transported though the vacuolar membrane. The transport into the vacuole is thought to be mediated via glutathione conjugation by glutathione S-transferase (GST) and the subsequent transport of these conjugates into the vacuole by a glutathione pump (Marrs 1996). Other modifications of the anthocyanins include further glycosylation, acylation and methylation, although the exact order or cellular location of these modifications is unknown.

1.7 Genes of the anthocyanin biosynthesis pathway

1.7.1 Introduction

Mutations have been extensively studied in order to understand the pathway of flavonoid biosynthesis. Since the mutations affect an easily selectable phenotype - that of tissue pigmentation - and the fact that this pigmentation is not essential for the viability of the plants, there is much material available. This is especially true for mutations which affect flower colour, as these have been largely selected for by horticulturalists as well as geneticists.

Colour can be altered in many different ways due to mutations. Those which affect early stages in the biosynthesis of anthocyanins often result in colourless phenotypes. However, if a mutation exists later in the pathway, it may only affect the types of anthocyanins produced. Others may disrupt the transport of the colour compounds into the vacuoles or the tissue distribution of anthocyanin production. As has been discussed above, there are

many variables which influence plant coloration. Therefore, changes in factors such as the pH of the vacuoles could change flower colour (de Vlaming *et al.* 1983).

Mutations have proved useful for the analysis of the phenylpropanoid biosynthesis pathway. Complementation experiments may reveal the gene which has been mutated. The order of the genes in the pathway may also be elucidated by studying the precursors which, presumably, build up if a gene is mutated. Kho *et al.* (1975) used this technique when studying two white flowering mutants of *Petunia hybrida*, *an1/an1* and *an3/an3*. When bud extracts from *an1/an1* flowers were added to a medium in which flowers from *an3/an3* mutants were growing, anthocyanins were produced. However, when bud extracts from *an3/an3* were added to *an1/an1* flowers, anthocyanins were not produced. This suggested that the *An3* gene codes for an enzyme which catalyses a reaction earlier in the pathway than the *An1* gene product.

Studies of pigmentation mutants have resulted in the characterisation of two main types of genes involved in anthocyanin synthesis. Some have been shown to only effect one structural gene of the biosynthetic pathway (e.g. Reddy *et al.* 1987). However, other mutations may affect the expression of one or more of the structural genes but do not map to these loci (e.g. Chandler *et al.* 1989; Quattrocchio *et al.* 1993). These may be involved in the regulation of the anthocyanin pathway and are thus classified as regulatory genes. As is discussed later, the regulatory genes encode transcription factors. Below is a summary of the isolation and characterisation of genes involved in anthocyanin biosynthesis, with a particular emphasis on maize, petunia and snapdragon as the pathway in these species is well characterised.

1.7.2 Anthocyanin pathway structural genes

1.7.2.1 Chalcone synthase (CHS)

A gene encoding chalcone synthase was first isolated from parsley (Kreuzaler *et al.* 1983). This was in fact the first flavonoid gene isolated, and was isolated using differential screening. The parsley clone was then used to isolate two CHS clones from petunia (Reif *et al.* 1985). Koes *et al.* (1989) found that there were at least eight different CHS genes in petunia, however only four were expressed in UV-irradiated seedlings (*ChsA, ChsB, ChsG* and *ChsJ*) and two of these are expressed in floral tissues (*ChsA* and *ChsJ*). In petunia, structural gene mutations in CHS genes have yet to be found, probably because more than one gene is expressed in the petals (Reif *et al.* 1985). Two genes encode the enzyme in maize. One, *C2*, is expressed in the kernel (Dooner 1983; Wienand *et al.* 1986), whereas the other, *Whp* (Franken *et al.* 1991), is expressed in the pollen (and some other floral tissues) and is necessary for pollen viability (Coe *et al.* 1981). The *C2* gene was isolated using transposon tagging (Wienard *et al.* 1986) and its identity confirmed using enzyme activity measurements, feeding experiments and sequence homology. A maize *C2* clone was used by Sparvoli *et al.* (1994) to isolate a homologue from grape seedlings. There are numerous reports of CHS mutants of *Antirrhinum* (e.g. Bonas *et al.* 1984, Coen and Carpenter 1988, Hudson *et al.* 1990) and these tend to result in pure white flowers. These are mutations of the *nivea* gene and are usually caused by the insertion of a transposable element, predominantly in the promoter region. A full-length snapdragon CHS cDNA clone was isolated by Sommer and Saedler (1986) using a partial genomic clone isolated from snapdragon by transposon tagging (Wienand *et al.* 1982).

1.7.2.2 Chalcone-flavanone isomerase (CHI)

Because of the ability of chalcononaringenin (the substrate for CHI) to isomerise non-enzymatically, mutations in CHI are leaky. Thus, the mutants found in *Dianthus* (Forkmann and Dangelmayr 1980) and *Callistephus* (Kuhn *et al.* 1978) produce flowers with pale pigmentation. The first CHI clone was isolated from French bean using antibodies (Mehdy and Lamb 1987) and a similar method was used to isolate CHI cDNA clones from petunia (van Tunen *et al.* 1988). Two CHI genes were isolated from petunia: *ChiA* which is expressed in floral tissues and UV-irradiated seedlings; and *ChiB* which is only expressed in immature anthers (van Tunen *et al.* 1988). The *ChiA* gene was linked to the *Po* gene in petunia, which controls CHI expression in anthers, and also complements the *po/po* mutant which accumulates chalcones in the pollen (van Tunen *et al.* 1991). *Antirrhinum* and maize CHI clones have been isolated using homology to previously isolated CHI clones (Martin *et al.* 1991; Grotewold and Peterson 1994) and an *Antirrhinum* clone was used to isolate a homologue from grape (Sparvoli *et al.* 1994).

1.7.2.3 Flavanone-3-hydroxylase (F3H)

Lines which have mutated F3H genes have been identified in several species, for example petunia (*an3*; Froemel *et al.* 1985) and *Antirrhinum* (*incolorata*; Forkmann and Stolz 1981). These plants can produce anthocyanins when fed a possible product of this step called dihydroquercetin, showing that there are no blocks further on in the pathway. The *incolorata* locus was cloned using differential screening and genetic mapping (Martin *et al.* 1991) and Sparvoli *et al.* (1994) used this snapdragon F3H clone to isolate a grape homologue. The F3H enzyme was purified from petunia and shown to be a 2-oxyglutarate-

dependent dioxygenase (Britsch and Grisebach 1986; Britsch 1990). Britsch *et al.* (1992) isolated a F3H cDNA clone from petunia petals and, using sequence analysis and a prokaryotic expression system, showed that it encoded an active hydroxylase.

1.7.2.4 Dihydroflavonol 4-reductase (DFR)

Maize DFR is encoded by the *A1* gene, which was tagged by the *Spm/En* transposable element (O'Reilly *et al.* 1985). *In vitro* translation of the *A1* cDNA clone produced an active enzyme and showed that the gene did indeed code for DFR (Reddy *et al.* 1987). The DFR gene has been localised to the *Pallida* locus in *Antirrhinum* and blocks in this gene result in ivory or white flowers (Coen *et al.* 1986). Martin *et al.* (1985) cloned the *Pallida* locus when they isolated an unstable insertion allele using a *Tam3* transposable element probe. Sparvoli *et al.* (1994) cloned a grape DFR homologue by screening a seedling cDNA library with a snapdragon DFR clone. Three DFR homologues were previously isolated from petunia using the snapdragon cDNA clone (Beld *et al.* 1989). Only *DfrA* was expressed in petunia flowers and was shown to correspond to the *An6* locus (Huits *et al.* 1994). The enzyme in petunia is interesting in that it shows substrate specificity, and prefers dihydroquercetin and dihydromyricetin to dihydrokaempferol as substrates (Forkmann and Ruhnau 1987). This specificity is not seen in the maize enzyme, coded for by the *A1* gene (Reddy *et al.* 1987). Thus, when the *A1* gene was introduced into petunia the resulting transformants could utilise dihydrokaempferol and produce pelargonidin resulting in flowers with a brick red colour (Meyer *et al.* 1987).

1.7.2.5 Genes involved in the conversion of leucoanthocyanidins to anthocyanidins (LDOX and dehydratase)

The genes involved in this section of the biosynthetic pathway are not well characterised. It is believed that there are at least two enzymes involved in this conversion - a hydroxylase and a dehydratase. There exist mutants in *Antirrhinum* which are unable to produce anthocyanidins if fed leucoanthocyanidins and these have been called *candica* and *incolorata* I (Martin and Gerats 1993a). A similar mutation exists in maize, and has been traced to a gene called *A2*. *Candica* and the *A2* gene have been cloned and show homology both to each other, and to α-ketoglutarate-dependent dioxygenases and F3H (Menssen *et al.* 1990; Martin *et al.* 1991). Weiss *et al.* (1993) cloned the petunia gene *Ant17* which shows 73% identity to *Candica* and 48% identity to *A2*. An LDOX cDNA has been cloned from grape using the *Candica* cDNA as a probe (Sparvoli *et al.* 1994). It is thought that these genes encode a dioxygenase required for anthocyanin synthesis. The *Incolorata* I gene may encode the other enzyme predicted to be required for the synthesis of anthocyanidins, a dehydratase, but the gene has yet to be cloned.

1.7.2.6 Flavonoid 3'-hydroxylase and flavonoid 3'5'-hydroxylase (F3'H and F3'5'H)

These enzymes catalyse the hydroxylation of the B ring (see Fig. 1.5). Flavonoid 3'-hydroxylase activity results in plants accumulating cyanidin-like anthocyanins rather than pelargonidin-like species. Flavonoid 3'5'-hydroxylase activity produces delphinidin species which result in blue colours. Thus, the lack of this enzyme is thought to be the reason for

the absence of blue shades in tulips or roses, to name two well-known examples. Both of these enzymes seem to possess a flexible substrate specificity, with both naringenin or dihydrokaempferol being able to be used as substrates (Stolz *et al.* 1985; Menting *et al.* 1994).

In petunia flowers the F3'H activity was linked to the genetic loci *Ht1* and *Ht2* and the F3'5'H activity was linked to the loci *Hf1* and *Hf2* (Wiering 1974). Two different cytochrome P-450 clones were isolated from petunia and shown to encode F3'5'H (Holton *et al.* 1993a). These mapped to the *Hf1* and *Hf2* loci and complemented mutant petunia lines. Holton and Cornish (1995) also reported the isolation of a P-450 F3'H, however it is not linked to either *Ht1* or *Ht2*. F3'H activity has also been linked to the *Pr* locus in maize (Coe *et al.* 1988) and the *Eos* gene in snapdragon (Forkmann and Stolz 1981), yet neither have been cloned.

1.7.2.7 UDP glucose-flavonoid 3-o-glucosyl transferase (UFGT), rhamnosyl transferase (RT) and other structural genes

Anthocyanidins are unstable, and glycosylation may increase the stability and the solubility of the molecule (Swain 1965). Marty *et al.* (1980) suggested that the sugar group may also prevent the anthocyanin from "leaking" out of the vacuole. Mutations of the enzyme responsible for glycosylation may be difficult to identify in flowers as the phenotype can be similar to wild type or other mutants. However, in maize, a mutation in the *Bronze1* (*Bz1*) gene leads to bronze coloured kernels rather than purple kernels. This coloration is due to the substrate of UFGT complexing with proteins. Larson and Coe (1977) showed that this gene encoded UFGT, and the *Bz1* gene was later isolated using transposon tagging

(Fedoroff *et al.* 1984; Dooner *et al.* 1985). Martin *et al.* (1991) used this maize gene to isolate a homologue from snapdragon and the snapdragon gene was in turn used by Sparvoli *et al.* (1994) to isolate a partial UFGT cDNA from grape seedlings.

Often another sugar is attached to the glucose added by UFGT, and this sugar is often rhamnose. Mutations of this gene in *Silene* and petunia produce phenotypes with less intense coloration than wild type (Kamsteeg *et al.* 1979). The UDP-rhamnose:anthocyanidin-3-glucoside rhamosyltransferase (*Rt*) gene from petunia has been cloned by two research groups. Brugliera *et al.* (1994) used differential screening of *Rt* versus *rt* petals to isolate a clone which showed homology to plant and non-plant glycosyl transferases. Both complementation and antisense analysis suggested that the clone coded for *Rt*. Kroon *et al.* (1994) used a mutant to the regulatory locus *an1* and differential screening to isolate a set of genes upregulated by *An1*. Seven classes of differentially expressed genes which were not homologous to previously cloned anthocyanin pathway genes were isolated and one was shown to map to the *Rt* locus. Further confirmation of the identity of the clone came from antisense experiments, the analysis of *rt* mutant alleles and sequence homology to maize *Bz1*.

Other genes are involved in the transport of anthocyanins into the vacuole and further modification of the anthocyanin species, but few have been cloned. Quattrocchio *et al.* (1993) reported the cloning of an anthocyanin-*o*-methyltransferase, but have not released the sequence or identified its genetic locus and there are no reports of the cloning of any anthocyanin acyltransferases. Mutations in the *Bronze2* (*Bz2*) gene from maize result in a change in colour of the tissue from purple or red to bronze as cyanidin 3-monoglucoside accumulates in the cytoplasm (Marrs *et al.* 1995). The *Bz2* gene has been shown to encode

glutathione *S*-transferase (GST) which is probably involved in tagging the anthocyanin for transport into the vacuole by a glutathione pump (Marrs *et al.* 1995). The petunia *An13* gene reportedly has homology to GSTs (E. Souer and R. Koes pers. comm. cited in Marrs 1996) and is able to complement a maize *bz2* mutant (MR Alfenito pers. comm. cited in Marrs 1996). This transport system is similar to that used by plants to recognise, transport and metabolise herbicides and xenobiotics, and seems to be the method that the plant uses to protect itself from anthocyanins which are in fact toxic to plant cells (Marrs 1996).

1.7.3 Anthocyanin pathway regulatory genes

1.7.3.1 Introduction

The regulation of the anthocyanin biosynthesis pathway has also attracted much study. Possible mechanisms for this regulation could include the control of transcription, mRNA processing or mRNA degradation. Jackson *et al.* (1992) point out that the research to date suggests that the regulation of transcription is the major form of control, and regulatory genes are the controllers (Coen *et al.* 1986; Almeida *et al.* 1989; Martin *et al.* 1991). As mentioned previously, some pigmentation mutants are caused by the change in expression in more than one structural gene, or cannot be mapped to specific structural genes. It is thought that such mutants have lesions in genes which regulate the expression of the structural genes. These regulatory genes have been studied extensively in maize and studies involving promoter fusions and heterologous plant systems indicate that the mechanisms controlling expression of the anthocyanin biosynthetic genes are in some way conserved in plants (e.g. Bevan *et al.* 1989; Schmid *et al.* 1990; Lloyd *et al.* 1992; Mooney *et al.* 1995).

1.7.3.2 Regulatory genes of maize

Some maize genes alter the production of anthocyanins in the plant tissues, but do not seem to encode structural genes. There is a substantial list of these genes (*R*, *Lc*, *C1*, *B*, *Sn*, *Pl*, *Vp*, *In* and *P*) and the similarity in function of some of these genes complicates the situation further. For example, similar regulatory genes are thought to be encoded by *R* (S and P), *B*, *Sn* and *Lc*. However, each gene has a different pattern of expression and thus the various patterns of anthocyanin production are determined by the specificity of production of each regulatory gene. Similarly, *C1* and *Pl* have been shown to be related in sequence and function (Cone *et al.* 1993).

In maize which is homozygous recessive for *r*(S) or *c1*, neither CHS nor DFR transcripts were detected in the aleurone, and only basal levels of UFGT were detected (Dooner and Nelson 1977; Dooner 1983; Cone *et al.* 1986; Ludwig *et al.* 1989). The *R* gene product has been shown to also influence F3H expression in aleurone (Larson 1989) and *Sn* can influence the activity of PAL, CHS, CHI and UFGT (Consonni *et al.* 1987). Therefore, these genes could affect at least five structural genes involved in anthocyanin biosynthesis. It has been proposed that *R* regulates all of the pathway, although *R* may not regulate CHS expression in seedlings as much as in kernels (Taylor and Briggs 1990). Further studies of the regulatory gene products revealed that an *R*-type gene is homologous to the *myc* protooncogene from animals which are basic helix-loop-helix (bHLH) transcription factors (Ludwig *et al.* 1989) and the *C1* gene encodes a *myb* homologue (Paz-Ares *et al.* 1987). Both gene products have domains which are typical of transcriptional activators (Goff *et al.* 1991), and have been shown to functionally interact and activate a *Bz1* promoter/luciferase

reporter construct (Goff *et al.* 1992). Thus the expression of these regulatory genes appear to affect the expression pattern of the structural genes by interacting with the promoters of the structural genes. Martin and Gerats (1993a) suggest that in the aleurone the *R* gene (or its homologues) and *C1* together regulate all or most of the structural genes involved in anthocyanin production. However, in other tissues, other regulatory genes probably function in a similar way to the two discussed above (Ludwig and Wessler 1990). Although these gene products are seen as being functionally equivalent, they probably have subtle differences in the way they regulate anthocyanin biosynthetic genes, and in some cases the environment can be a factor (e.g. light can substitute for *Pl*). Overall, it appears that both *R*-type and *C1*-type regulatory factors are required for anthocyanin production in maize (Dooner *et al.* 1991).

The maize regulatory genes have been shown to be functional in other plant species, suggesting that anthocyanin synthesis is controlled by similar mechanisms in these species. Lloyd *et al.* (1992) transformed tobacco and *Arabidopsis* with the *R* gene allele *Lc* and *C1*. The *C1* transformants in both species were indistinguishable from wild type, but the *Lc* transformants generally showed higher anthocyanin levels in normally pigmented tissues and the *Arabidopsis* transformants also produced extra trichomes in tissues where they are usually found. Crosses were also made between *Lc* and *C1* expressing *Arabidopsis* and some of the progeny possessed pigment in tissues which do not normally accumulate anthocyanins. Lloyd *et al.* (1992) suggested that the *C1* gene may only be functional in *Arabidopsis* when it interacts with the *Lc* gene and that the *R* (*Lc*) homologue in both tobacco and *Arabidopsis* is limiting for pigment synthesis in those tissues which usually accumulate anthocyanins. In petunia the introduction of the *Lc* gene results in the synthesis of anthocyanins in parts of the plant not normally pigmented (Quattrocchio *et al.* 1993;

Bradley *et al.* 1995). Similarly *Lc* mediates enhanced pigmentation in all vegetative tissues in tomato (Goldsbrough *et al.* 1996). Either *Lc* alone can regulate the expression of anthocyanin structural genes in these species, or *C1*-like homologues, with which *Lc* can interact and induce gene expression, are present in all tissues. Transient expression experiments using particle bombardment have also demonstrated the ability of *R*-like and *C1*-like genes to induce anthocyanin accumulation in tissue from orchid (Griesbach and Klein 1993) and sugarcane (Bower *et al.* 1996).

1.7.3.3 Regulatory genes in other species

In *Antirrhinum* there are mutants which have been suggested to involve regulatory genes. The *delila* (*del*) mutants have a loss of pigment in the floral tube. There is little effect on the expression of early genes (CHS and CHI) in these mutants, but there is inhibition of the expression of F3H, DFR, LDOX and UFGT (Martin *et al.* 1991). So it seems that the mutant only affects the later genes of the pathway. The *Eluta* mutant results in the floral pigmentation being restricted to the base of the corolla tube, the central region of the face and the inner edges of the back lobes (Martin *et al.* 1991). Again the control of anthocyanin production seems to be influenced by the expression of the structural genes later in the pathway (Bartlett 1989 cited in Martin and Gerats 1993a). It is suggested that *Eluta* competes for an activator that usually interacts with *Delila* and thus expression is reduced in some of the anthocyanin structural genes (Martin *et al.* 1991). Two alleles of the *Rosea* locus, *rosea*^{colorata} and *rosea*^{dorsea} affect anthocyanin production. Pigmentation is reduced in the central face, the outer epidermis and the upper part of the tube in *rosea*^{colorata} mutants (Stubbe 1966, cited in Martin and Gerats 1993a) and the *rosea*^{dorsea} mutants have pigmentation restricted to the outer epidermis of the lobes and the ring at the base of the

tube. Bartlett (1989, cited in Martin and Gerats 1993a) found that expression of F3H, DFR, LDOX and UFGT genes were reduced in these mutants, but CHI expression was increased. Thus, it seems that the "key regulatory point" for anthocyanin production in *Antirrhinum* is the activity of F3H (Martin and Gerats 1993a).

The *Delila* gene has been studied further, and mutational analysis of DFR promoters revealed that the *Delila* product, or a protein activated by *Delila* may interact with sequences in this region to control DFR gene expression (Almeida *et al.* 1989). Goodrich *et al.* (1992) isolated the *Delila* gene and found that it encoded a transcription factor similar to the *R* gene family in maize. Like the *Lc* gene from maize, *Delila* is able to enhance pigmentation of tobacco flowers and vegetative tissues of tomato (Mooney *et al.* 1995). The *Eluta* gene also interacts with *Delila* to control patterns and levels of gene expression of the anthocyanin biosynthesis genes (Martin *et al.* 1991), but the cloning of *Eluta* has not been reported. A *myb*-like partner for *Delila* has not been identified, although Jackson *et al.* (1991) isolated six genes with homology to *myb*-like genes that are expressed in snapdragon flowers.

The petunia mutants *an1*, *an2*, *an4* and *an11* have been shown to be mutations in anthocyanin regulatory genes (Beld *et al.* 1989; Quattrocchio *et al.* 1993). These mutants lack DFR mRNA, and also have reduced UFGT or RT activity but the expression of CHS, CHI and F3H is not altered (Beld *et al.* 1989; Quattrocchio *et al.* 1993). Thus it seems that the control point for the flavonoid pathway in petunia is one step further on (DFR) compared to *Antirrhinum* (F3H), and three steps further on compared to maize (CHS). The *An1* gene has been cloned and shown to encode a bHLH *myc*-like protein (C. Spelt unpublished data cited in Mol *et al.* 1996) and the *An2* gene encodes a *myb*-like

transcription factor (Quattrocchio 1994 cited in Mol *et al.* 1996). These perhaps represent analogues to the *R* and *C1* genes which are known to control anthocyanin synthesis in maize. Quattrocchio (1994 cited in Mol *et al.* 1996) has also cloned another *myc*-like gene from petunia called *Jaf13* which has been shown to be involved in the regulation of DFR in petunia. The *An11* gene has been cloned and encodes a protein which contains the WD-repeat motif common to other regulatory proteins from yeast, plants and animals (de Vetten *et al.* 1997). *An2* overexpression could restore anthocyanin gene expression in *an11* mutants in transient assays which suggested that *An11* works upstream of *An2* (de Vetten *et al.* 1997).

Thus, it seems that anthocyanin biosynthesis is controlled by similar regulatory factors in different plant species which show homology to *myc*-like and *myb*-like transcription factors. The manner in which the pathway is controlled in different plant species with regards to the tissue specificity of anthocyanin synthesis and how much of the pathway is upregulated upon anthocyanin synthesis seems to be influenced by both the promoters of the anthocyanin structural genes and the properties of the endogenous and/or exogenous regulatory genes involved.

1.8 Aims of this research

Although the ripening processes of climacteric fruit have been well studied, those of nonclimacteric fruit have not. The grape berry is a nonclimacteric fruit of great importance to Australia, however, little is understood about the molecular control of ripening processes in grape berries. There are problems associated with the non-ripening of grapes in some cooler Australian regions and the phenomenon of uneven ripening amongst grapes in a

bunch (Fig. 1.1), bunches on a vine and vines in a vineyard. A number of dramatic changes occur during the ripening of grapes and these are all major determinants of grape quality. One of these processes involves the accumulation of anthocyanins which are the colour components of black and red grape varieties. In general terms, the goal of my research was to understand the control of the genes involved in anthocyanin accumulation in the grape berry with the long term goal of using genetic transformation techniques to adjust both the quantity and quality of these pigment compounds to improve wine quality. The accumulation of anthocyanins and the change in anthocyanin biosynthesis gene expression were also used as ripening related markers in the investigation of the hormonal control of grape berry ripening.

The grape anthocyanin cDNA clones used in this study were isolated by Sparvoli *et al.* (1994). They were obtained by screening a cDNA library constructed from mRNA from grape seedlings grown in light for 48 hours using snapdragon and maize heterologous probes. Sparvoli *et al.* (1994) confirmed the identity of these clones by sequence analysis and showed that that PAL belongs to a large multigene family in grapevine, whereas the other anthocyanin biosynthesis genes are present in one to four copies per haploid genome. The expression of the anthocyanin biosynthesis genes was studied in etiolated seedlings either exposed to continuous white light, which induces the accumulation of anthocyanins, or kept in darkness. PAL was constitutively expressed in darkness and light and the other anthocyanin pathway genes were expressed at low levels in darkness, but their expression dramatically increased upon exposure to light (Sparvoli *et al.* 1994). However, Sparvoli and co-workers (1994) did not extend their research of grape anthocyanin gene expression to the accumulation of anthocyanins in grape berries.

This thesis describes the investigation of anthocyanin biosynthesis during berry development and the expression of anthocyanin biosynthesis pathway genes during this time (Chapter 2). The work also includes studies on the expression of these genes in other grapevine tissues and in the berry skins of black and white-berried varieties (Chapter 3). Grape berry colour mutants were characterised with regard to their anthocyanin profiles and the expression of the genes from the biosynthesis pathway (Chapter 4). Results from the berry development work (Chapter 2) were extended in a study into the effects of a synthetic auxin on berry ripening and the expression of ripening-regulated genes (Chapter 5). Finally, I attempted to isolate cDNA clones coding for anthocyanin regulatory genes by probing a cDNA library with heterologous probes (Chapter 6) and by isolating the UFGT promoter and using this to screen for specific DNA-binding proteins in a yeast-based complementation system (Chapter 7).

Chapter 2

Anthocyanin biosynthesis during Shiraz berry development

2.1 Introduction

Anthocyanin biosynthesis has been extensively studied in petunia, snapdragon and maize, resulting in the elucidation of the biosynthesis pathway by which the various anthocyanin pigments are synthesised from phenylalanine. Control of anthocyanin biosynthesis appears to be exerted at the level of transcription (Martin and Gerats 1993a), but the point at which the pathway is controlled differs in the three plant species mentioned above. In maize, it appears that the first major control point is CHS, whereas in snapdragon and petunia the major control points are further on in the pathway, at F3H and DFR respectively (for reviews, see Martin and Gerats 1993a; Martin and Gerats 1993b; Holton and Cornish 1995). As yet, the control of the expression of the anthocyanin pathway genes in fruit tissues has not been studied.

The colour of red and black grapes results from the accumulation of anthocyanins which are usually only located in the skin of the berry. The quantity and quality of colour in grape berries at harvest are crucial factors influencing wine making. Each species or variety of grapes has a unique set of anthocyanins, and the anthocyanin profiles of many *Vitis* species and varieties have been described (for review, see Mazza and Miniati 1993). *Vitis vinifera* varieties usually produce 3-monoglucoside, 3-acetylglucoside and 3-*p*-coumaroylglucoside derivatives of the aglycones delphinidin, cyanidin, peonidin, petunidin and malvidin, with malvidin derivatives often being the major forms present. However, there are exceptions, Pinot Noir being notable as it only produces non-acylated anthocyanins (Fong *et al.* 1971),

and many muscat cultivars produce less malvidin derivatives than other anthocyanins (Cravero *et al.* 1994). There are no reports of any pelargonidin derivatives isolated from grape berry skins. Limited enzymatic studies of anthocyanin accumulation in grapes (Kataoka *et al.* 1983; Hrazdina *et al.* 1984; Roubelakis-Angelakis and Kliewer 1986) have not revealed a great deal about the control of this biosynthesis pathway and what causes anthocyanin production to be switched on during ripening.

To further investigate the regulation of anthocyanin production in grape berries, grapevine cDNAs encoding enzymes of the anthocyanin biosynthesis pathway, isolated by Sparvoli *et al.* (1994) from etiolated grape seedlings were utilised. Berries from the cultivar Shiraz were sampled throughout development, and the tissue used to study anthocyanin profiles and the expression of seven structural genes from the anthocyanin biosynthesis pathway.

2.2 Materials and methods

2.2.1 Plant tissue

The berries used in this study were sampled during the 1994-95 growing season from *Vitis vinifera* L. cv Shiraz vines grown at a commercial vineyard in Willunga, South Australia (see Fig. 2.1). The vines from the 'source block' of John Harvey's Slate Creek vineyard were planted in 1975. The time of flowering was defined as the date when 50% of the flowers on the vines had dropped their caps. A sample was taken at this time by harvesting a random selection of flowers and freezing the tissue immediately in liquid nitrogen. Further samples were taken at fortnightly intervals. In all cases except the two weeks postflowering sample, the berries were deseeded in the field and immediately frozen in liquid nitrogen.

The two weeks postflowering sample was not deseeded until after being frozen. All the samples were stored at -80°C pending further analysis. Separate skin and flesh samples were obtained by peeling frozen berries.

To define the stage of berry development, a sample of 100 randomly selected berries from 30 bunches was individually labelled and scored each week for deformability, length and width using a Harpenden (British Indicators, Burgess Hill, West Sussex, UK) skinfold calliper gauge as described by Coombe and Bishop (1980). The volumes of the Shiraz berries were calculated using the formula for an ellipsoid ($4/3\pi abc$, where a, b and c are the semiaxes of the ellipsoid). Another random sample of 50 berries was measured for soluble solids (°Brix) with a refractometer (model 10430; Reichert, Vienna, Austria).

2.2.2 Anthocyanin extraction

To prepare samples for HPLC analysis, 10 to 20 frozen berries were removed from storage and peeled. The skin tissue was ground in liquid nitrogen using a mortar and pestle. A 0.5 g subsample of the tissue was then added to 1 mL of methanol and the anthocyanins were extracted for 1 h at -20°C. The grape tissue was pelleted by centrifugation at 10,300*g* for 15 min at 4°C, and the supernatant was retained for HPLC analysis. A 5 µL aliquot of this sample was diluted to 1 mL in methanol and 1% (v/v) HCl, and total anthocyanins were measured by reading absorbance at 520 nm.

2.2.3 HPLC analysis of anthocyanin extracts

A 5 µm Gold Pack *C18* column (4.6 × 25 mm; Activon, Sydney, Australia) and Varian (Melbourne, Australia) equipment consisting of the Vista 5500 pumps and solvent programmer, a Rheodyne (Cotati, California, USA) injector, and a UV-200 detector (Varian, Melbourne, Australia) operating at 520 nm were used. The signal was received and analysed using a data acquisition, plotting and analysis package from DAPA Scientific (Kalamunda, Australia), which measured retention times and peak areas. The weak solvent A was 1.4% (v/v) perchloric acid, the strong solvent B was 100% methanol, and solvent C was water. Solvent A was maintained at 30% throughout the analyses, and the flow rate was 1.5 mL min^{-1}. The initial condition of solvent B was 20%, increased to 35% in 5 min, and then increased to 55% in 35 min. In all cases 100 µL of extract (Section 2.2.2) were injected.

2.2.4 Proanthocyanidin quantification

Proanthocyanidins were detected in the berry skin and flesh samples using a vanillin-HCl assay (pers. comm. Dr. G. Tanner CSIRO, Canberra) based on the method described by Broadhurst and Jones (1978). A 5 g sample of frozen plant material was ground to a powder in a pre-chilled coffee grinder. A subsample of this powder (0.1 g) was then added to 0.5 mL of 70% acetone, 0.1% (w/v) sodium ascorbate and vortexed briefly to mix. The grape tissue was then pelleted by centrifugation at 10,000*g* for 10 min at 4°C and the supernatant retained on ice. In 1.5 mL tubes 10, 20 and 50 µL of the supernatant were added to 70% acetone, 0.1% ascorbate to make a final volume of 200 µL. A 0.8 mL aliquot of a freshly made vanillin-HCl reagent (5 mL of 5% vanillin in methanol, 3 mL HCl)

was added to the extracts, mixed and incubated at room temperature in the dark for 15 min. The absorbance at 500 nm was then measured and compared to standards of known catechin and proanthocyanidin concentrations.

2.2.5 Isolation of total RNA

Total RNA was isolated from grape berry skin and flesh tissue using the perchlorate method of Rezaian and Krake (1987) with modifications. A 4 g sample of tissue was removed from -80°C storage and ground to a powder using a coffee grinder. The powder was added to 16 mL of extraction buffer (0.3 M Tris-HCl [pH 8.3], 2% [w/v] PEG 4000, 5 M sodium perchlorate, 1% [w/v] SDS, 8.5% [w/v] PVPP, and 1% [v/v] β-mercaptoethanol) and stirred rapidly for 30 min at room temperature. This slurry was then centrifuged at 200*g* for 15 min at 4°C through Centriflo cone column holders (Amicon, Massachusetts, USA) packed with glass wool, and the 'raft' was discarded. The eluate was collected, and nucleic acids were precipitated with 2.5 volumes of ethanol, incubated at -20°C for 20 min, and then pelleted by centrifugation at 7700*g* for 15 min at 4°C. This pellet was rinsed with 70% ethanol, dried under vacuum, and resuspended in 1 mL of 0.1 mM Tris/1 mM EDTA (pH 7.6) and 0.2% (v/v) β-mercaptoethanol. The suspension was then extracted three times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and once with an equal volume of chloroform:isoamyl alcohol (24:1, v/v). The RNA was precipitated by adding 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol to the aqueous phase and incubating at -20°C for at least 20 min. Finally, the RNA was pelleted by centrifugation at 7700*g* for 15 min at 4°C, washed with 70% ethanol, dried under vacuum, and resuspended in 300 µL of water.

2.2.6 Grape anthocyanin genes and probe preparation

The probes used for the northern analysis of the anthocyanin genes were gifts from Dr. C. Tonelli, Universita Degli Studi Di Milano, Italy. They were isolated from a cDNA library constructed using mRNA purified from 14 day old seedlings (*Vitis vinifera* L. var. lambrusco a foglia frastagliata [f.f]) grown in continuous light for 48 h (Sparvoli *et al.* 1994). Heterologous probes from *Antirrhinum majus* (PAL, CHI, F3H, DFR, LDOX and UFGT) or maize (CHS) were used to screen the library for grape homologues (Sparvoli *et al.* 1994). Each cDNA clone had been cloned into the *Eco* RI site of pBluescriptSK and the insert was excised as described in Table 2.1 below. Table 2.1 also shows the name of the plasmid and a brief indication of the size and completeness of the clone.

The specific DNA fragments were isolated after digestion and electrophoresis in TBE buffer (90 mM H_3BO_3, 2 mM EDTA, 90 mM Tris-HCl [pH 8.0]) and low melting point agarose gels by cutting them out of the gel using a sterile scalpel blade. The DNA was purified from the gel slice using a QIAquick Gel Extraction kit (QIAGEN) according to the manufacturer's instructions.

A Gigaprime random primer labelling kit (Bresatec) was used to prepare radioactive DNA probes. Reactions containing 50-100 ng of template DNA and 5 µL of α-^{32}P-dATP (10 mCi mL^{-1}; Bresatec) were incubated at 37°C for at least 30 min. Labelled DNA was separated from unincorporated nucleotides by passing the reaction mixture through a 1 mL Biogel P-50 (Bio-Rad) column. To measure ^{32}P-dATP incorporation into DNA, aliquots of the probe were counted in a liquid scintillation counter. The probes were of approximately equal specific activities of at least 3×10^6 cpm ng^{-1}.

Table 2.1 Brief description of the clones used for probing the northern blots

Gene	Plasmid	Description	Digest for gene excision
PAL	pBS204	1368 bp, 3' end	*Eco* RI
CHS	pBS305	1377 bp, full-length	*Eco* RI
CHI	pBS407	979 bp, full-length	*Bam* HI/*Hin*d III
F3H	pBS710	1419 bp, full-length	*Eco* RI
DFR	pBS510	1315 bp, full-length	*Eco* RI
LDOX	pBS603	1334 bp, full-length	*Eco* RI
UFGT	pBS801	532 bp, 3' end	*Eco* RI

2.2.7 Northern blot analysis

Total RNA was extracted from grape tissues as described in Section 2.2.5. Aliquots of 4 μg were heated at 70°C for 10 min in 3 volumes of Mops buffer (20 mM Mops, pH 7.0, 5 mM sodium acetate, 1 mM EDTA) containing 70% (v/v) formamide and 8.9% (v/v) formaldehyde. The RNA was then run on a 1.2% agarose gel containing a final concentration of 7.4% (v/v) formaldehyde with Mops buffer. RNA loadings were checked on ethidium bromide-stained gels to confirm that they were equal. The RNA was transferred by capillary action to a ZetaProbe membrane (Bio-Rad) for at least 15 h and then prehybridized for 2 h at 65°C in 0.25 M sodium phosphate (pH 7.0), 1 mM EDTA (pH 8.0), and 7% (w/v) SDS. Membranes were hybridised for 15 h at 65°C in the same buffer with the addition of denatured ^{32}P-labelled probes of the anthocyanin genes (see Section 2.2.6). The membrane was then washed twice for 10 min in 2 × SSC (150 mM NaCl and 15 mM tri-sodium citrate, pH 7.0) and 0.1% (w/v) SDS (65°C) and

then for 15 min in 1 × SSC and 0.1% (w/v) SDS (65°C). The membranes were exposed to Kodak XAE film with intensifying screens at -80°C.

2.3 Results

2.3.1 Grape berry development

Data from the measurement of various ripening parameters throughout the development of the Shiraz berries sampled (Fig. 2.1) are presented in Figure 2.2 and show that berry growth followed the typical double sigmoid curve. The volume of the berries (Fig. 2.2A) increased during the first seven weeks of development to approximately 650 mm^3, followed by a cessation in the berry expansion until nine weeks after flowering, after which time the volume began to increase again. Berry volume peaked at week 11 (1183 mm^3) and then decreased to a final value of 765 mm^3 at harvest. The onset of ripening (termed véraison by viticulturists) is indicated by an increase in softness, sugar content, berry size and, in red and black grapes, the development of skin colour. Deformability (a measure of berry softness) began to increase after eight weeks postflowering (Fig. 2.2B). By 16 weeks postflowering the berries were very soft, deforming by 3.1 mm on average. Soluble solids (measured as °Brix) also began to increase after eight weeks postflowering and continued to rise, reaching a value of 24 °Brix 16 weeks after flowering (Fig. 2.2C). Proanthocyanidin levels were highest early in berry development and decreased throughout the growing season on a fresh weight basis (Fig. 2.2D). When compared on a per berry basis, the levels of proanthocyanidins still decrease throughout berry development in both the skin and the flesh, but the initial decrease is less rapid (data not shown). The amount of

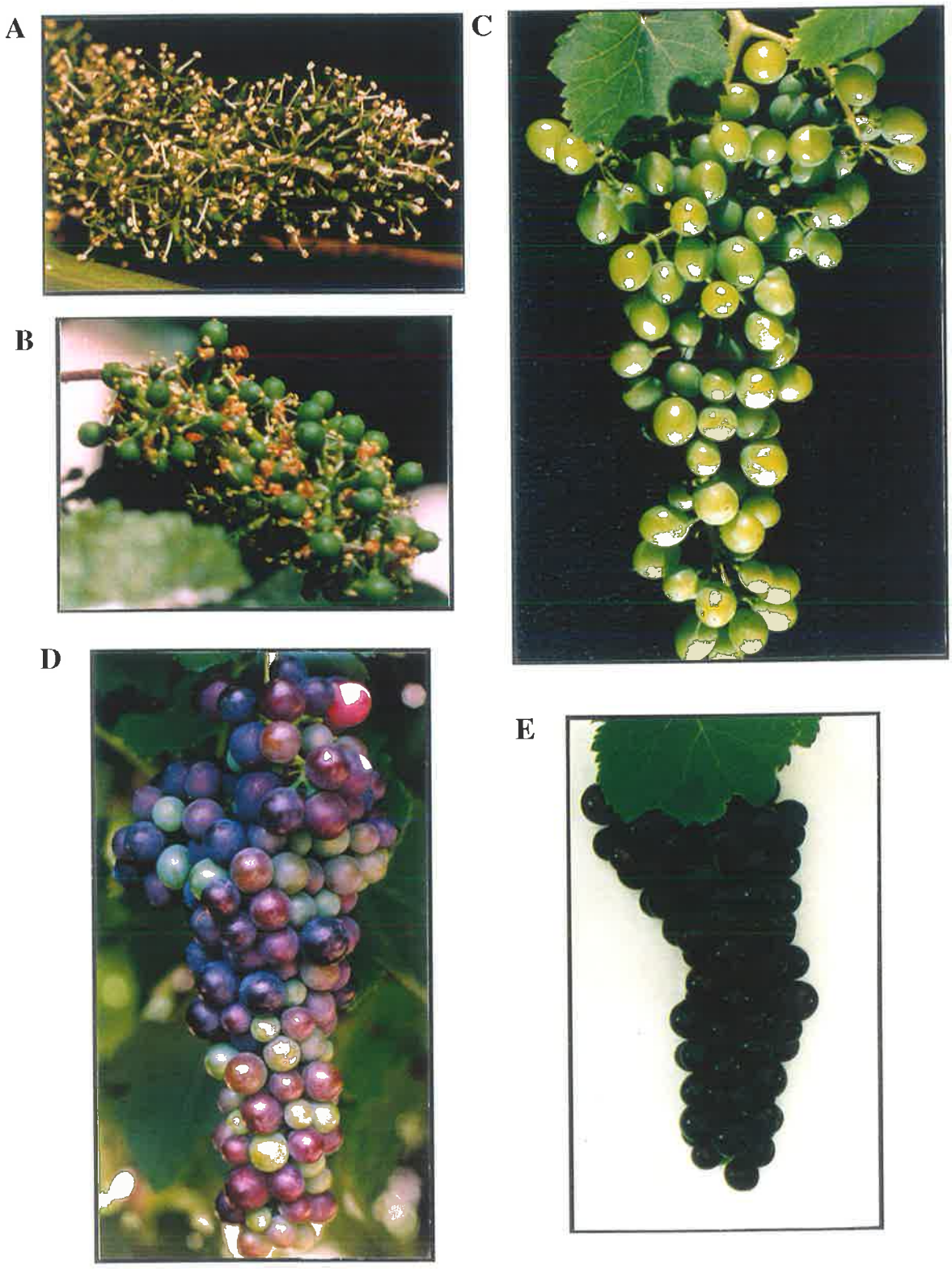


Figure 2.1 Shiraz grapes at various stages of development. **A**, full-flowering; **B**, two weeks postflowering; **C**, six weeks postflowering; **D**, just after véraison, approximately ten weeks postflowering; **E**, 14 weeks postflowering.

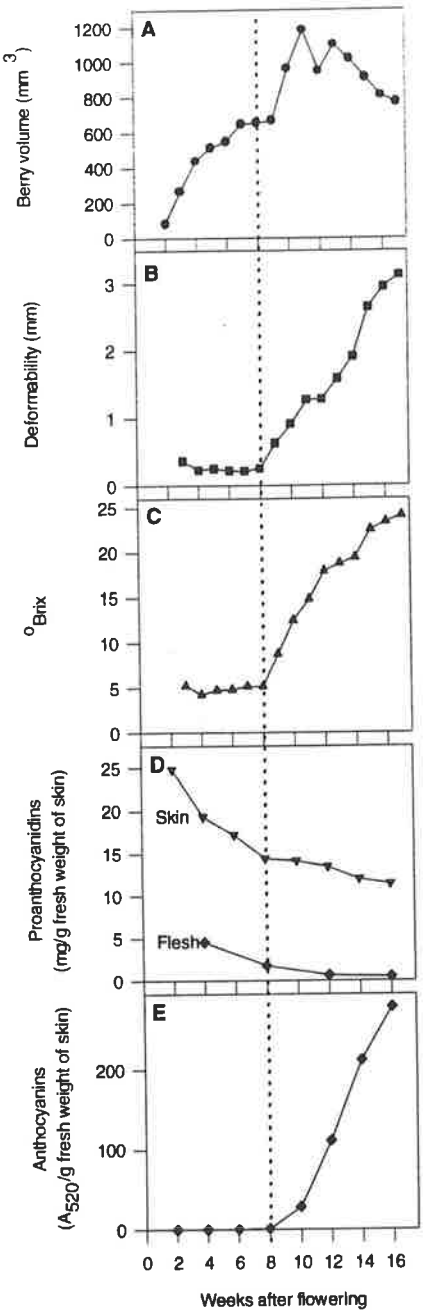


Figure 2.2 Changes in various parameters measured during the development and ripening of cv. Shiraz grape berries. **A**, berry volume; **B**, berry deformability; **C**, total soluble solids in the berry juice, measured as °Brix; **D**, proanthocyanidins per gram fresh weight of berry skin or flesh; **E**, total anthocyanins per gram fresh weight of skin. The vertical, dotted line represents véraison.

proanthocyanidin per gram fresh weight was five times greater in the skin than the flesh at four weeks postflowering, but at harvest there was approximately 30 times more proanthocyanidin in the skin than in the flesh. Anthocyanins were first detected in the sample taken ten weeks postflowering (Fig. 2.2E), although field observations indicated that some pigmentation was present after nine weeks (samples for anthocyanin and RNA extractions were only taken on even-numbered weeks). Thus there seemed to be a slow accumulation of anthocyanins between nine and ten weeks postflowering, followed by a substantial increase in anthocyanin levels up to harvest, 16 weeks postflowering.

2.3.2 HPLC analysis of anthocyanin accumulation

HPLC techniques were used to follow the accumulation of the individual anthocyanins present in Shiraz berry skin tissue. Seventeen anthocyanins were present in all samples and 16 of these were identified by comparing their retention times and elution order with previous studies of grape and wine anthocyanins (Wulf and Nagel 1978; Roggero *et al.* 1986; Gonzalez-SanJose *et al.* 1990). Figure 2.3 shows a typical elution pattern of Shiraz pigments under the HPLC conditions used. The most abundant anthocyanins present in all samples were malvidin 3-monoglucoside, malvidin 3-acetylglucoside, and malvidin 3-*p*-coumaroylglucoside (Fig. 2.4). Of the peonidin, delphinidin, and petunidin anthocyanins, the 3-monoglucoside derivatives were the major contributors, and there was very little of any cyanidin-based anthocyanins. Although the anthocyanin 3-monoglucosides increased in concentration, their percentage of the total anthocyanins decreased during ripening, probably because of the more rapid rate of accumulation of malvidin 3-acetylglucoside and

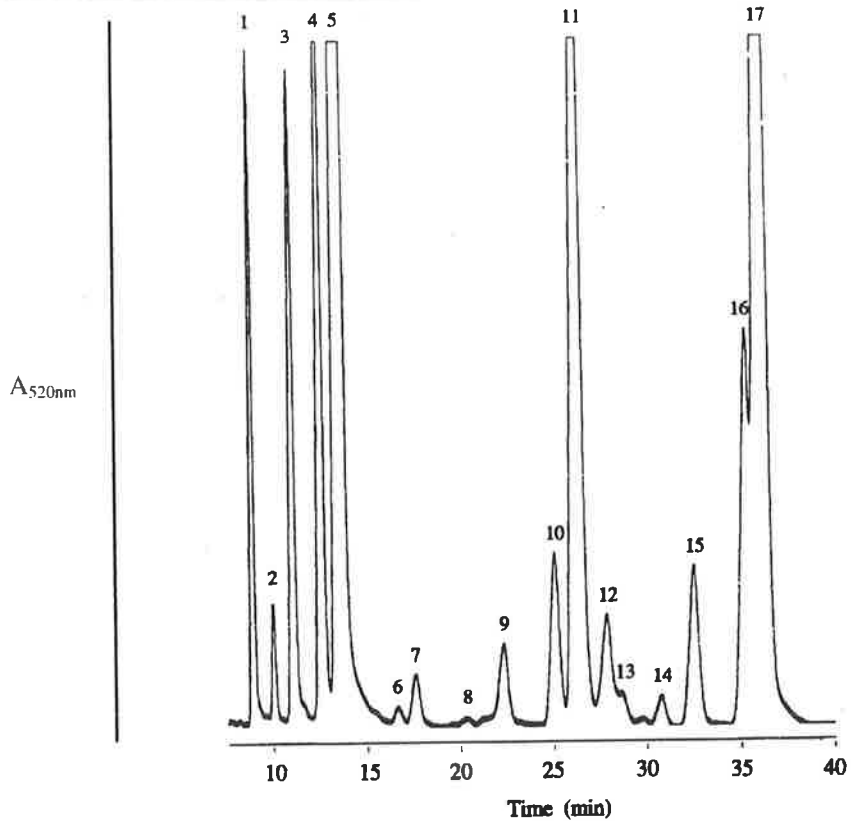


Figure 2.3 Typical separation of the anthocyanins of cv Shiraz grapes. Peak 1, Delphinidin-3-monoglucoside; peak 2, cyanidin-3-monoglucoside; peak 3, petunidin-3-monoglucoside; peak 4, peonidin-3-monoglucoside; peak 5, malvidin-3-monoglucoside; peak 6, unknown; peak 7, delphinidin-3-acetylglucoside; peak 8, cyanidin-3-acetylglucoside; peak 9, petunidin-3-acetylglucoside; peak 10, peonidin-3-acetylglucoside; peak 11, malvidin-3-acetylglucoside; peak 12, delphinidin-3-*p*-coumaroylglucoside; peak 13, malvidin-3-caffeoylglucoside; peak 14, cyanidin-3-*p*-coumaroylglucoside; peak 15, petunidin-3-*p*-coumaroylglucoside; peak 16, peonidin-3-*p*-coumaroylglucoside; peak 17, malvidin-3-*p*-coumaroylglucoside

malvidin 3-*p*-coumaroylglucoside (Figs. 2.5 and 2.6A). During this time the percentage of the total 3-acetylglucosides and 3-*p*-coumaroylglucosides increased between ten and 16 weeks postflowering (Fig. 2.6A). The malvidin derivatives increased as a percentage of the total anthocyanins from 62 to 73% over the ripening period, whereas the percentage of the other compounds decreased slightly during this time (Fig. 2.6B). The percentage of trihydroxylated derivatives (delphinidin, petunidin and malvidin) remained relatively

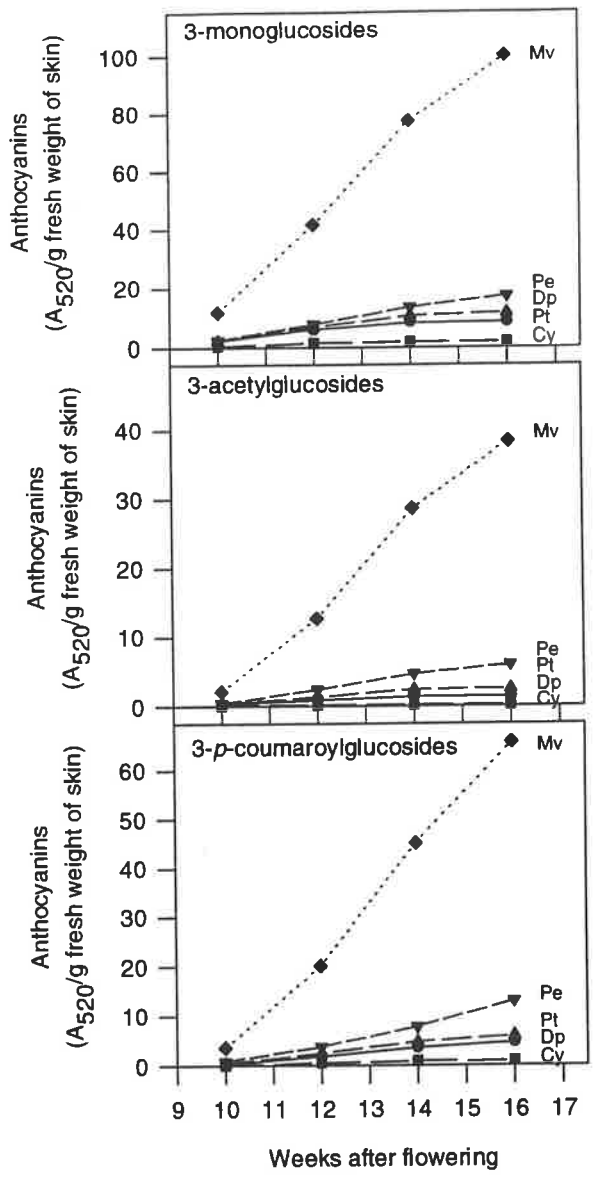


Figure 2.4 Changes in the total amounts of the major individual anthocyanin species found in grapes during development. The data are grouped as anthocyanin 3-monoglucosides, 3-acetylglucosides and 3-*p*-coumaroylglucosides as indicated. Individual species within the groups are denoted by the abbreviations: Cy, cyanidin; Dp, delphinidin; Mv, malvidin; Pe, peonidin; Pt, petunidin.

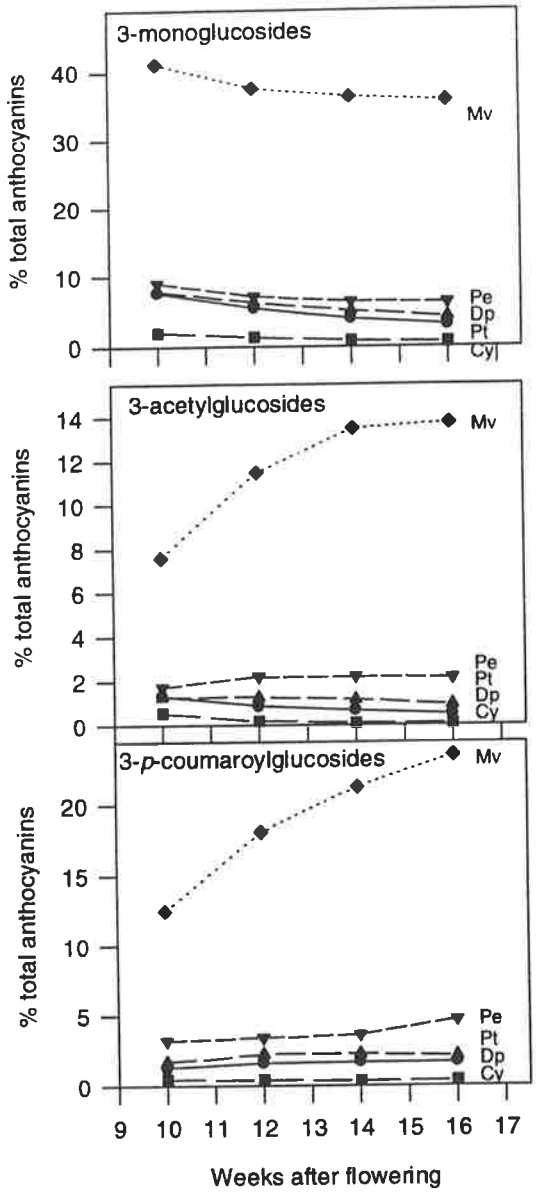


Figure 2.5. Changes in the percentage of the major individual anthocyanin species found in grapes during development. The data are grouped as anthocyanin 3-monoglucosides, 3-acetylglucosides and 3-*p*-coumaroylglucosides as indicated. Individual species within the groups are denoted by the abbreviations: Cy, cyanidin; Dp, delphinidin; Mv, malvidin; Pe, peonidin; Pt, petunidin.

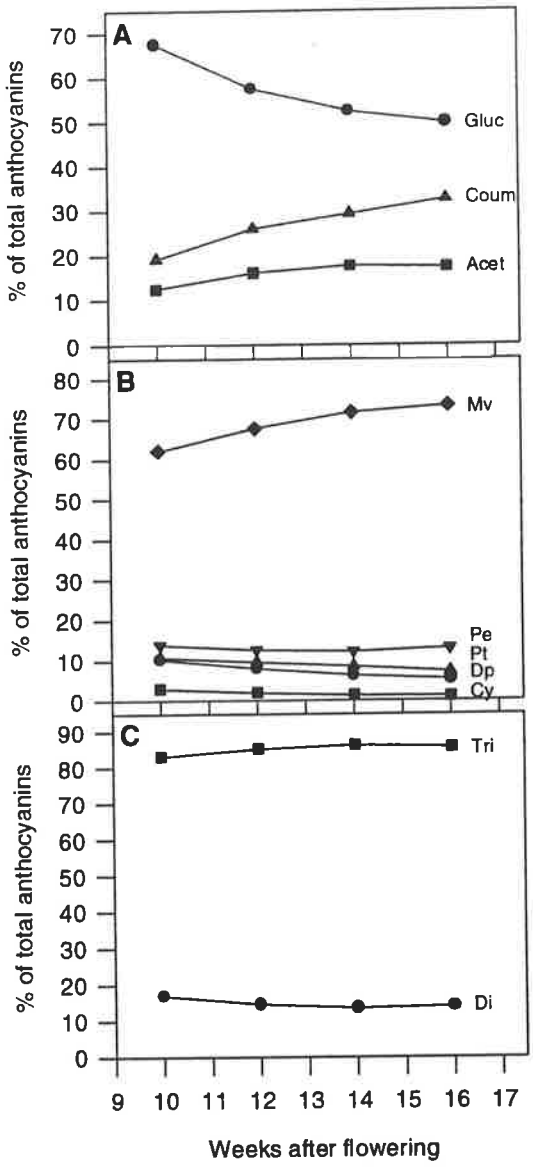


Figure 2.6 Changes in the percentage of various anthocyanin species groups during grape berry development. **A.** Changes in acylation of the anthocyanins. Acet = acetylglucosides; Coum = coumaroylglucosides; Gluc = monoglucosides. **B.** Changes in anthocyanidin species. Cy = cyanidins; Dp = delphinidins; Mv = malvidins; Pe = peonidins; Pt = petunidins. **C.** Changes in hydroxylation of the anthocyanins. Di = dihydroxylated; Tri = trihydroxylated.

constant during ripening as did the percentage of dihydroxylated (cyanidin and peonidin) derived anthocyanins (Fig. 2.6C). Thus, there was an increase in the concentration of anthocyanins throughout ripening of the berries but no major changes in the proportion of the 3'-substituted and the 3',5'-substituted anthocyanins.

2.3.3 Expression of anthocyanin biosynthesis genes in Shiraz berry skin

The expression of seven anthocyanin biosynthetic genes (PAL, CHS, CHI, F3H, DFR, LDOX and UFGT) was investigated in samples taken throughout the development of grape berry skin tissues (Fig. 2.7). Northern blot analysis indicated that anthocyanin pathway gene expression occurred in two phases. Most genes in the pathway were briefly expressed early in berry development and again after véraison, when colour development occurred. All of the anthocyanin genes examined except UFGT were expressed in flowers and in the berry skin up to four weeks postflowering. In some cases (PAL, CHS and LDOX), maximum expression occurred in the flowers, whereas CHI, F3H and DFR showed maximum expression in the berry sample two weeks postflowering. There was then a reduction in expression of these genes six to eight weeks postflowering, which coincided with the observed lag phase in berry volume increase (see Section 2.3.1). It should be noted that high level expression of invertase genes was detected in the same RNA samples used in this study for this specific period of berry development (Davies and Robinson 1996). This demonstrates, first, that the total RNA samples extracted at these times were intact and, second, that the decline in expression of anthocyanin genes does not reflect a general reduction in mRNA production in grape berry skin at this stage of development. Following

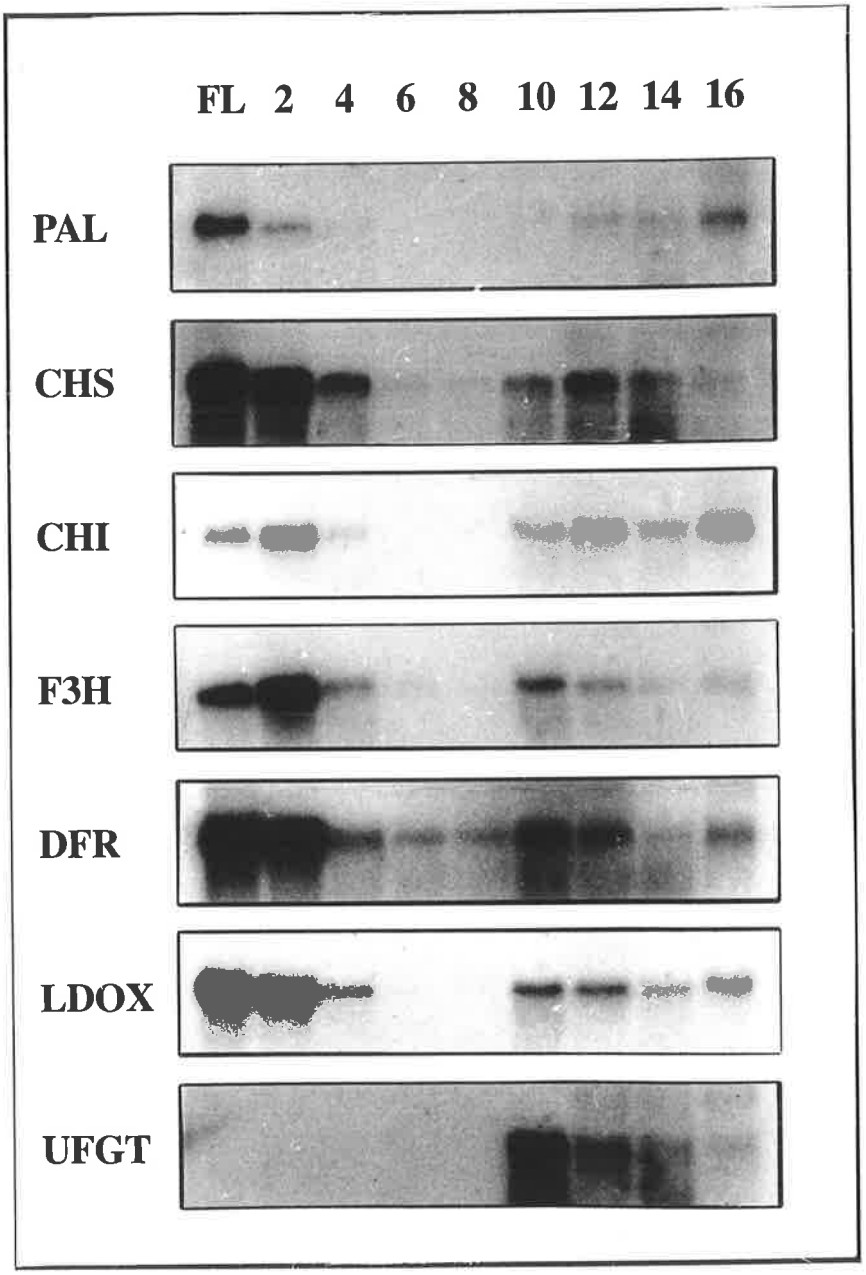


Figure 2.7 Temporal expression of anthocyanin biosynthesis genes in grape berry skin during berry development. Northern blots are of total RNA from grape flowers and grape berry skin samples taken at eight different stages during development, probed with grape cDNA clones for PAL, CHS, CHI, F3H, DFR, LDOX, and UFGT. FL denotes flower RNA and the numbers indicate weeks postflowering at which berry skin RNA was extracted.

this period of little or no gene expression, there was a coordinate increase in expression of all the genes except PAL in the ten weeks postflowering sample at approximately the time of véraison. Expression of these genes then continued throughout the remainder of berry development. The expression of PAL showed a similar increase following véraison but did not commence until 12 weeks postflowering. Thus, all of the genes of the anthocyanin pathway showed a similar pattern of expression except for the UFGT gene, which was found to be expressed only ten to 16 weeks postflowering and this expression coincided precisely with the accumulation of anthocyanin pigments in the berry skins (Fig. 2.2E).

2.3.4 Expression of anthocyanin biosynthesis genes in Shiraz berry flesh

The expression of the seven anthocyanin biosynthesis genes was also studied in four Shiraz berry flesh samples taken four, eight, 12 and 16 weeks postflowering (Fig. 2.8). These tissues contained no detectable anthocyanins. No PAL or UFGT mRNA was detected in any of the samples tested. Both CHS and LDOX mRNA levels were at a maximum four weeks postflowering and subsequently decreased during development. The other genes tested (CHI, F3H, and DFR) showed maximum mRNA levels four weeks postflowering and a minimum expression four weeks later. Expression was again detected in the 12 and 16 week postflowering samples but at a lower level than observed four weeks postflowering.

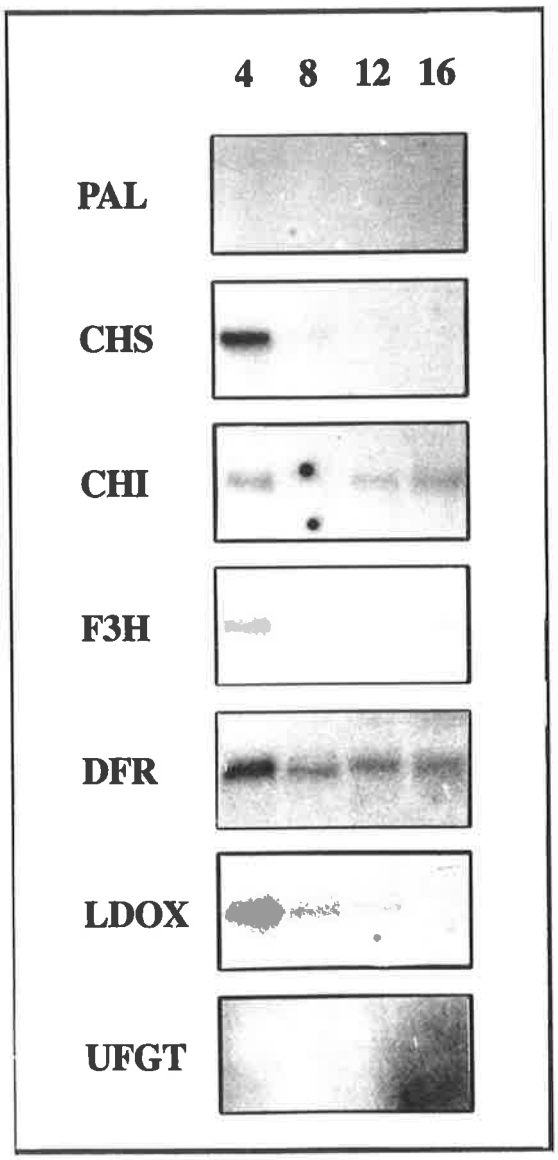


Figure 2.8 Temporal expression of anthocyanin biosynthesis genes in grape berry flesh tissue during berry development. Northern blots are of total RNA from grape berry flesh samples taken at four different stages during development, probed with grape cDNA clones for PAL, CHS, CHI, F3H, DFR, LDOX, and UFGT. Numbers indicate weeks postflowering at which berry flesh RNA was extracted.

2.4 Discussion

The increase in volume of the developing grape berries displayed a double sigmoid growth curve. This is typified by two periods when berry volume increases separated by a lag phase of little or no change (Fig. 2.2A). The onset of ripening (véraison) at the end of the lag phase, eight weeks postflowering, is characterised by an increase in the deformability of the berries (Fig. 2.2B) and an accumulation of both soluble solids and anthocyanins (Figs. 2.2C and 2.2E). Proanthocyanidin levels are at their greatest early in berry development and these levels decrease through to harvest in both berry skins and flesh (Fig. 2.2D).

HPLC analysis showed that the anthocyanin accumulation during the ripening period was predominantly due to the production of malvidin-based anthocyanin derivatives, although a broad range of anthocyanins are produced. The concentration of anthocyanins present in the berry skins did not decrease towards the end of ripening and there was no evidence for the conversion of cyanidin-based anthocyanins to delphinidin derivatives as reported by Roggero *et al.* (1986). However, there was evidence for the increased accumulation of acylated anthocyanins during ripening (Fig. 2.6A). The rate of flux down either "branch" of the anthocyanin biosynthesis pathway (Fig. 1.5) appeared to be almost constant throughout ripening (Fig. 2.6C).

Northern analysis of the expression of the anthocyanin biosynthetic genes in Shiraz berry skins supports the finding that anthocyanin accumulation continues throughout ripening. All the genes studied were expressed in samples taken at the four different time points after véraison. This suggests that the enzymes of the anthocyanin biosynthetic pathway encoded by these genes are being translated and catalysing their respective reactions. Surprisingly,

expression of all these genes, except UFGT, was detected in flowers and in berry skin up to at least two weeks after flowering. No anthocyanins could be detected in these samples, presumably because they were not being synthesised due to a lack of UFGT. It is possible that these flavonoid pathway genes are expressed in tissues not producing flavonoids. Nevertheless, the early steps of the pathway depicted in Fig. 1.4 are common to other biosynthesis pathways, notably those involved in the biosynthesis of aurones, flavones, flavonols, isoflavonoids and proanthocyanidins, and this may explain expression of these genes in the absence of anthocyanin synthesis in the flowers and young berries. In the floral tissues, it is likely that flavonols are being produced, as they are known to accumulate in the ovaries of petunia flowers (Koes *et al.* 1990), and are essential for pollen viability in maize and petunia (Mo *et al.* 1992). It is also possible that any flavonols produced could be protecting the developing tissues from UV damage (Schmelzer *et al.* 1988; Li *et al.* 1993). The young developing berries could also be producing a number of flavonoid derived compounds. For example, isoflavonoids (flavonoid derivatives) may play a role in protecting the young berries from various phytopathogens (Lamb *et al.* 1989) or as a feeding deterrent to insects (Caballero *et al.* 1986). Young developing seeds (which may be present in the flower sample) may also accumulate leucoanthocyanidins as appears to occur in petunia (Koes *et al.* 1990). Expression of the anthocyanin genes up to and including DFR would be necessary for proanthocyanidin production, and these compounds were detected in young berries and flowers (Fig. 2.2D). Nevertheless, the observed expression of LDOX in these early samples is puzzling. LDOX is the putative leucoanthocyanidin dioxygenase required for the first of two enzymatic steps between leucoanthocyanidins and anthocyanidins (Martin and Gerats 1993a), the other step probably being catalysed by a putative dehydratase (Heller and Forkmann 1988). Any intermediates between these

reactions are presumed to be unstable (Heller and Forkmann 1988) and no coloured anthocyanidins were detected. ◆

The expression pattern seen for PAL in the skin of the developing Shiraz berries (Fig. 2.7) is consistent with the changes in PAL activity seen in the skins of two coloured grape varieties studied by Kataoka *et al.* (1983). Hrazdina *et al.* (1984) also found PAL activity early in berry development and after véraison. Thus, the gene expression and activity data suggest that the activity of PAL is under transcriptional control in grape berries. Hrazdina *et al.* (1984) also measured CHS, CHI and UFGT activity during berry development, and the CHI and UFGT activities are consistent with the patterns of gene expression observed (Fig. 2.7). However, CHS activity was not detected early in berry development, which is a surprising result given that CHI activity, an enzyme after CHS in the flavonoid pathway, was observed during this stage of development (Hrazdina *et al.* 1984), and that flavonoids (such as flavonols and proanthocyanidins) may be synthesised in these tissues. The clarification of the relationship between CHS gene expression and activity during grape berry development awaits further study.

In Shiraz grape berries, anthocyanins accumulate in the skin, but not in the flesh. The pattern of expression seen in the berry flesh samples was similar to that in the berry skin, except no PAL and UFGT expression was detected, and CHS was not expressed late in development. Both PAL and CHS are encoded by multigene families in grapes (Sparvoli *et al.* 1994), and thus other gene family members, not detected by the probes used in this northern analysis study, may be expressed in this tissue. Only one UFGT gene seemed to be present in the grape genome (Sparvoli *et al.* 1994) and this was not expressed in the berry flesh tissue. The data presented in Figure 2.2D suggest that proanthocyanidins are synthesised in berry flesh early in development and that their concentration decreases during

◆see page 75

ripening.◆ The northern data supports this hypothesis in that expression of all the genes except PAL and UFGT is detectable in the early flesh samples but not in the samples taken late in development. If CHS substrates were not already present, PAL gene expression would be required for proanthocyanidin production, therefore our inability to detect PAL mRNA may be due to the origin of the PAL probe which was used as discussed above.

Anthocyanins begin to accumulate at about véraison and this coincides with the increase in expression of all seven genes tested from the anthocyanin biosynthesis pathway. This suggests that there is coordinate regulation of all these genes at this time in the developing grape berry skin and that the control of anthocyanin biosynthesis in grape berry skins is mainly transcriptional. Sparvoli *et al.* (1994) have also shown that as anthocyanins accumulate in dark-grown grape seedlings subsequently exposed to light, there is a coordinate induction of the genes from the committed steps of the anthocyanin biosynthetic pathway (CHS, CHI, F3H, DFR, LDOX, UFGT). This is similar to the control of the anthocyanin biosynthesis pathway in maize aleurone, which is regulated by the *R* and *C1* gene families (Dooner *et al.* 1991). Nevertheless, the pattern of expression seen in the flower and young berry skin samples prior to véraison suggests that UFGT expression is under a different regulatory regime. In these samples, all of which did not possess anthocyanins, UFGT was the only structural gene tested which was not induced.

The start points for the control of the anthocyanin pathway in the species most studied (maize, snapdragon and petunia) are different but all appear to be controlled by *myc*- and *myb*-like transcription factors (see Section 1.7.3). The structural genes from the grape anthocyanin biosynthesis pathway may also be controlled by *myc*- and *myb*-like regulatory genes. However, the way in which the structural genes are regulated in grape berry skins appears to be different to the patterns observed in snapdragon, petunia and maize. The

◆see addendum

pattern of gene expression in grape berry skins could be explained in relation to regulatory genes in two ways. First, two types of regulatory genes may be active in the berry skin, one of which is expressed early and which induces expression of all the structural genes except UFGT and another that is expressed later and results in the induction of expression of all the structural genes. Alternatively, two types of regulatory genes may be present, one that controls expression of PAL, CHS, CHI, F3H, DFR and LDOX, and another that induces UFGT gene expression. In this case the regulatory gene that controls expression of PAL, CHS, CHI, F3H, DFR, LDOX is expressed early in berry development, whereas both the regulatory genes are expressed as the grape ripens, resulting in induction of all the genes and thus anthocyanin biosynthesis. In either case, it appears that the major control point of anthocyanin biosynthesis in grape berry skins is UFGT and this control is later in the pathway than has been observed in the studies into maize, petunia and snapdragon anthocyanin biosynthesis.

Chapter 3

Flavonoid gene expression in various grapevine tissues and in the berry skins of different grape cultivars

3.1 Introduction

The results presented in Chapter 2 suggest that the control point of anthocyanin biosynthesis in grape berry skins is beyond the LDOX step in the anthocyanin synthesis pathway. Interestingly, it was also found that all of the genes tested except UFGT were expressed in grape flowers and early in berry development in the absence of anthocyanin production. The genes early in the anthocyanin synthesis pathway are required for proanthocyanidin synthesis, and these compounds were found in the non-pigmented flowers and young berries. The goal of the work presented in this chapter was to investigate the pattern of expression of the genes from the flavonoid synthesis pathway in other grapevine tissues to see if the expression of 'early' pathway genes observed in flowers and young berries also occurs in other unpigmented grapevine tissues. The expression of flavonoid genes in white grape berry skins was also investigated. It is thought that the progenitors of all modern grape varieties possessed black berries (Slinkard and Singleton 1984), and that white varieties have been derived from black varieties as bud sports or by other somatic mutations. The grape anthocyanin cDNA clones described in Section 2.2.6 were used to study the expression of the flavonoid synthesis pathway genes in the skins of various black and white grape cultivars. The expression patterns of these genes may reveal information about the nature of mutations leading to the loss of anthocyanin synthesis in white grape berry skins.

3.2 Materials and methods

3.2.1 Plant material

Samples were taken from various Shiraz grapevine tissues from John Harvey's Slate Creek vineyard in Willunga, South Australia. In all cases the tissue was immediately frozen in liquid nitrogen, transported back to the laboratory on dry ice and stored at -80°C pending further analysis. These grapevine tissues included: unexpanded leaves (young leaf); leaves expanded to ca. 5 cm (mid leaf); fully expanded leaves (old leaves); young tendrils; green cane tissue; seeds collected four weeks after flowering; and flowers. The berry skin and flesh tissue were obtained by peeling whole berries (14 weeks postflowering) as described in Section 2.2.1. To obtain root tissue, Shiraz canes were rooted and young root tips were harvested for extraction.

Skins from the berries of eight different grape cultivars were obtained from several sites in South Australia as listed below. In all cases, whole bunches were cut from the vine, and transported to the laboratory on ice. Individual berries were then scored for total weight and skin weight, with the skins being separated from seeds and flesh by squeezing whole berries. The juice was pooled and a combined measurement of total soluble solids (°Brix) taken using a refractometer (model 10430, Reichert, Vienna, Austria). Skins were then frozen in liquid nitrogen and stored at -80°C pending further analysis. Leaf tissue was also obtained from the same grapevines as a source of material for DNA extraction.

Grapes from the cultivars Riesling, Semillon, Chardonnay and Shiraz were obtained from John Harvey's Slate Creek vineyard in Willunga, South Australia. Sultana and Muscat Gordo were sampled from the Waite Agricultural Research Institute's collection at Urrbrae, South Australia. Pinot Noir grapes were obtained from the collection of the South Australian Research and Development Institute at Nuriootpa and the Cabernet Sauvignon grapes were collected from a commercial vineyard in Langhorne Creek, South Australia owned by Mac Cleggett.

3.2.2 Total RNA extraction and northern analysis

Total RNA was extracted from the various grapevine tissues and the skin samples from the red and white cultivars using the method described in Section 2.2.5. Radiolabelled probes were prepared and northern analysis carried out as described in Sections 2.2.6 and 2.2.7.

3.2.3 Anthocyanin and proanthocyanidin extraction and quantification

Anthocyanins were extracted and quantified by following the methods in Section 2.2.2 and proanthocyanidin analysis was carried out as described in Section 2.2.4.

3.2.4 Grape genomic DNA extraction

The method of Thomas *et al.* (1993) was used to extract genomic DNA from grape leaves which had been stored at -80°C. Two grams of leaf tissue were ground to a

powder with sand and liquid nitrogen using a mortar and pestle. The frozen powder was then transferred to 25 mL of crude nuclei chromatin buffer (0.25 M NaCl, 0.2 M Tris-HCl [pH 7.6], 2.5% PVP, 0.05 M EDTA, 0.1% β-mercaptoethanol) at 4°C and mixed by inversion. The plant material was pelleted by centrifugation at 4100*g* for 10 min at 4°C and the supernatant discarded. The pelleted was resuspended in 5 mL of DNA extraction buffer (0.5 M NaCl, 0.2 M Tris-HCl [pH 8.0], 2.5% PVP, 0.05 M EDTA, 3% sarkosyl, 20% ethanol, 1% β-mercaptoethanol) by incubating at 65°C for 30 min with occasional shaking. An equal volume of chloroform:isoamyl alcohol (24:1) was then added and the phases separated by centrifugation at 4100*g* for 10 min at 20°C. The upper aqueous phase was collected and the nucleic acid precipitated following the addition of 0.54 volumes of isopropanol. The nucleic acid was then pelleted by centrifugation at 10000*g* for 10 min and the pellet washed briefly with 70% ethanol. The pellet was resuspended in 600 µL of TE buffer and the contaminating RNA removed by incubation with RNase A (10 µg mL^{-1}) at 37°C for 30 min. The mixture was then spun at 10000*g* for 10 min, the supernatant recovered and mixed with 0.5 volumes of 7.5 M ammonium acetate and centrifuged again at 10000*g* for 15 min. The DNA in the supernatant was then precipitated with 0.54 volumes of isopropanol and pelleted at 10000*g* for 10 min following a 10 min incubation at room temperature. The DNA pellet was washed in 70% ethanol, dried under vacuum and resuspended in 200 µL of water.

3.2.5 Southern blot analysis

DNA aliquots of 5 µg were digested with restriction endonucleases and fractionated on a 0.8% agarose gel in 1 × TAE (40 mM acetic acid, 1 mM EDTA, 40 mM Tris-HCl

[pH 8.0]) buffer. To aid transfer, DNA fragments were depurinated by incubating the gel in 0.2 M HCl for 20 min. The DNA was transferred to ZetaProbe membrane (Bio-Rad) according to the manufacturer's instructions. Membranes were prehybridised for 2 h at 65°C in 0.25 M sodium phosphate (pH 7.0), 1 mM EDTA (pH 8.0), 7% SDS and then hybridised for 15 h under the same conditions following the addition of denatured ^{32}P-labelled UFGT probe, prepared by random primer labelling (see Section 2.2.6). The UFGT probe used was a grape cDNA clone called Vv*ufgt*2, which is described in detail in Section 7.3.1. The membranes were then washed twice for 10 min in 2 × SSC (150 mM NaCl and 15 mM tri-sodium citrate, pH 7.0) and 0.1% (w/v) SDS (65°C) and then for 15 min in 1 × SSC and 0.1% (w/v) SDS (65°C). The membranes were exposed to Kodak XAE film with intensifying screens at -80°C.

3.3 Results

3.3.1 Anthocyanin and proanthocyanidin content and expression of anthocyanin synthesis pathway genes in various Shiraz grapevine tissues

Total RNA was extracted from different tissues of the red grape cultivar Shiraz as described in Section 2.2.5 in order to investigate the expression of seven genes from the anthocyanin synthesis pathway. The pattern of expression of each gene tested was similar in each of the tissues with the exception of UFGT (Fig. 3.1). Seeds and mid-leaves showed the highest levels of expression of PAL, CHS, CHI, F3H, DFR and LDOX, but significant expression of these six genes was also detected in the young leaf, tendril, green cane, root and flower RNA samples. Lower levels of expression were also

detected for these six genes in fully expanded leaves and berry skin tissue, but in the berry flesh tissue only DFR expression was detected. UFGT was detected only in the berry skin sample, and this was also the only sample in which anthocyanins were detected (Table 3.1). Together with the data presented in Chapter 2, these results suggest that the main point of control of the anthocyanin production in grapevine is after the LDOX catalysed step in the synthesis pathway (Fig. 1.4). Expression of all the flavonoid synthesis pathway genes, except UFGT, was detected in all the unpigmented tissues except berry flesh. Most of the intermediates of the anthocyanin pathway (Fig. 1.4) are precursors of other biosynthesis pathways, and this may explain why the expression of these genes occurs in the absence of anthocyanin synthesis in unpigmented tissues. For example, expression of the flavonoid synthesis pathway genes up to and including DFR would be necessary for production of proanthocyanidins, which are precursors to tannin synthesis. Proanthocyanidins were detected in all of the above grapevine tissues (Table 3.1) using a vanillin-HCl assay. There was a qualitative correlation between the level of expression of the flavonoid synthesis pathway genes (other than PAL) and the concentration of proanthocyanidins in the various tissues. For example, seeds and flowers contained large amounts of proanthocyanidins (Table 3.1) and also showed high levels of flavonoid synthesis pathway gene expression (Fig. 3.1), whereas roots and flesh had low concentrations of proanthocyanidins and little flavonoid synthesis pathway gene expression.

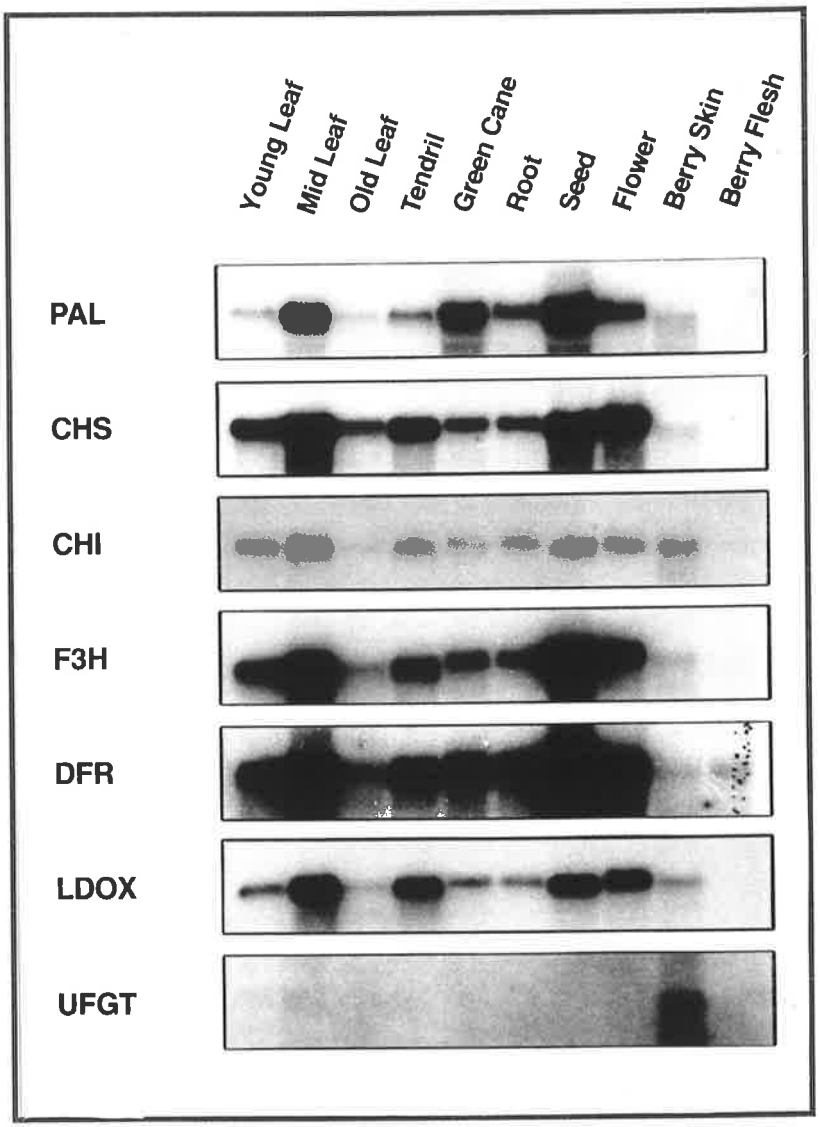


Figure 3.1 Expression of anthocyanin biosynthesis pathway genes in various grapevine tissues. Northern blots are of total RNA from grapevine tissue samples probed with grape cDNA clones as indicated on the left side of the figure.

Table 3.1 *The concentration of proanthocyanidins and anthocyanins in various Shiraz grapevine tissues. n/d = not detected (<2A_{520}/g fresh weight)*

Grapevine Tissue	Proanthocyanidins (mg/g fresh weight)	Anthocyanins (A_{520}/g fresh weight)
Young Leaf	5.2	n/d
Mid Leaf	18.0	n/d
Old Leaf	23.8	n/d
Tendril	13.5	n/d
Green Cane	9.9	n/d
Root	2.1	n/d
Flower	46.8	n/d
Seed	57.0	n/d
Berry Skin	3.3	212.4
Berry Flesh	0.6	n/d

3.3.2 Anthocyanin and proanthocyanidin content and expression of anthocyanin synthesis pathway genes in red and white grape cultivars

Having observed that the major control point of anthocyanin biosynthesis in red Shiraz grape tissues was beyond LDOX, the expression of the various flavonoid synthesis pathway genes in the skins of red and white grapes was investigated to determine why white grapes do not synthesise anthocyanins. Skin tissue was sampled from the ripening berries of five white cultivars and three red cultivars as described in Section 3.2.1 above. Expression of flavonoid synthesis pathway genes is dependent on the stage of berry ripening. Soluble solids increase throughout the ripening of grapes (see Fig. 2.2C) and °Brix is used as a measure of the stage of ripening. Soluble solids in these samples

ranged from 13.5 °Brix for Muscat Gordo to 22.7 °Brix for Sultana (Table 3.2). The data in the previous chapter shows that flavonoid synthesis pathway genes are expressed in Shiraz berry skins throughout this phase of berry ripening (Figs. 2.2C and 2.7). Using northern analysis, mRNAs homologous to each of the anthocyanin synthesis pathway genes were detected in the Pinot Noir and Shiraz samples, and all of the genes except PAL were expressed in the Cabernet Sauvignon sample (Fig. 3.2). In the white cultivars, expression of the genes was generally much lower than in the red cultivars, but the white cultivars could be divided into two different groups based on the observed patterns of expression (Fig. 3.2). One group, consisting of Riesling, Semillon and Chardonnay, showed moderate levels of expression of all the genes tested except UFGT. In the other group, containing Muscat Gordo and Sultana, all the genes were expressed at very low or non-detectable levels. PAL, F3H or UFGT expression could not be detected in the Muscat Gordo sample and PAL, CHS, F3H, LDOX or UFGT expression was not detected in the Sultana berry skins.

None of the white cultivars possessed any detectable anthocyanins, but they all contained proanthocyanidins (Table 3.2). Thus, it would be expected that the enzymes of the flavonoid synthesis pathway down to DFR might be present in the white berry skins. There was no qualitative correlation between the proanthocyanidin levels in the berry skin samples and the expression of the flavonoid genes (Table 3.2 and Fig. 3.2) as was seen for other grapevine tissues (Table 3.1 and Fig. 3.1). This may be due to the stage of development at which the skin tissue was sampled. It appears that proanthocyanidin levels are highest early in berry development and then decrease throughout ripening (Fig. 2.2D) which suggests that most of the proanthocyanidins are produced very early in

Table 3.2 *The concentration of proanthocyanidins and anthocyanins in the berry skins of white and red grape cultivars. Soluble solids (measured as °Brix) were also measured to give an indication of the ripeness of the berries. n/d = not detected (<2A_{520}/g fresh weight)*

Cultivar	°Brix	Proanthocyanidins (mg/g fresh weight)	Anthocyanins (A_{520}/g fresh weight)
Riesling	14.8	2.1	n/d
Muscat Gordo	13.5	6.2	n/d
Semillon	17.0	8.7	n/d
Chardonnay	21.3	5.1	n/d
Sultana	22.7	2.0	n/d
Cabernet Sauvignon	15.5	2.4	84.4
Pinot Noir	21.6	3.5	137.8
Shiraz	20.8	3.3	212.4

berry development. Thus, the expression seen after véraison may not have as great an influence on total proanthocyanidin levels as the expression of these genes seen immediately after fruit set (see Fig. 2.7).

Expression of UFGT was detected in all of the red grapes, but not in any of the white grapes. The absence of detectable UFGT mRNA suggests that the lack of anthocyanin production is due to the complete absence of this gene or a change in the transcriptional control of UFGT rather than a point mutation in UFGT or another gene of the pathway. Furthermore, the differences in the patterns of anthocyanin gene expression seen in Muscat Gordo and Sultana compared to Riesling, Semillon and Chardonnay suggests that the mutations leading to the lack of anthocyanin production affect the control of these genes in different ways and have thus arisen independently.

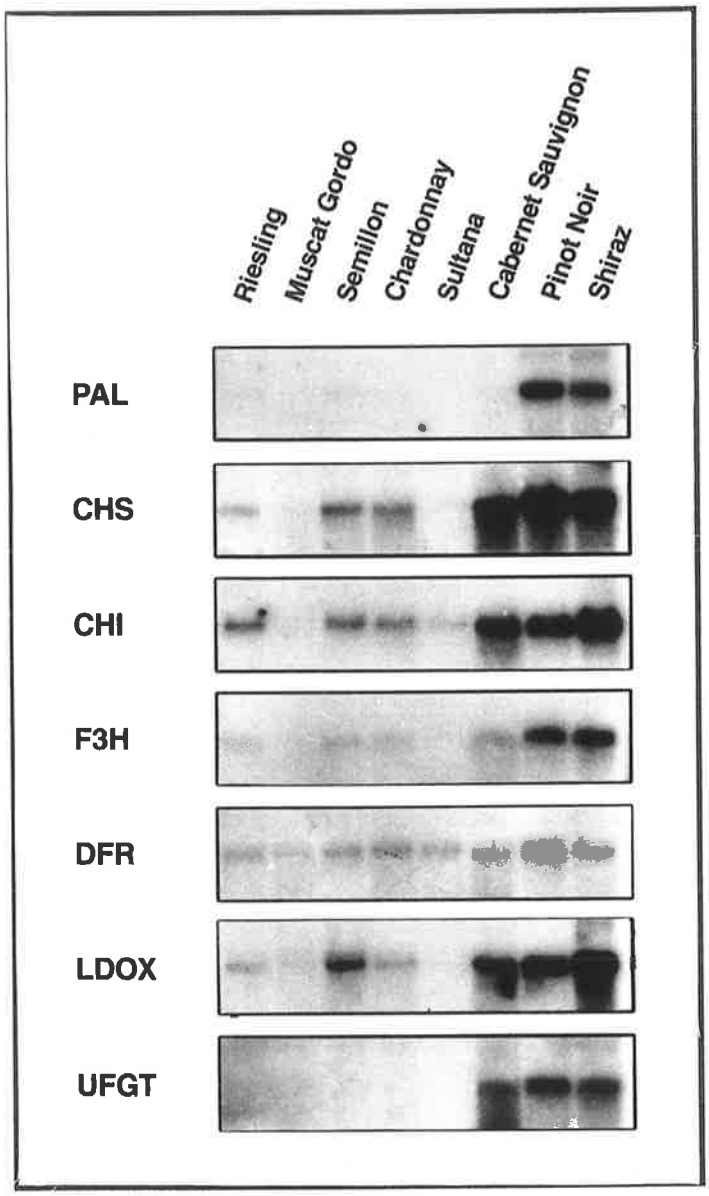


Figure 3.2 Expression of anthocyanin synthesis pathway genes in the skins of white and red grape cultivars. Northern blots are of total RNA from grape berry skin samples probed with grape cDNA clones as indicated on the left side of the figure.

3.3.3 Southern analysis of UFGT genes in red and white grape varieties

The gene expression data suggests that the major control of whether a grape berry will produce anthocyanins is the expression of UFGT. UFGT is the only gene tested which showed an absolute differential expression pattern between the white and coloured grape berry skins. This raises the possibility that this gene is absent in these grape varieties. To test this, the presence of UFGT genes in the genomes of the white-berried grapevines was investigated using Southern analysis (Fig. 3.3). Genomic DNA was isolated from each of the varieties investigated in this study, digested with the restriction enzymes *Eco* RV or *Dra* I and Southern blotted. When probed with a grape UFGT cDNA clone, hybridising bands were detected in each of the samples. This suggests that both the white- and coloured-berried varieties possess at least part of the UFGT gene(s). This indicates that lack of UFGT expression may be due to an aberration in the transcriptional control of this gene, perhaps due to the absence of a functional promoter rather than the absence of UFGT-related sequences in the genomes.

It is interesting to note the different restriction patterns that the UFGT genes possess in these varieties when *Eco* RV was used to digest the DNA. Both the restriction enzymes used are insensitive to ^{m5}CG and ^{m5}CNG methylation (McClelland and Nelson, 1988), nevertheless, *Eco* RV gave variable restriction patterns whereas *Dra* I digests gave exactly the same restriction pattern for all of the varieties.♦ This suggests that the *Eco* RV site in the UFGT gene in grapevines is in a 'hotspot' for mutations, or perhaps a crossover region, whereas the *Dra* I site is not.

♦ see addendum

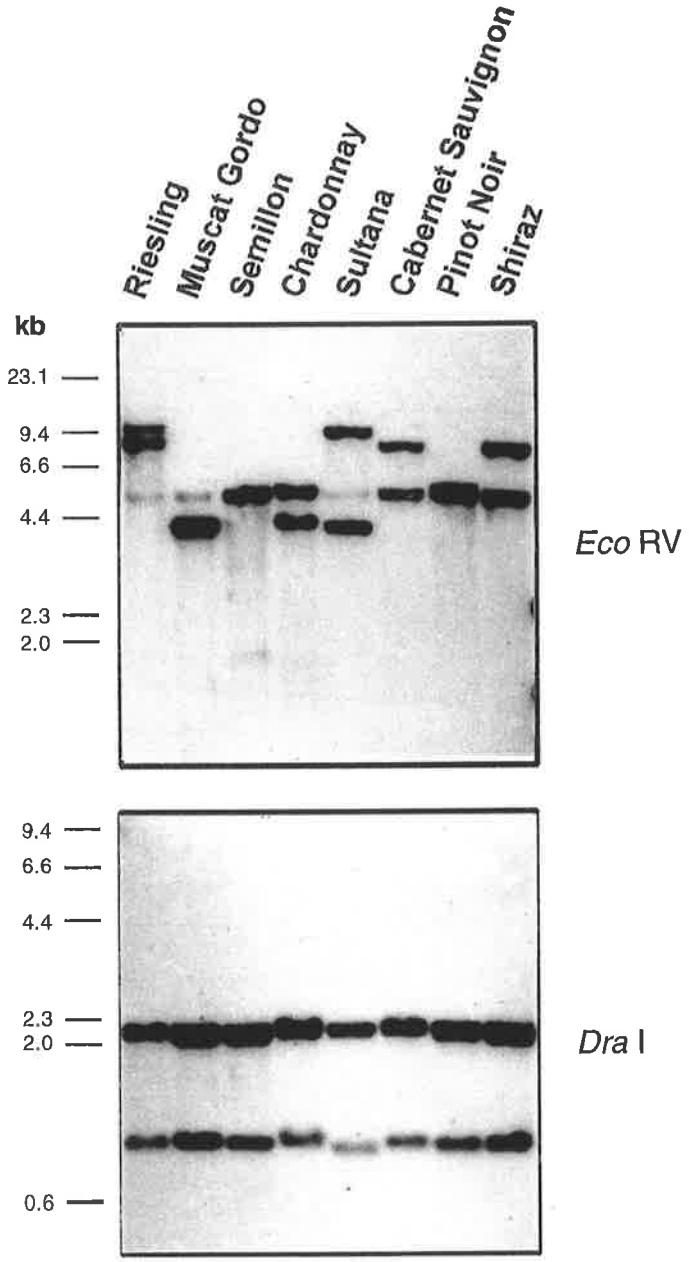


Figure 3.3 Southern analysis of genomic DNA from the various grapevine cultivars probed with a full-length UFGT cDNA clone. Southern blots are of genomic DNA from each of the grapevine cultivars, digested with *Eco* RV (top) or *Dra* I (bottom) and probed with a full-length cDNA clone coding for UFGT. Size standards are indicated on the left of each figure.

3.4 Discussion

The data presented in this chapter support the findings in Chapter 2 which suggest that the first major control point of anthocyanin biosynthesis in grapevine is beyond the LDOX step in the pathway. In all unpigmented grapevine tissues except berry flesh, expression of all of the genes from the pathway was detected with the exception of UFGT (Fig. 3.1). Also, in the skins of three white-skinned grape varieties (Riesling, Semillon and Chardonnay), a similar pattern, with only UFGT expression undetectable, was seen (Fig. 3.2). Muscat Gordo and Sultana berry skins had a much reduced expression of all of the anthocyanin synthesis pathway genes and no detectable UFGT mRNA expression (Fig. 3.2). The only gene that displayed an absolute differential expression pattern between pigmented and non-pigmented tissues was UFGT and it seems that this gene is under a different regulatory control than the rest of the anthocyanin synthesis pathway genes. This difference in control is both temporal, as is the case in the berry skin during development (Chapter 2), and spatial, as can be seen in the expression patterns of UFGT compared to the other anthocyanin synthesis pathway genes in various grapevine tissues (Fig. 3.1).

The expression of most of the anthocyanin pathway genes in unpigmented grapevine tissues (Fig. 3.1) mirrors that seen in the skin of young berries (Fig. 2.7). As is depicted in Figure 1.4, the intermediates of the anthocyanin pathway are also precursors of other biosynthesis pathways, the products of which have many functions in plants (reviewed by Koes *et al.* 1994). These genes may be expressed in unpigmented tissues as these flavonoid products are essential to the viability of the plant. The production of

proanthocyanidins in these unpigmented tissues could explain the expression of flavonoid synthesis pathway genes in the absence of anthocyanin synthesis. Proanthocyanidins were detected in all unpigmented tissues (Table 3.1), and their production requires the expression of the anthocyanin genes up to and including DFR. This would not explain why LDOX is expressed in these tissues given the pathway depicted in Figure 1.4. LDOX is believed to catalyse the first of two enzymatic steps between leucoanthocyanidins and anthocyanidins, and the second step is presumed to be catalysed by a dehydratase (Heller and Forkmann 1988). Detection of intermediates between these steps is difficult as they are believed to be unstable. The pathway in Figure 1.4 would only involve LDOX in the synthesis of anthocyanins, so it is not clear why this gene is expressed in tissues where anthocyanins are not synthesised. Perhaps the enzyme encoded by the LDOX gene has a loose specificity and thus is involved in other reactions involving flavonoids. However, there is also the possibility that flavonoid synthesis pathway genes can be expressed in tissues not producing flavonoids.

The simplest explanation for the difference in control of UFGT and the rest of the flavonoid synthesis pathway genes is that they can be controlled by different regulatory genes which are differentially expressed. The existence of several regulatory regimes for the flavonoid synthesis pathway genes has been confirmed in other species. In maize, it has been shown that anthocyanin synthesis requires both a *myb*-homologous gene and a *myc*-homologous gene (Dooner *et al.* 1991) and a physical interaction between the *R/B* (*myc*-like) and *C1*(*myb*-like) proteins has been demonstrated (Goff *et al.* 1992). However, a subset of the flavonoid synthesis pathway genes, which are required for phlobaphene synthesis, can also be under the transcriptional control of the maize *P* gene,

which is a *myb*-homologue (Grotewold *et al.* 1991). The *P* gene could bind to and activate the DFR gene but not the UFGT gene, and unlike the *C1 myb*-like gene, did not require the *R* gene product for activation (Grotewold *et al.* 1994). The *R* gene could not alleviate the inability of *P* to activate UFGT (Grotewold et al. 1994). This work showed that although *P* and *C1* both encode *myb*-like binding proteins and activate an overlapping set of genes, there is a difference in their specificity which seems to be influenced by their differential requirement for the *R*-like gene. It is possible that in post-véraison berry skin a transcription factor (or factors) which has the ability to activate all of the anthocyanin pathway synthesis genes is present, but in other tissues a different regulatory gene product is produced which activates only a subset of these genes. Nevertheless, there are different types of control seen for flavonoid synthesis pathway genes. In snapdragon floral tubes, the genes below CHI of the anthocyanin synthesis pathway are induced when anthocyanins accumulate and this process involves the *myc*-like regulatory gene *Delila* (Almeida *et al*, 1989; Martin *et al.* 1991). However, the *Delila* gene does not appear to be required for the expression of these same genes in snapdragon flower lobes. Martin and Gerats (1993b) suggest that combinations of transcription factors must be involved in determining the pattern of pigment production in snapdragon flowers. Two *myb*-like genes (*myb305* and *myb340*) were also shown to be able to regulate some of the flavonoid synthesis pathway genes (Moyano *et al.* 1996). These regulatory genes are expressed in the same tissues, seem to compete for the same DNA binding site and have differential affects on the activation of transcription. They may represent a close control of the production of a secondary metabolite that can be adjusted during developmental and environmental changes by the discreet action of each *myb*-like gene or via their interaction (Moyano *et al.* 1996). In the various grape tissues

there is much variation in the levels of expression of the flavonoid synthesis pathway genes. Perhaps there is a similar tight regulation of the genes in this pathway that can be adjusted depending on the development of the tissue and in response to external stimuli. This could be achieved by the same regulatory genes in the different tissues or by a family of regulatory genes, members of which are expressed in different tissues and have a differential affect on the activation of flavonoid synthesis pathway genes. Unfortunately, this complexity of control may make the identification of the genes required for the regulation of anthocyanin synthesis difficult as expression patterns alone may not indicate that a specific regulatory gene is produced and subsequently activating the structural genes. Also demonstrating that a transcription factor can bind to the promoters of structural genes *in vitro* does not mean that it is activating transcription *in vivo*.

It is quite likely that the white grape has arisen independently a number of times from different red varieties. The mutations could be different in each case, and it is not surprising that patterns of gene expression were different in the various white varieties (Fig. 3.2). The loss of UFGT gene expression in Riesling, Semillon and Chardonnay is not accompanied by the absence of expression of any of the other structural genes tested. It is possible that the UFGT promoter has been mutated in some way in each of these white-skinned varieties which has resulted in a loss of UFGT gene expression. The Southern analysis of UFGT in the various grape varieties (Fig. 3.3) suggests that one region of this DNA is very different in each variety and may represent a 'hotspot' for mutations or perhaps a crossover region. Thus, it is possible that the UFGT promoter is more likely to be mutated than other structural or regulatory genes and thus loss of

anthocyanin synthesis is quite often due to a loss of UFGT gene expression. However, it seems that the other structural genes in the pathway, although still expressed, show a reduced level of expression as compared to the black-skinned varieties. This may represent a feed-back inhibition of the early pathway genes when a product produced further down the pathway accumulates. The product beyond LDOX however has not been identified as it is extremely unstable and thus unlikely to accumulate. There was evidence of elevated proanthocyanidin levels in Muscat Gordo, Semillon and Chardonnay, but this was not the case for the other two white-skinned varieties, Riesling and Sultana (Table 3.2). Perhaps the loss of UFGT gene expression in these varieties is due to the absence of a regulatory gene product. Loss of anthocyanin synthesis can arise by mutations in individual structural genes in the flavonoid pathway, or by alterations of regulatory genes controlling expression of a number of the structural genes. In many instances, lack of production of anthocyanin pigments is the result of mutations in these regulatory genes resulting in decreased expression of a number of structural genes in the pathway. A similar picture is apparent in the skins of white grapes, where there were lower levels of expression of most of the genes in the flavonoid synthesis pathway (Fig. 3.2). This suggests that alterations of regulatory genes may have occurred in these varieties resulting in decreased synthesis of a number of enzymes of the pathway, preventing accumulation of anthocyanins. Expression of UFGT was not detected in any of the white grapes (Fig. 3.2) which may result from mutations of this gene or mutations of a separate regulatory gene controlling its expression, which appears to be independent of the other flavonoid genes in grapevine. The two patterns of expression shown by the white-skinned varieties suggest that there may be more than one regulatory factor involved in UFGT gene expression. With the pattern seen in Riesling, Semillon and

Chardonnay, it could be that a regulatory factor vital for UFGT expression, but which has a qualitative effect on the rest of the pathway is not present or active. However, Muscat Gordo and Sultana have most of the pathway repressed, which may be due to mutations in another regulatory factor which is necessary for the activation of gene expression for most of the early pathway genes as well as UFGT. These white-skinned varieties provide evidence that the anthocyanin synthesis pathway in grapevine has more than one control regulating expression of the structural genes. It is surprising that there were no cases where the expression of an early gene (for example CHS) alone was absent, or where all the genes were expressed, but anthocyanin synthesis was inhibited by a mutation in an early gene resulting in a loss of function of the resultant enzyme. This may reflect natural selection against these mutants as they may lack essential, flavonoid based compounds, or selection against such white-skinned grapevines by man as they may be less useful for wine-making or perhaps have other undesirable properties such as decreased disease resistance.

The northern analyses of anthocyanin gene expression in berry flesh tissues shown in Figs. 2.8 and 3.1 appear to be inconsistent. Expression of CHI, F3H and DFR was detected at 12 and 16 weeks postflowering in Figure 2.8, but only DFR expression was detected 14 weeks postflowering in Figure 3.1. This is probably due to the differences in exposure times of the different northern blots as those shown in Figure 3.1 were exposed for a shorter period (24 hours) than those shown in Figure 2.8 (72 hours) as the signal from mid-leaf and seed was so high that shorter exposure times were required. The UFGT northern blots were not affected by this difference in signal intensity as expression could only be detected in berry skin tissue and only after a long exposure time

(approximately one week). Another surprising result was the lack of PAL mRNA expression detected in Cabernet Sauvignon berry skins (Fig. 3.2). PAL, CHS and F3H have been reported to be encoded by multigene families in grapevine (Sparvoli *et al.* 1994). The cDNA clones used as probes were isolated from grape seedlings, so it is possible that other members of the gene families were not detected by these probes in the tissues tested. CHI, DFR, LDOX and UFGT were apparently present as single copies in the grape genome (Sparvoli *et al.* 1994), and therefore this problem should not arise for these genes in the flavonoid pathway. It is interesting that Cabernet Sauvignon should have a PAL gene expressed in berries which is less homologous to the clone isolated from seedlings than the other varieties. Sparvoli and co-workers (1994) estimated that there are 15-20 PAL genes in the grape genome, so perhaps the gene detected in most of the varieties is not induced in response to a developmentally controlled signal involved in anthocyanin accumulation. Northern analysis of different members of the PAL gene family in bean and parsley revealed differential expression patterns during development or due to environmental stimuli (Liang *et al.* 1989; Lois and Hahlbrock 1992). The promoters of two members of the bean PAL gene family were fused to the GUS gene and transformed into *Arabidopsis*, potato and tobacco, and the constructs were found to have different temporal and spatial expression patterns during development as well as a differential response to environmental stimuli (Shufflebottom *et al.* 1993). Perhaps the Cabernet Sauvignon grapes were not subject to the same environmental stresses as the other varieties sampled and thus PAL genes homologous to the one isolated from grape seedlings were not expressed in this sample. This is indeed possible as the Cabernet Sauvignon sample was taken from Langhorne Creek, an area far removed from where

the other samples were taken, with the vines under a different pruning and irrigation regime than those vines sampled for the other grape varieties.

Chapter 4

Grape anthocyanin mutants

4.1 Introduction

Sports or mutations are common in plants, and there are many mutants with modified flower- or fruit-colour (Forkmann 1993). The reason for the observed predominance of these kinds of mutants over others is probably because they are both non-lethal and easily seen. Grapevines are no exception as many examples of berry-colour sports have been reported (Galet 1979).

The work described in this chapter involves comparisons of the anthocyanin composition of a number of grape varieties and their sports. Some of the sports involve black varieties exhibiting a reduction in anthocyanin biosynthesis (Bronze and White Cabernets), and others a reversion of white-skinned varieties to anthocyanin producing varieties (Pink Sultana and Red Chardonnay). The anthocyanin profiles of these grapes were determined to investigate if there was any difference in the profiles between sports where pigment synthesis is diminished and those where it has been regained. The expression of six genes from the flavonoid synthesis pathway was also analysed in these sports to compare the genetic control of the pathway in grapevines with white or coloured berries.

4.2 Materials and methods

4.2.1 Plant material

Grape berries were obtained from several sites in South Australia as listed below. In all cases, whole bunches were cut from the vine and transported to the laboratory on ice. Individual berries were scored for total weight and skin weight, with the skins being separated from seeds and flesh by squeezing whole berries. The juice was pooled and a combined measurement of total soluble solids (°Brix) taken using a refractometer. Skins were then frozen in liquid nitrogen and stored at -80°C pending further analysis. Young leaves from each variety were also harvested, transported to the laboratory on ice, frozen in liquid nitrogen and stored at -80°C as a source of tissue for DNA extraction.

Grapes from the varieties Muscat à Petits Grains Blanc (hereafter referred to as Muscat Blanc) and Muscat à Petits Grains Rouge (hereafter referred to as Muscat Rouge), Pinot Blanc, Pinot Noir, Chardonnay, Red Chardonnay, Pink Sultana and Shiraz were obtained from the collection of the South Australian Research and Development Institute at Nuriootpa. Sultana grapes were sampled from the Waite Agricultural Research Institute's collection at Urrbrae. Cabernet Sauvignon, and the subsequent sports which we have named Bronze Cabernet and White Cabernet, were collected from the commercial vineyard of Mac Cleggett in Langhorne's Creek, South Australia.

4.2.2 Anthocyanin and proanthocyanidin extraction and HPLC analysis

Anthocyanins were extracted from the grape berry skins and total anthocyanin content was measured as described in Section 2.2.2. The individual anthocyanins in each extract were analysed following the protocol in Section 2.2.3. Proanthocyanidin analysis was carried out as described in Section 2.2.4.

4.2.3 Total RNA extraction and northern analysis

Total RNA was extracted from the various grape skin samples from the red and white varieties using the method described in Section 2.2.5. Radiolabelled probes were prepared and northern analysis carried out as described in Sections 2.2.6 and 2.2.7.

4.2.4 Genomic DNA extraction and Southern analysis

DNA was extracted from young leaf samples as described by Thomas *et al.* (1993) and outlined in Section 3.2.4. This DNA was then used for Southern analysis following the protocol in Section 3.2.5.

4.2.5 Grapevine DNA-typing

Grapevine DNA was extracted using the method described in Section 3.2.4 (Thomas *et al.* 1993). DNA-typing was performed as outlined in Thomas *et al.* (1994). Six pairs of primers which flank grapevine microsatellite sequences (Thomas and Scott 1993) were used in the PCR reactions. One of the primers from each pair had a fluorescent dye attached to allow the detection of the specific primer products within a mixed PCR reaction. The

20 µL PCR mix contained 50 ng of grapevine DNA, 1 U of *Taq* DNA polymerase, reaction buffer (20 mM Tris-HCl [pH 8.4], 50 mM KCl), 2 mM $MgCl_2$, 125 pmol or 250 pmol of each primer, 200 µM of dGTP, dCTP, dTTP and dATP. Following an initial denaturation cycle of 3 min at 95°C, 26 cycles of PCR were performed (denaturation 45 s at 94°C; annealing 30 s at 50°C; extension 1.5 min at 72°C) followed by a 7 min elongation step. Each PCR reaction was then quantified on an 8% acrylamide gel and appropriate aliquots mixed with formamide and a red DNA size standard. These samples were then denatured and run on an automated DNA sequencing apparatus running GENESCAN software. The PCR products were automatically sized by this software.

4.3 Results

4.3.1 Total anthocyanins and proanthocyanidins

The results of the analyses of total anthocyanins and proanthocyanidins in the berry skins are presented in Table 4.1. The table also includes °Brix measurements to indicate the stage of ripening of each berry sample. It has been shown in Chapter 2 that the total level of anthocyanins in grape berries increased throughout ripening and so this should be considered when comparing total anthocyanin measurements in Table 4.1.

Skins were sampled from Muscat Rouge, which is a coloured Muscat à Petits Grain with a deep-red hue (Fig. 4.1). When anthocyanins were extracted from these skins they had an A_{520} per gram fresh weight of 17.6 (Table 4.1), whereas skins harvested from Muscat Blanc grapes had no detectable anthocyanins. The Muscat Blanc grapes were more ripe than the Muscat Rouge, having an average °Brix measurement of 22.2 compared to 17.9 (Table

4.1). It is uncertain if the Muscat Blanc is a sport of Muscat Rouge or *vice versa*. It should be noted that there are at least two white-berried Muscats (Kerridge and Antcliff 1996) and a number of different coloured Muscat cultivars, and the variation in the anthocyanin profiles amongst these is great (Cravero *et al.* 1994).

Pinot Blanc is a sport of Pinot Noir, and has been grown mainly in Alsace and northern Italy (Kerridge and Antcliff 1996). Thus the Pinot Noir vine we used is unlikely to be the mother vine of the Pinot Blanc sport. Pinot Blanc did not contain any anthocyanins, whereas the Pinot Noir had much pigment, with an A_{520} of 137.8 per gram fresh weight (Table 4.1).

Table 4.1. *Total anthocyanins and proanthocyanidins in the skins of various grape varieties. n/d = not detected (<2A_{520}/g fresh weight)* (<0.5A/berry)

Variety	°Brix	Proanthocyanidins		Anthocyanins	
		mg/g skin	mg/berry	A_{520}/g skin	A_{520}/berry
Muscat Blanc	22.2	4.9	2.8	n/d	n/d
Muscat Rouge	17.9	4.4	2.2	17.6	8.8
Pinot Blanc	20.8	4.8	1.9	n/d	n/d
Pinot Noir	21.6	5.0	1.7	137.8	46.6
Chardonnay	20.6	4.7	1.3	n/d	n/d
Red Chardonnay	21.4	3.7	1.1	17.6	5.0
Sultana	22.7	2.1	1.5	n/d	n/d
Pink Sultana	21.4	1.7	1.1	4.8	3.0
White Cabernet	17.5	4.4	2.1	n/d	n/d
Bronze Cabernet	17.0	4.3	1.9	9.2	4.1
Cabernet Sauvignon	15.5	3.4	1.2	84.4	30.6
Shiraz	19.3	4.7	1.2	212.4	53.3

The Red Chardonnay sport arose as a bud sport of a Chardonnay vine in the Clare Valley, South Australia and the grapes sampled were obtained from vines vegetatively propagated from the mother vine and the bud sport. Such Chardonnay sports have been described previously (Galet 1979). The colour of the berries is red (Fig. 4.1), with not as deep a hue as the Muscat Rouge berries. Leaves from these vines were subjected to DNA-typing analysis using the method described by Thomas *et al.* (1994). The DNA-typing method involves the analysis of the alleles present at six sequence-tagged microsatellite sites. Polymorphisms are detected by differences in the sizes of the microsatellites when they are separated on a polyacrylamide gel. Cultivars can be identified by comparing the size of the alleles in a sample with those in a database. The analysis of DNA-typing of the Red Chardonnay is consistent with it being a sport of Chardonnay (Table 4.2).

Pink Sultana sports have been described previously (Galet 1979). The berries have a light pink pigmentation (Fig. 4.1), probably because they contain little anthocyanin. No anthocyanins were detected in the Sultana berry skins, but skin from Pink Sultana at the same degree of ripening had an A_{520} of 4.8 per gram fresh weight (Table 4.1).

The two Cabernet Sauvignon berry-colour sports described below represent previously unreported mutants. The sports arose at a vineyard in Langhorne's Creek, South Australia. The Bronze sport is a bud sport of a normal Cabernet Sauvignon vine with bronze-grey appearance (Fig. 4.1). Twelve vines were vegetatively propagated from it and all had bronze coloured fruit. One of the Bronze Cabernet vines subsequently generated another bud sport which lacked pigment, and this White Cabernet was subsequently vegetatively propagated. The Bronze sport had approximately one ninth of the total anthocyanins of Cabernet Sauvignon even though the Bronze Cabernet berries were slightly more ripe than the Cabernet Sauvignon berries (Table 4.1). The White Cabernet did not contain detectable

levels of anthocyanins (Table 4.1). Leaves taken from these vines have been subjected to DNA-typing analysis, and the results (Table 4.2) are consistent with these mutants being bud sports of Cabernet Sauvignon.

The amount of anthocyanins in the skins of the red or black grapes varied from an A_{520} of 4.8 per gram fresh weight of skin in Pink Sultana to A_{520} of 212.4 per gram fresh weight of skin in Shiraz, a 44-fold difference. Both samples were obtained from the same vineyard with the Pink Sultana sample riper than the Shiraz sample, which suggests that this significant difference is due to varietal differences rather than environmental or management factors. Muscat Rouge, Pink Sultana, Red Chardonnay and Bronze Cabernet all possessed much less anthocyanin than the more commonly cultivated black varieties Pinot Noir, Cabernet Sauvignon and Shiraz. Although the varieties with coloured berries were at different stages of ripening, the least ripe was Cabernet Sauvignon and this variety still possessed far more anthocyanins than the Muscat Rouge, Pink Sultana, Red Chardonnay and Bronze Cabernet. No anthocyanins were detected in any of the white-skinned grapes, although some may possess the ability to produce low levels of anthocyanins very late in ripening (Galet 1979, Gholami and Coombe 1995).

***Table 4.2** Results of DNA-typing analysis of Cabernet Sauvignon, Chardonnay and their respective mutants. The data is presented in base pairs, and a difference <2 bp is insignificant.*▼

Variety	VVS1 alleles	VVS29 alleles	VVS2 alleles	VVS5 alleles	VVS1006 alleles	VMD007 alleles
Cabernet Sauvignon	182:-	178:180	140:153	102:123	286:-	239:-
Bronze Cabernet	182:-	178:180	140:153	103:124	286:-	239:-
White Cabernet	182:-	178:180	140:153	103:124	286:-	239:-
Chardonnay	184:191	171:178	138:145	89:148	286:292	239:243
Red Chardonnay	184:190	171:178	138:144	89:148	286:292	239:243

▼ >2 bp is also insignificant

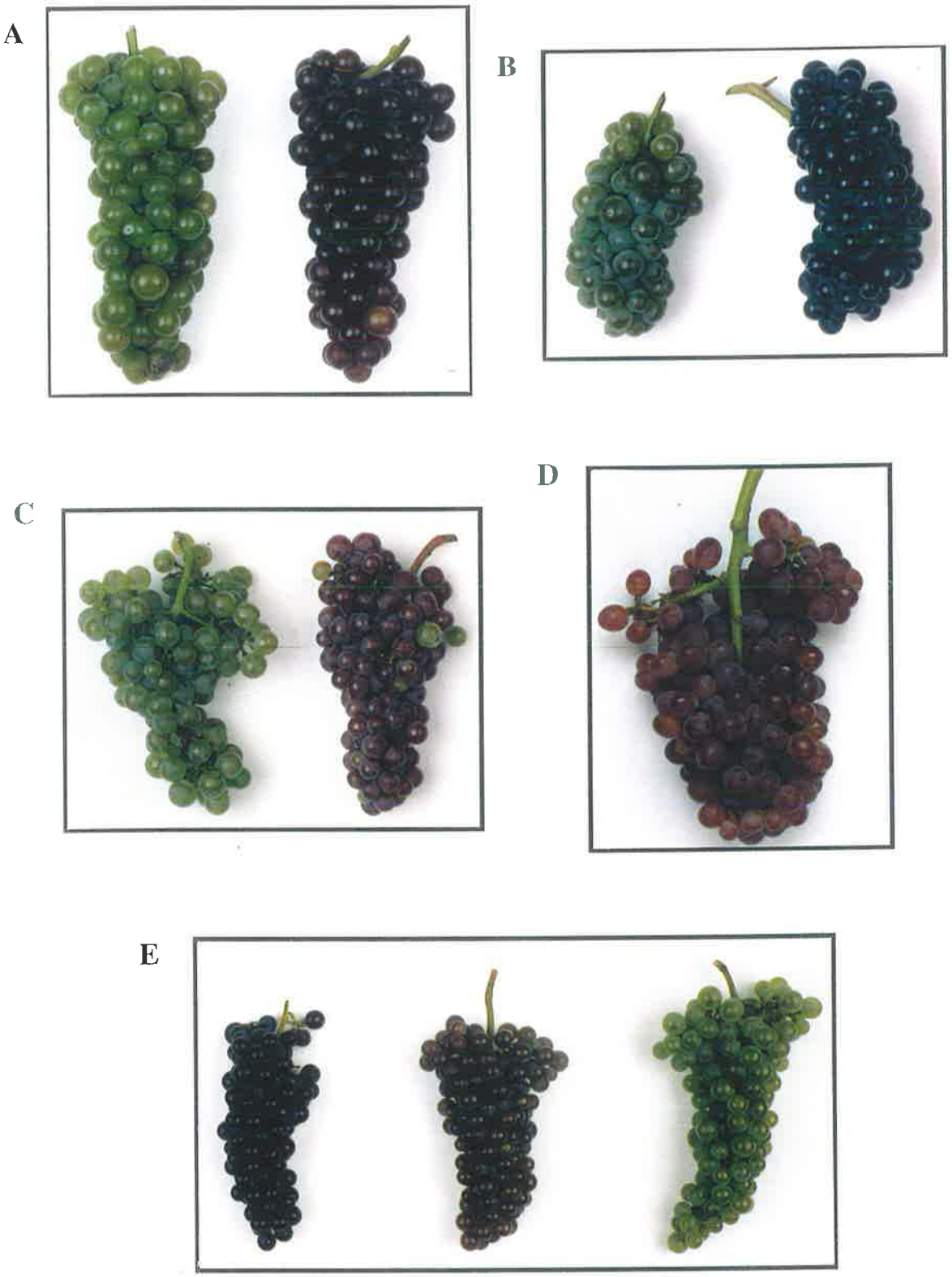


Figure 4.1 Grape anthocyanin mutants. **A**, Muscat Blanc (left) and Muscat Rouge (right); **B**, Pinot Blanc (left) and Pinot Noir (right); **C**, Chardonnay (left) and Red Chardonnay (right); **D**, Pink Sultana; **E**, Cabernet Sauvignon (left), Bronze Cabernet (centre) and White Cabernet (right).

4.3.2 Anthocyanin profiles of the sports

Results of the HPLC analysis of the anthocyanin contents of the skins of the coloured varieties are shown in Figures 4.2 and 4.3. Extracts of Shiraz and Cabernet Sauvignon berry skins exhibited HPLC profiles similar to those reported previously (Wulf and Nagel 1978; Roggero *et al.* 1986) and contained predominantly malvidin derivatives with significant amounts of acylated anthocyanins, whereas Pinot Noir contained only 3-monoglucosides. Muscat Rouge, Red Chardonnay and Pink Sultana had similar patterns to Pinot Noir in that they contained mainly 3-monoglucosides with minimal amounts of acylated anthocyanins. However, there was significant variation in the proportions of the various 3-monoglucosides found in Muscat Rouge, Red Chardonnay and Pink Sultana. In Muscat Rouge, peonidin 3-monoglucoside and malvidin 3-monoglucoside, both of which are methylated in the B-ring of the molecule (Fig. 1.3), constituted 76.6% of the anthocyanins. Red Chardonnay had predominantly cyanidin 3-monoglucoside and peonidin 3-monoglucoside (81.7%) pigments, which are found on the dihydroxylated 'branch' of the anthocyanin pathway (Fig. 1.5). The major anthocyanins of Pink Sultana were the non-methylated forms cyanidin 3-monoglucoside and delphinidin 3-monoglucoside (78.7%). The Bronze Cabernet sport had a very similar anthocyanin profile to the wild type Cabernet Sauvignon plant from which it arose despite the much lower level of total anthocyanins (Fig. 4.3). The most significant differences seen in the Bronze sport were an increase in the proportion of peonidin 3-monoglucoside, peonidin 3-acetylglucoside and malvidin 3-*p*-coumaroylglucoside, and a reduction in malvidin 3-monoglucoside.

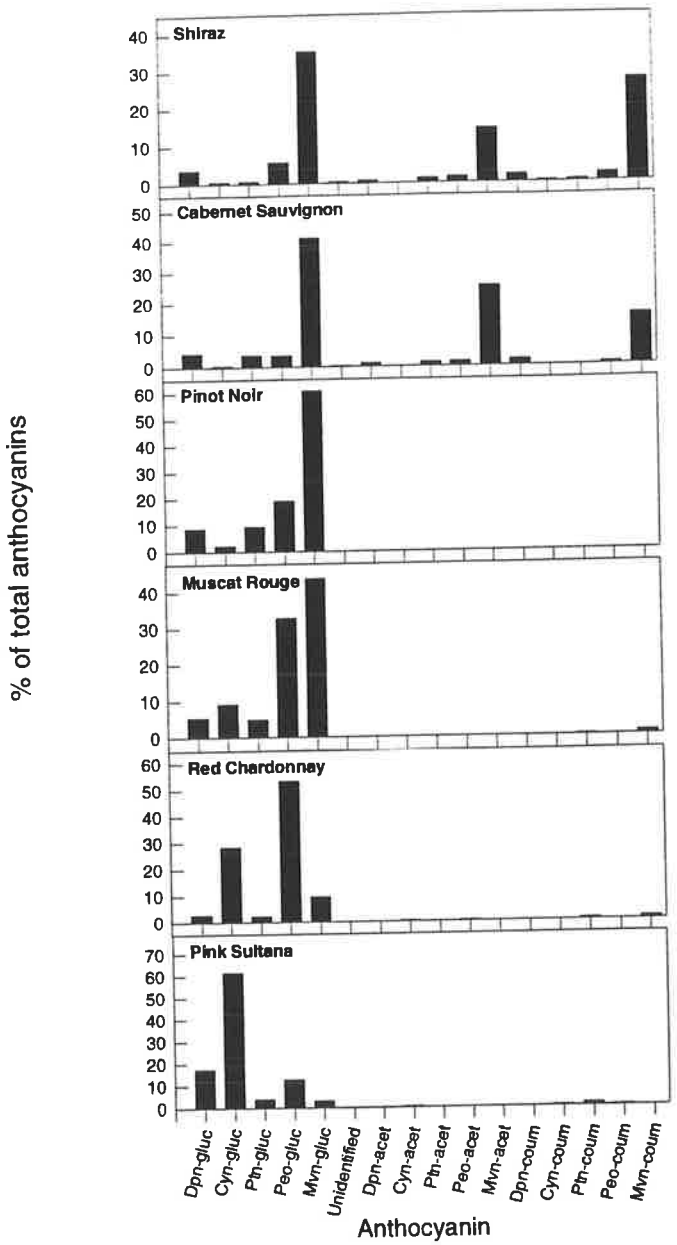


Figure 4.2 Anthocyanin profiles of six grapevine varieties. The graphs show the amount of each anthocyanin species as a percentage of the total anthocyanins found in each grapevine variety. Dpn = delphinidin; Cyn = cyanidin; Ptn = petunidin; Peo = peonidin; Mvn = malvidin; gluc = 3-monoglucoside; acet = 3-acetylglucoside; coum = 3-*p*-coumaroylglucoside.

The anthocyanin profiles of the coloured varieties reflect the complexity of the genetics of the pathway. Pinot Noir has been well documented as a variety which lacks acylated anthocyanins (Rankine *et al.* 1958; Fong *et al.* 1971). Presumably this is due to alterations in the levels and/or characteristics of the enzymes which are involved in adding acetyl or coumaroyl groups onto the 3-monoglucosides. The anthocyanin profiles of Muscat Rouge, Red Chardonnay and Pink Sultana suggest that the activity of other modifying enzymes in the pathway (e.g. flavonoid 3'-hydroxylase [F3'H], flavonoid 3'5'-hydroxylase [F3'5'H] and methyltransferases [MT]) have been altered. Muscat Rouge has 43% malvidin derivatives, compared to 80% in Shiraz and Cabernet Sauvignon (Fig. 4.2). Thus the flux down the trihydroxylated branch of the pathway (Fig. 1.5) is proportionally less in the Muscat Rouge grapes. This effect is even more marked in Red Chardonnay and Pink Sultana, where the percentages of trihydroxylated anthocyanins were only 16.4% and 25% respectively (Fig. 4.2).

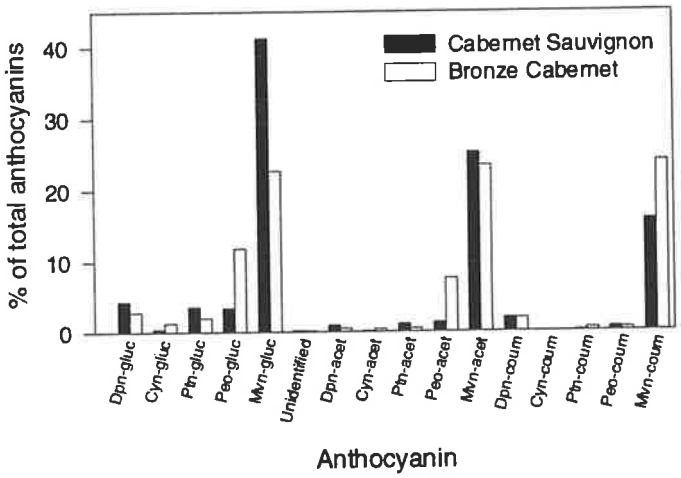


Figure 4.3 Anthocyanin profiles of Cabernet Sauvignon and the Bronze Cabernet mutant. The amount of each anthocyanin species is represented as a percentage of the total anthocyanins. Dpn = delphinidin; Cyn = cyanidin; Ptn = petunidin; Peo = peonidin; Mvn = malvidin; gluc = 3-monoglucoside; acet = 3-acetylglucoside; coum = 3-*p*-coumaroylglucoside.

4.3.3 Expression of the anthocyanin biosynthesis genes and Southern analysis of UFGT

Analysis of the expression of six genes from the anthocyanin biosynthesis pathway in the berry skins is presented in Figure 4.4. Using northern analysis, expression of each of the anthocyanin biosynthesis pathway genes was detected in the red- or black-skinned berries. The level of anthocyanin gene expression in the berries of all the varieties with coloured fruit correlates well with the total amounts of anthocyanins isolated from the skins (Fig. 4.4). This is especially so when the expression of leucoanthocyanidin dioxygenase (LDOX) or UFGT are compared amongst the grape varieties. In the varieties with white-skinned berries, expression of all the genes was generally much lower than in those with red- or black-skinned berries. The most notable difference in the expression patterns of the coloured and white grape skins was that UFGT expression was detected in all the coloured berries, but in none of the white-skinned varieties. LDOX also has a distinct pattern of expression, with expression always being higher in the coloured berries. A good example of this is the expression of LDOX in the Cabernet grape series, which shows increasing expression with increased anthocyanin levels (Fig. 4.4).

The gene expression data suggests that the major control of whether a grape berry will produce anthocyanins is the expression of UFGT. UFGT is the only gene tested which showed an absolute differential expression pattern between the white and coloured grape berry skins. This raises the possibility that this gene is absent in the white-skinned grape varieties. The presence of UFGT genes in the genomes of the white-berried grapevines was investigated using Southern analysis (Fig. 4.5). Genomic DNA was isolated from each of the varieties investigated in this study, digested with the restriction enzymes *Eco* RV or *Dra* I and Southern blotted. When probed with a full-length UFGT cDNA clone,

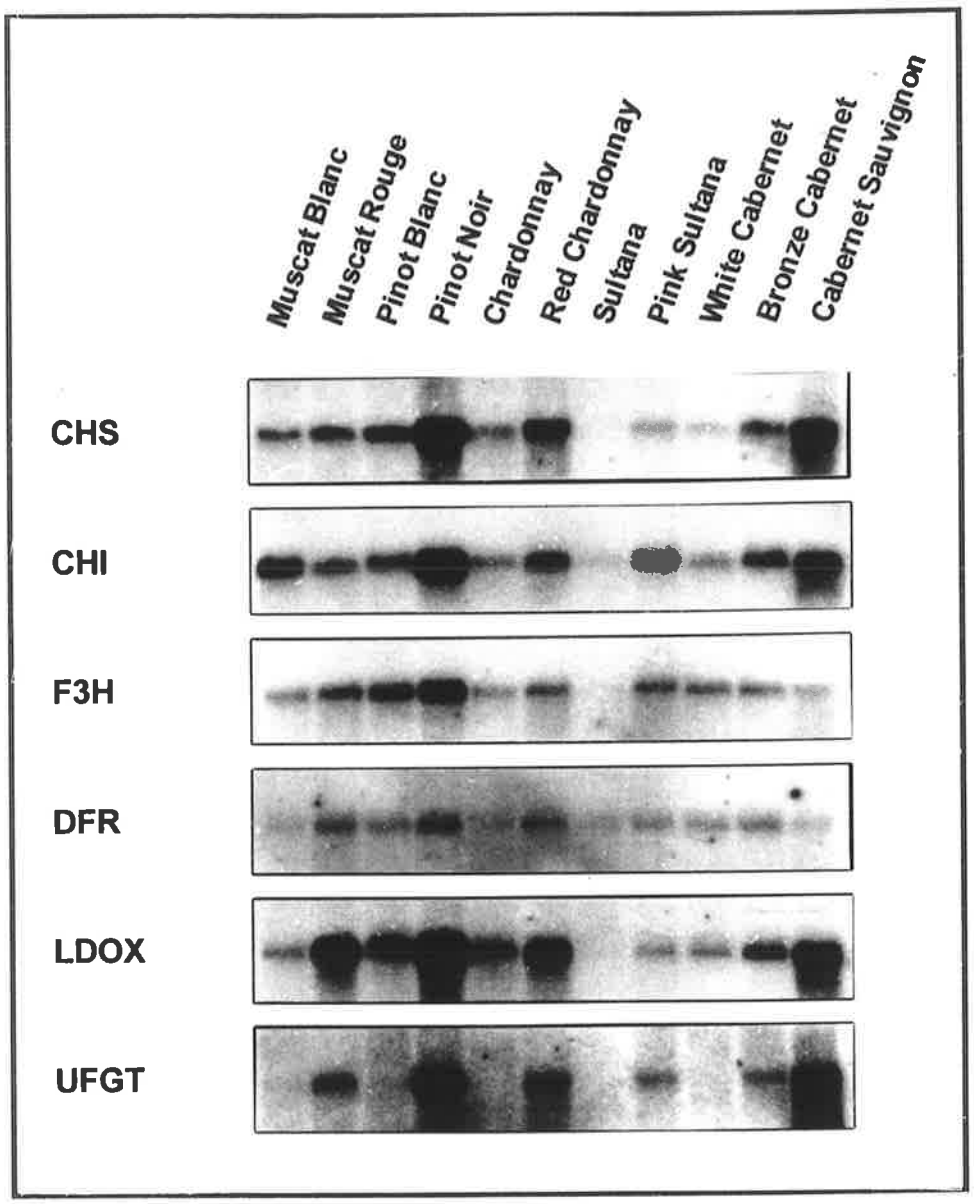


Figure 4.4 Expression of anthocyanin biosynthesis genes in the skins of the various grapevine varieties. Northern blots are of total RNA from grape berry skin samples from each variety and probed with grape cDNA clones as indicated on the left side of the figure.

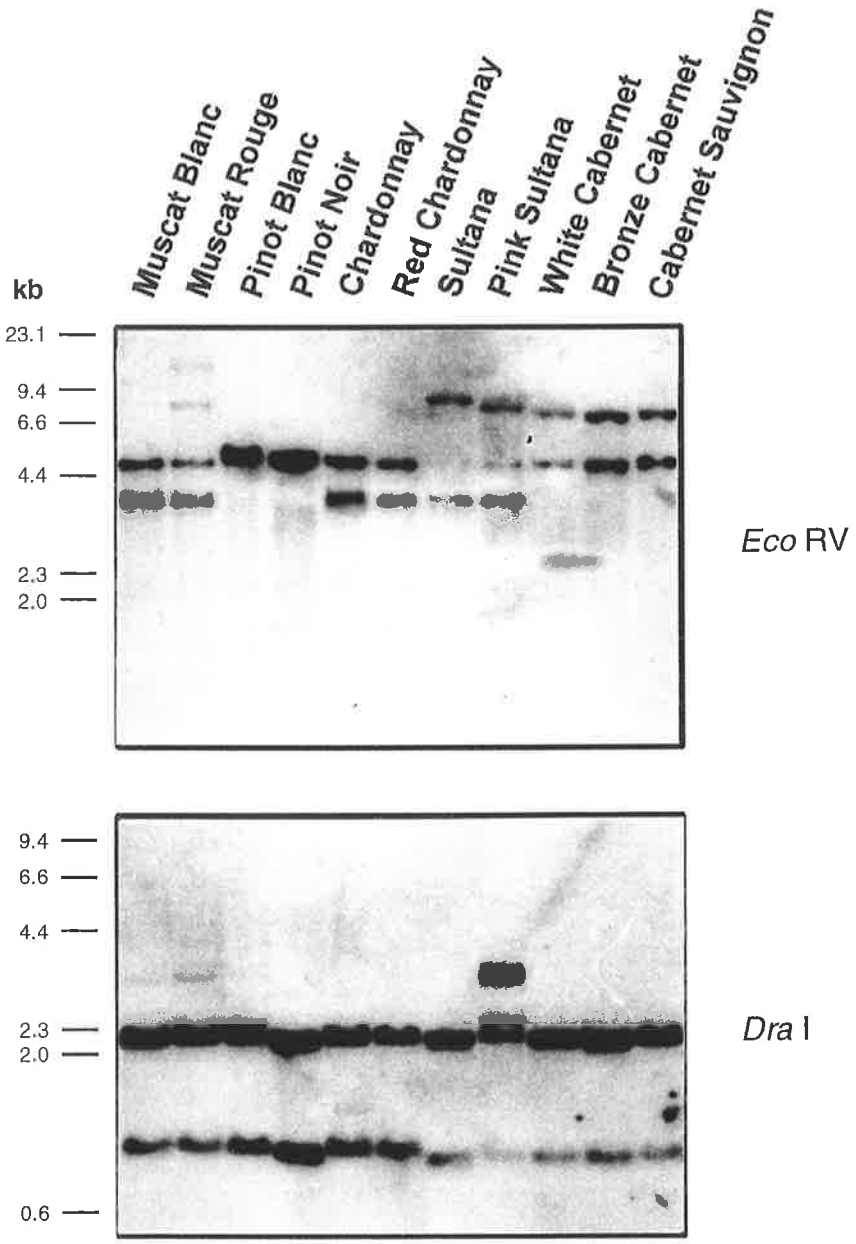


Figure 4.5 Southern analysis of genomic DNA from the various grapevine cultivars probed with a full-length UFGT cDNA clone. Southern blots are of genomic DNA from each of the grapevine cultivars, digested with *Eco* RV (top) or *Dra* I (bottom) and probed with a full-length cDNA clone coding for UFGT. Size standards are indicated on the left of each figure.

hybridising bands were detected in each of the samples (Fig. 4.5). This suggests that these varieties all possess at least part of the UFGT gene(s). The Southern analysis indicates that the lack of UFGT expression may result from an aberration in the transcriptional control of this gene, due to the absence of a functional promoter or related transcription factor rather than the absence of UFGT-related sequences in the genomes.

4.4 Discussion

This chapter describes several grapevine colour mutants, some of which have been observed to occur previously (Pink Sultana, Red Chardonnay) and some which are first reported here (Bronze and White Cabernets). To ensure that the Chardonnay and Cabernet mutants were indeed sports of the 'wild-type' variety, DNA typing was carried out and this confirmed the origins of the mutants (Table 4.2). Some of the sports show a loss of anthocyanin pigmentation in the berry skins (Cabernet sports), and others display a reversion in berry skin pigmentation (Pink Sultana, Red Chardonnay). The sports arising from white cultivated varieties all had red-hued berries as opposed to the black, dark-pigmented berries of most cultivated pigmented varieties. The coloured sports all contained less total anthocyanins than cultivated coloured varieties, and this was not due to a significant difference in the maturity of the berries (Table 4.1). This is also true for the Bronze Cabernet sport, however, the colour of the berries is very different from those of the Red Chardonnay or Pink Sultana sports (Fig. 4.1). The colour is very much more orange than red and is comparable to the colour of Pinot Gris berries. When the anthocyanin profile of the Bronze Cabernet sport is compared to the Red Chardonnay or Pink Sultana sport, the immediate difference noted is the amount of acylated anthocyanins in the Bronze Cabernet as compared to the Red Chardonnay and Pink Sultana sports. However, factors other than

the anthocyanin species present may influence the perceived colour of the berries. Certain genes in petunia are known to affect the colour of the flower by changing the pH (presumably of the vacuoles) of the flower limbs (de Vlaming *et al.* 1983). The pH of the vacuole may also influence the amount of each anthocyanin species that accumulate (Gerats and Martin 1992). The cellular localisation of the anthocyanins also influences the colour of the plant tissue. The *Bronze2* gene of maize is involved in the tagging of anthocyanins for their subsequent transport into the vacuole (Marrs *et al.* 1995) and mutations in this gene results in bronze coloration in the cells as anthocyanins accumulate in the cytoplasm (Marrs 1996). This gene may also affect the total amount of anthocyanins that accumulate and thus the colour of the plant tissue, as pale pink pigmentation is seen in some *bz2* mutants which are still able to accumulate small amounts of anthocyanins in the vacuoles (Walbot *et al.* 1994). Furthermore, the hue of the berries could be altered by the presence or absence of co-pigments in the vacuole, or the ability of each anthocyanin species to interact with possible co-pigments.

It could be argued that the mechanism for the loss of pigmentation in berry skins is different from the mechanism for regaining anthocyanin production. From this it would perhaps be expected that a coloured mutant from the White Cabernet would produce red berries as opposed to bronze berries, and the progenitors of Chardonnay or Sultana (if it is accepted that white varieties derived from coloured varieties) would have black or bronze berries. The Muscat Rouge therefore, would appear to be derived from a white Muscat variety, and the Pinot Blanc variety would appear to be a mutant of Pinot Noir, via the Pinot Gris intermediate mutant, very much like the Cabernet series of mutants described here.

Perhaps the most fascinating anthocyanin profile seen is that of Pinot Noir, where there is a complete absence of any acylated anthocyanins (Fig. 4.2). The coordinate shutdown of all

acylation suggests that (a) one enzyme catalyses the primary acylation of all the anthocyanins and it has been mutated, or (b) all the genes involved in acylation have been mutated, or (c) a regulatory gene controlling the expression of acylating genes has been mutated. The Muscat Rouge, Red Chardonnay and Pink Sultana sports show an increased flux down the dihydroxylated branch of the anthocyanin pathway (Fig. 4.2). Reasons for this may include: increased F3'H activity, decreased F3'5'H activity, increased affinity of DFR for dihydroxylated dihydroflavonols or decreased affinity of DFR for trihydroxylated dihydroflavonols. Glycosylation of the various anthocyanidins may also be affected by UFGT mutations which could govern the level of various species of anthocyanins finally accumulating in the vacuole. The properties of GSTs and glutathione pumps could also affect the type of anthocyanins which are able to accumulate in the vacuole. Pink Sultana is unusual in that it has low levels of methylated anthocyanins, which may indicate a mutation in the genes involved in methylation or in their controlling genes. Another possibility is that there is not enough substrate for the methylation enzymes, as Pink Sultana possessed the least anthocyanins of all the sports (Table 4.1).

The Bronze Cabernet sport has an anthocyanin profile almost identical to Cabernet Sauvignon (Fig. 4.3). It seems that the major difference in the hue of these berries is due to the lower levels of total anthocyanins. It is possible that the Bronze Cabernet sport is a chimaera, with some cell layers in the skin 'wild-type' and some mutant. Anthocyanins accumulate in the dermal cell layers (Winkler *et al.* 1974), and Considine and Knox (1981) showed that in Muscat Gordo there are 6-7 dermal cell layers in mature berries. Cell lineage analysis showed that two cell layers gave rise to the seven in the mature berries and that one of the two contributed six of these through periclinal divisions (Considine and Knox 1981). If the cell line contributing six cell layers was mutated with respect to anthocyanin synthesis, we would expect a greater reduction in total anthocyanins than if the

cell layer remaining single had been mutated. This may explain the large reduction in anthocyanins in the Bronze Cabernet sport. The White Cabernet sport could subsequently arise due to periclinal divisions in the mutated cell layer, or due to another mutation in the 'wild-type' cell layer.

The northern analyses of the various sports provide evidence that the UFGT step is the control point for anthocyanin production in grape berry skins as it is the only gene that displays an absolute differential expression pattern between pigmented and non-pigmented grape berries (Fig. 4.4). Previous studies looking at expression of anthocyanin genes during grape development (Chapter 2) and in other grape tissues (Chapter 3) also suggest that the controlling point of anthocyanin production in grapes is beyond the LDOX step of the pathway. This is in contrast to results in snapdragon, petunia and maize where the key regulatory points are F3H, DFR and CHS respectively (Martin and Gerats 1993a; Holton and Cornish 1995). All of the white-skinned varieties also show less expression of all the genes of the anthocyanin biosynthesis pathway with Sultana having the greatest reduction in expression (Fig. 4.4). This may reflect the manner in which regulatory genes control the anthocyanin structural genes in grapes and this is discussed extensively in Section 3.4.

Southern analysis showed that all the varieties reported in this chapter contained at least part of the UFGT gene (Fig. 4.5). Thus the lack of UFGT gene expression in the white-berried varieties seems to be due to a lack of transcription of the gene and not simply an absence of UFGT in the genome. These results indicate that changes in anthocyanin synthesis in grapevine sports occur by alteration at the level of gene transcription, as has been observed in other plants (Martin and Gerats 1993b). The anthocyanin structural genes in grapes are, presumably, controlled by two groups of regulatory genes which show homology to *myc* and *myb* transcription activators as they are in other species (Section

1.7.3). It is possible that mutations in either of these genes may alter anthocyanin production. Mutations in the promoter of UFGT which alter the ability of *myc* and/or *myb* transcription activators to control UFGT expression may also result in a loss of berry skin pigmentation.

Although the grapes which lack anthocyanins all lack detectable UFGT gene expression and the reversion to pigment-producing berries involves a restoration of UFGT expression, the various sports have arisen independently. Therefore, the phenotypes may have arisen due to different mechanisms. As stated above, a loss of UFGT expression may be due to a mutation in the promoter of the gene or a mutation in a transcription factor which induces expression of the gene. The differences in the proportions of the various anthocyanins in the Pink Sultana, and Red Chardonnay sports may be due to these different mechanisms. The theory that white-skinned grapevine varieties have arisen from black-coloured progenitors raises interesting possibilities about the subsequent reversion of the white grape berries to red-skinned berries. Reversion is commonly seen in maize kernels, and is due to the presence of transposable elements (for review see Fedoroff 1983). The insertion of the element into an anthocyanin biosynthesis or regulatory gene may result in a lack of anthocyanin production; however, the subsequent excision of the transposable element may revert the gene expression to a regular pattern and thus a wild type phenotype (Fedoroff 1983). Retrotransposon-like sequences have been found in grapes (pers. comm., Dr. M.R. Thomas, CSIRO, Adelaide), however, these appear not to excise and thus would probably not be responsible for the reversion seen in some sports. Nevertheless, it is possible that other transposable elements could be responsible for the loss and/or reversion of anthocyanin production.

Possible manipulations of the anthocyanin profiles of grape skins may have oenological benefits. Different anthocyanin species have been shown to have different extraction capabilities during the early stages of wine making and the rate of loss of individual anthocyanins in wine may depend on their structure (see Section 1.5.4). Unfortunately, many studies into the stability of anthocyanins during wine-making and the subsequent storage of wine only measure the loss of each anthocyanin species. Complicating factors are the ability of an anthocyanin to complex with several compounds found in grape juice (most significantly tannins) and the subsequent hue and stability of these complex compounds. The formation of these complexes makes the study of the stability of individual anthocyanin species in wine very difficult. Perhaps the use of radiolabelled molecules may enable the fate of each anthocyanin species to be followed during wine-making. Following this, the isolation of genes involved in the modification of the B-ring substitution of the anthocyanins (F3'H, F3'5'H, MT) and the acylation of these molecules (acyltransferases) will be necessary before the ability to change the grape skin anthocyanin profiles in a qualitative manner is possible.

Chapter 5

The effects of an auxin-like compound on grape berry ripening

5.1 Introduction

Most studies into the development and ripening of fruit have focused on climacteric fruit. Ripening in climacteric fruit is accompanied by a burst in respiration which usually follows an increase in ethylene production. In contrast, non-climacteric fruit often show a decline in respiration during ripening and ripening is not induced by ethylene. This is indeed true for grape berries which are considered to be non-climacteric, and the 'trigger' for ripening in these fruit remains unidentified. Ethylene, considered the signal that initiates ripening in climacteric fruit, does not effect grapes the same way. Ethylene levels in grape berries have been shown to decline during ripening (Alleweldt and Koch 1977; Weaver and Singh 1978), and when grapes are treated with ethylene or ethephon, ripening can be advanced or delayed depending on the timing of application (Hale *et al.* 1970; Coombe and Hale 1973).

Studies into the effects and endogenous levels of other plant growth hormones do not support a single factor being involved in initiating grape berry ripening. Abscisic acid (ABA) concentration increases as berries ripen and treatments which delay the onset of ripening also cause a delay in the increase of ABA (Coombe and Hale 1973; Scienza *et al.* 1978; Cawthon and Morris 1982; Kataoka *et al.* 1982). Hale and Coombe (1974) were able to reduce the time between flowering and ripening through the application of ABA at specific times during development, but this need for the berries to be 'responsive' suggests that ABA is not the only factor involved.

There is evidence that auxins play a role in fruit ripening. Many fruit are auxin sensitive with respect to ripening and these include avocado (Tingwa and Young 1975), banana (Vendrell 1969), pear (Frenkel and Dyck 1973) and strawberry (Given *et al.* 1988). Strawberries ripen when the auxin levels fall below a certain concentration (Given *et al.* 1988) and auxins have also been shown to induce and repress the expression of specific mRNAs and polypeptides in strawberry (Veluthambi and Poovaiah 1984; Reddy and Poovaiah 1990; Manning 1994). Recent studies by Cohen (1996) using tomato fruit grown in tissue culture suggest that elevated auxin levels can delay ripening in this climacteric fruit.

Indole acetic acid (IAA) levels in grape berries are at their highest just after anthesis and then decline to extremely low levels after véraison (Cawthon and Morris 1982). Grape berry ripening can be delayed by the addition of the synthetic auxin analogue benzothiazole-2-oxyacetic acid (BTOA) prior to véraison (Weaver 1962; Hale 1968; Hale *et al.* 1970; Coombe and Hale 1973; Hale and Coombe 1974). Thus, it is possible that ripening in grape berries is not simply due to the increase in a ripening initiator, but may come about due to the decrease in the level of a ripening inhibitor or a combination of both processes. The results presented in this chapter show the effect of BTOA on the expression of a number of developmentally controlled genes.

5.2 Materials and methods

5.2.1 Plant material

The berries used in this study were sampled during the 1995-1996 growing season from *Vitis vinifera* L. cv. Shiraz vines grown in a commercial vineyard in Willunga, South

Australia. Forty bunches were selected, each from a different vine, which were at similar stages of development and similar positions on each vine. These bunches were dipped for 30 secs in a solution of 20 ppm benzothiazole-2-oxyacetic acid (BTOA; American Cyanamid Co., Princeton, New Jersey) containing 0.05% Tween 20 at 6 and 8 weeks postflowering.

5.2.2 Sampling and measurement of berries

Control and BTOA-treated berries were measured and sampled every two weeks, with the sampling of BTOA-treated grapes beginning 8 weeks postflowering. A subsample of 30 berries were measured for deformability and °Brix as described in Section 2.2.1 and the skins frozen in liquid nitrogen for total anthocyanin analysis. Another sample of 30 berries was deseeded and immediately frozen in liquid nitrogen pending further analysis. These berries were assayed for invertase activity and reducing sugars as described by Ruffner *et al.* (1995), and anthocyanins as described in Section 2.2.2.

5.2.3 Quantification of berry ABA levels

Quantitative analysis of ABA was carried out by stable isotope analysis. Frozen berries were thawed rapidly and 1 g of chopped berries was homogenised in 20 mL of cold methanol. An internal standard of $^{2}H_{3}$-(±)ABA was added at a rate of 1 μg g^{-1} fresh weight. The extract was then spun at 20,000*g* for 10 min at 2°C. The resulting pellet was washed with 20 mL of cold methanol, pelleted again, and the two methanol supernatants were pooled. Twenty mL of water was added and the methanol was removed under vacuum at 20°C. This aqueous extract was adjusted to pH 2.5 with 1 N 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 residue was dissolved in 200 µL of methanol, mixed with 800 µL of water, and clarified by centrifugation. The extract was subjected to HPLC, the zone corresponding to ABA was dried *in vacuo* and methylated with ethereal diazomethane, dried under nitrogen and dissolved in 200 µL of acetone. GC-MS was conducted using a Hewlett-Packard HP-GC series 6809. Ions at 162, 165, 190, 193 were monitored. Quantification was by reference to a calibration curve.

5.2.4 Isolation of grape berry RNA and northern analysis

Total RNA was isolated from the BTOA-treated and control berries, which had their seeds removed, using the method described in Section 2.2.5 and northern analysis followed the protocol of Section 2.2.7. DNA probes to CHS, UFGT (Section 2.2.6), a putative vacuolar invertase (GIN1; Davies and Robinson 1996), chitinase (Vv*Chi*4; Robinson *et al.* 1997) and a ripening related cDNA clone encoding an extensin-like protein (GRIP4; Dr. C. Davies, CSIRO, Adelaide) were prepared by random primer labelling (Section 2.2.6).

5.3 Results

5.3.1 Changes in berry physiology due to BTOA treatment

The treatment of grape berries with BTOA retarded ripening (Fig. 5.1). Berry softening, which was measured by an increase in deformability, was delayed by approximately two weeks in the BTOA-treated berries, however the rate of softening was then similar to that of the control berries (Fig. 5.2A). The increase in soluble solids which begins at the onset

of ripening was also delayed by approximately two weeks in the BTOA-treated berries (Fig. 5.2B). The rate of increase in soluble solids was slightly less in the BTOA-treated berries as compared to the control berries, and the final °Brix measurements taken 16 weeks postflowering were 14.8 °Brix for the BTOA-treated sample and 21.4 °Brix for the control sample.

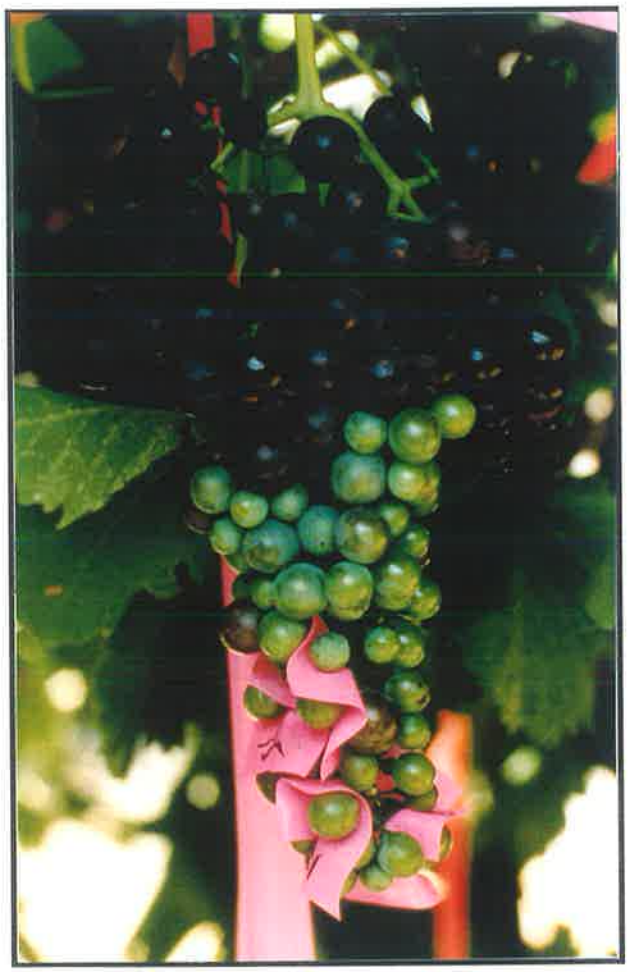


Figure 5.1 The effect of BTOA treatment on the ripening of Shiraz grape berries. The lower half of the bunch of Shiraz grapes pictured above was dipped for 30 secs in a solution of 20 ppm BTOA containing 0.05 % Tween 20 at six and eight weeks postflowering. The upper half of the bunch has not been treated and has thus ripened normally. The photograph was taken at approximately 13 weeks postflowering.

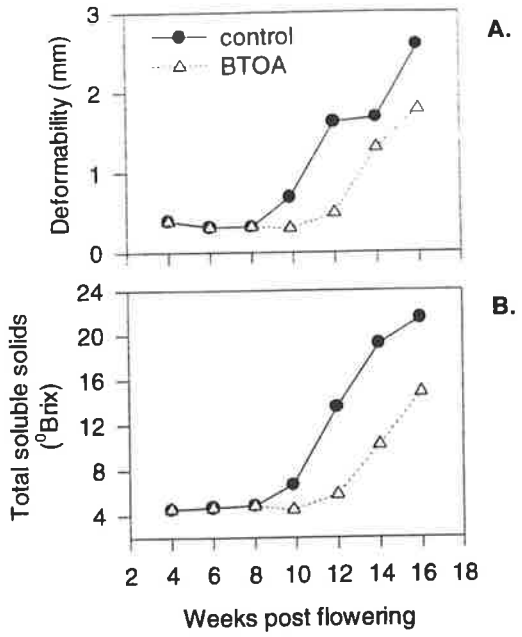


Figure 5.2 Effect of BTOA treatment on grape berry ripening parameters. **A**, berry deformability; **B**, total soluble solids in the berry juice, measured as °Brix. Measurements were taken at two weekly intervals throughout berry development as described in Section 5.2.2.

5.3.2 Changes in invertase activity and gene expression

Figure 5.3 shows the effect of BTOA treatment on the accumulation of reducing sugars, berry weight and invertase activity in the berries. The reducing sugar accumulation in the BTOA-treated and control berries was similar to the changes in soluble solids, as would be expected. By the end of the sampling period, reducing sugars had accumulated to 21.3 g/100 g fresh weight in the control berries and to 16 g/100 g fresh weight in the BTOA-treated fruit. In the control berries, invertase activity on a per gram fresh weight basis increased rapidly early in development until véraison, after which activity declined. This

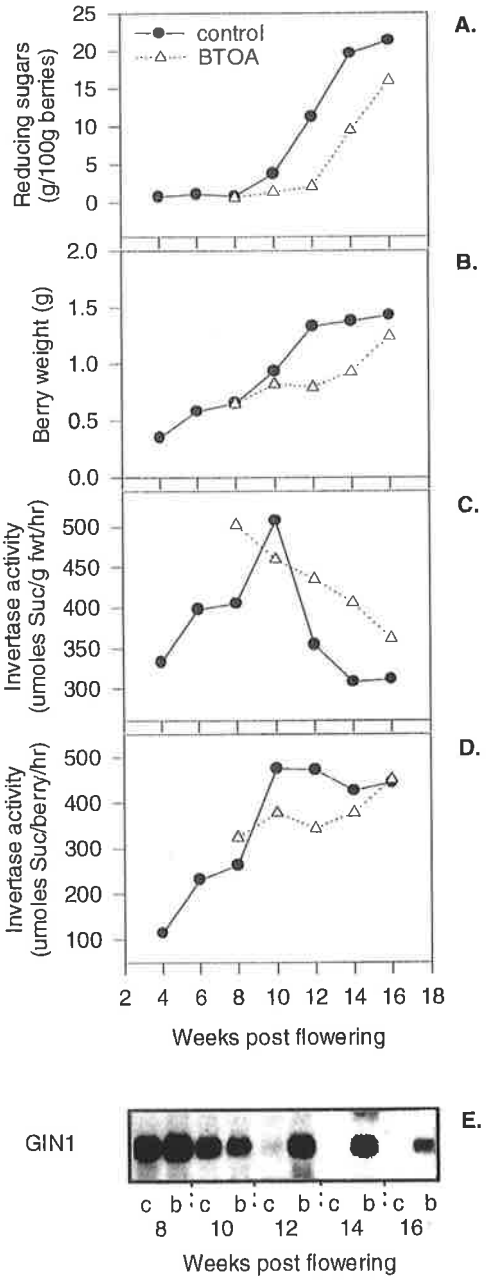


Figure 5.3 Effect of BTOA treatment on berry weight increase and sugar accumulation. **A**, reducing sugars; **B**, berry weights; **C**, invertase activity on a per g basis; **D**, invertase activity on a per berry basis; **E**, invertase mRNA expression assayed by northern blot analysis using the GIN1 cDNA as a probe; c = control; b = BTOA-treated.

decline in activity per gram fresh weight was due to an increase in berry weight rather than a reduction in total activity. If invertase activity was plotted on a per berry basis the maximum was again reached at véraison in the control berries, however the activity then levelled off throughout the rest of berry ripening. Invertase activity was higher, on a per gram fresh weight basis, in the BTOA-treated fruit than in the control berries for all samples except the ten week postflowering sample. There was also a decline in activity per gram fresh weight in the treated berries after véraison as was seen in the control samples. However, the activity per berry in the BTOA-treated samples continued to rise after véraison rather than remain stable as in seen in the control samples.

The expression of invertase mRNA was also investigated and the northern blot is shown in Figure 5.3E. The probe used was the most abundant form of the putative vacuolar invertase genes (GIN1) isolated by Davies and Robinson (1996). High levels of invertase mRNA were detected in the control berries until around véraison (ten weeks postflowering) after which the message rapidly declined in abundance from 12 weeks postflowering throughout the rest of berry development. In contrast, invertase message was easily detected in all the BTOA-treated berry samples and was still expressed in BTOA-treated fruit long after it could not be detected in the control berries. It is interesting that the prolonged expression of this invertase gene extends beyond the two-week period when ripening is delayed in the BTOA-treated grape berries. This expression of invertase mRNA may explain the elevated levels of invertase activity after véraison in the BTOA samples compared to the control berries.

5.3.3 Changes in anthocyanin accumulation and the expression of anthocyanin biosynthesis genes

Anthocyanin accumulation was measured by the increase in A_{520} in skin extracts. As can be seen in Figures 5.1 and 5.4A, the BTOA treatment retarded anthocyanin accumulation. Control berries contained anthocyanins at 12 weeks postflowering, whereas anthocyanins were not detected in the BTOA-treated samples until 14 weeks postflowering. The rate of accumulation of anthocyanins in the BTOA-treated fruit was less than the rate in the control berries. As a result the levels of anthocyanins in the treated berries were much lower than that in the control samples.

As presented in Chapter 2, anthocyanin synthesis begins at the onset of véraison and coincides with an increase in the expression of several genes in the flavonoid pathway. Northern analysis was carried out on total RNA extracted from BTOA-treated and control fruit at various stages of ripening to see if the delay in anthocyanin accumulation is accompanied by a delay in anthocyanin gene expression. Two genes from the anthocyanin biosynthesis pathway (CHS and UFGT) known to be involved in the accumulation of anthocyanins in ripening Shiraz berry skins were used in the northern analysis and the results are presented in Figure 5.4B. In untreated Shiraz berries CHS expression was detected at low levels prior to véraison, however, the level of CHS mRNA increased considerably at véraison when anthocyanins began to accumulate in the berries. BTOA treatment decreased the level of CHS expression before véraison and delayed the increase seen after véraison in the control berries by approximately four weeks, with expression of CHS not increasing until 14 weeks postflowering in the BTOA-treated berries (Fig. 5.4B). UFGT expression was also affected by BTOA treatment. In the control fruit, UFGT expression was not detected until ten weeks postflowering, as anthocyanins began to accumulate, and the

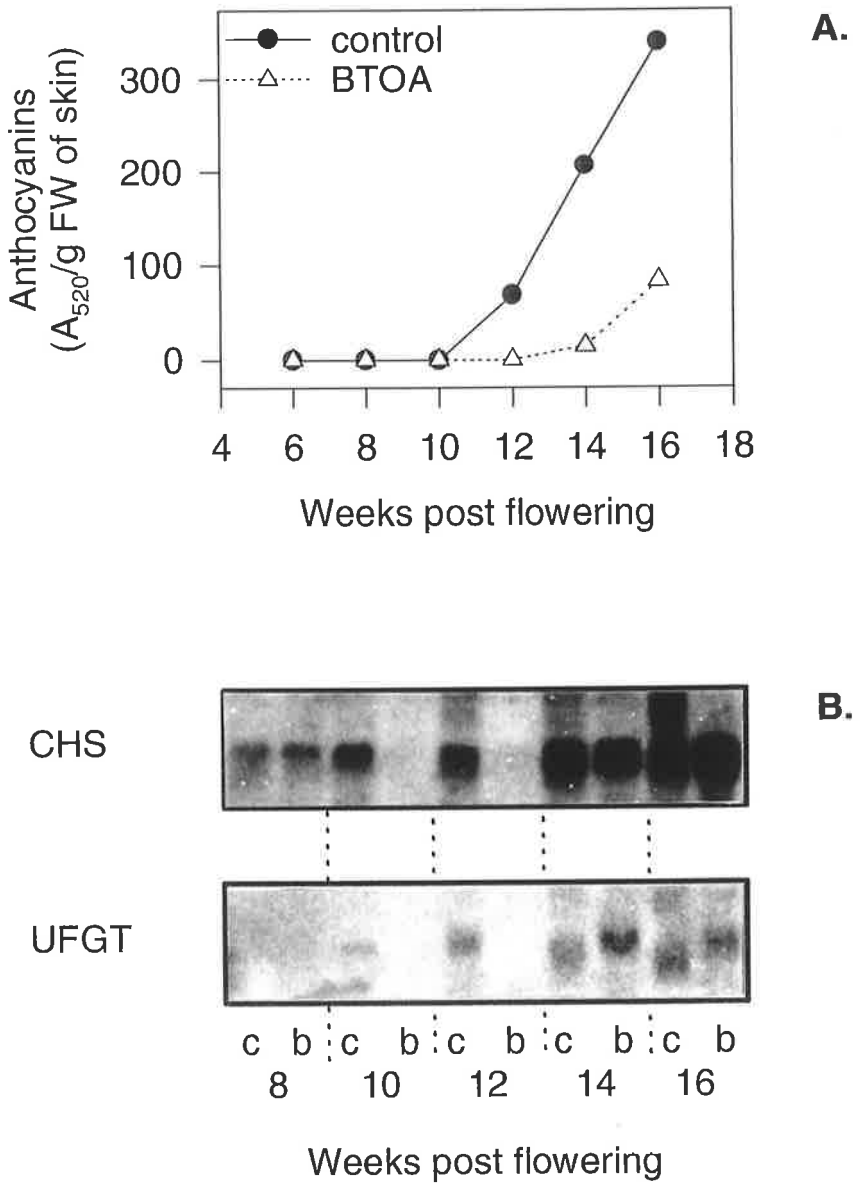


Figure 5.4 Effect of BTOA treatment on anthocyanin accumulation and anthocyanin gene expression. Anthocyanin content was determined by measuring the A_{520} of berry skin samples taken at two weekly intervals. CHS and UFGT gene expression were determined by northern blot analysis. c = control; b = BTOA-treated; FW = fresh weight.

UFGT message was then present through to harvest. However, in BTOA-treated grape berries, UFGT mRNA was not detected until 14 weeks postflowering and thus was delayed four weeks compared to the control sample (Fig. 5.4B).

5.3.4 Changes in the expression of other ripening-related genes

Other projects conducted in the CSIRO, Division of Horticulture laboratory in Adelaide have identified genes that are regulated in a ripening specific manner. These were used as probes to test the effects of BTOA on ripening related expression of other genes in grapes.

The GRIP4 clone was highly represented in a cDNA library made from post-véraison berries (pers. comm., Dr. C. Davies, CSIRO, Adelaide). The expression of GRIP4 in control fruit is very high just after véraison (ten weeks postflowering) but was undetectable before véraison and later in berry development. In BTOA-treated berries the GRIP4 message was not detected until 12 weeks postflowering and remained expressed at a high level throughout the rest of berry ripening (Fig. 5.5).

Vv*Chi*4 is a berry specific chitinase gene that is induced during grape berry ripening (Robinson *et al.* 1997). As Figure 5.5 shows, Vv*Chi*4 mRNA was first detected ten weeks after flowering in the control berries with expression increasing by 12 weeks postflowering. This high level of expression was maintained throughout the rest of berry development. However, BTOA treatment retarded the onset of Vv*Chi*4 gene expression for two weeks. Vv*Chi*4 mRNA was first detected in the 12 week postflowering BTOA-treated sample and remained highly expressed throughout ripening (Fig. 5.5).

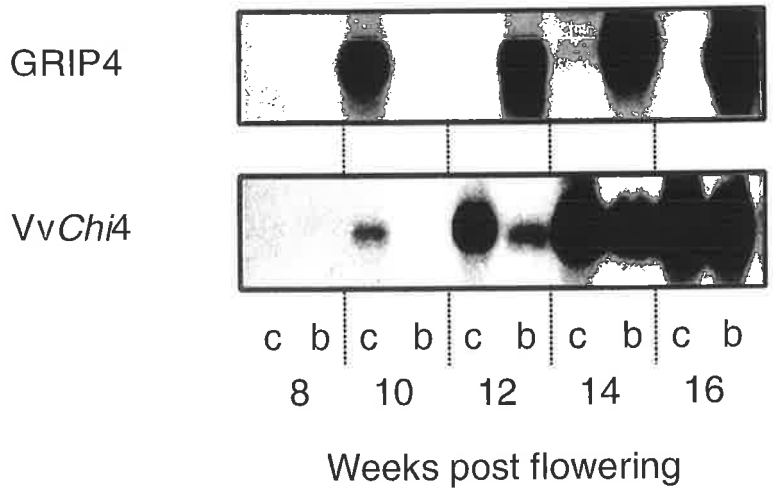


Figure 5.5 Effect of BTOA treatment on the expression of two ripening regulated genes. The ripening related genes GRIP4 and Vv*Chi*4 were used to probe total RNA from control (c) and BTOA-treated (b) berries.

5.3.5 ABA levels in the control and BTOA-treated berries

The level of ABA in the control berries was high in the sample taken two weeks after flowering but had decreased to a low level by six weeks postflowering (Fig. 5.6). After véraison (eight weeks postflowering) the level of ABA began to rise, reaching a maximum at 12 weeks postflowering after which the level again decreased. In the BTOA-treated fruit, the ripening related increase in ABA levels was delayed by two weeks when compared to the control fruit, which coincided with the delay in véraison in these fruit. In BTOA-treated fruit the ABA level increased at 12 weeks postflowering, reaching a maximum at 14 weeks postflowering and then declining.

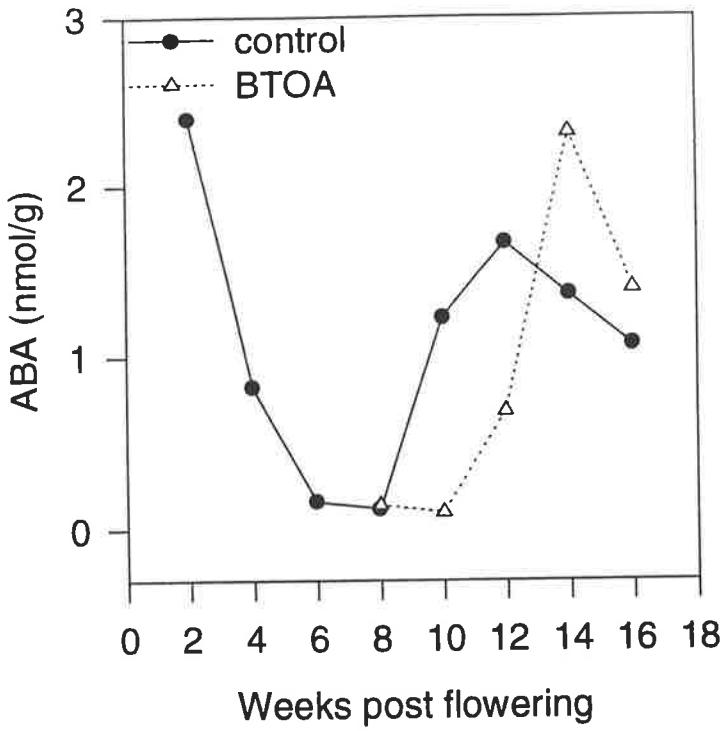


Figure 5.6 ABA levels in control and BTOA-treated berries. ABA levels were measured by GC-MS analysis as described in Section 5.2.3.

5.4 Discussion

The treatment of grape berries with the synthetic auxin-like compound BTOA before véraison resulted in a delay in the onset of ripening as has been observed previously (Weaver 1962; Hale 1968; Hale *et al.* 1970; Coombe and Hale 1973; Hale and Coombe 1974). Anthocyanin and hexose accumulation, berry softening, and an increase in berry weight are processes associated with berry ripening and each was delayed by approximately two weeks in the BTOA-treated fruit (Figs. 5.2, 5.3 and 5.4). The onset of the

accumulation of skin colour was later than the changes in the other ripening parameters by approximately two weeks in both samples. The final extent of the changes caused by the BTOA treatment was different for the ripening processes monitored. The final level of anthocyanins in the BTOA-treated fruit was 47% of the level in the control sample (Fig. 5.4A), the level of reducing sugars in BTOA-treated fruit was also reduced, being 75% of the control value (Fig. 5.3A). The final berry weight was the least effected ripening parameter measured, the BTOA-treated berries having 87% of the weight of the control berries (Fig. 5.3B). Although BTOA treatment of the berries delayed ripening and affected the final degree of ripening related changes, it did not appear to upset the coordination between the various ripening processes. Nevertheless, it has been noted that exogenous growth regulators can differentially affect colour development and the other ripening related processes. The application of ABA to developing grape berries can increase anthocyanin production without altering the changes in total soluble solids and titratable acids that occur in control berries (Kataoka *et al.* 1982). It appears that ripening in grape berries may consist of a set of processes that are coordinated, but not absolutely linked to each other by a single controlling factor.

The delay in the accumulation of hexoses in the BTOA-treated berries cannot be linked to the changes this treatment induces in the expression of a putative vacuolar invertase. The level of invertase mRNA seemed to be higher in the treated samples than the control samples at eight weeks postflowering (Fig. 5.3E). The continued elevated expression of invertase mRNA after ten weeks postflowering in the BTOA-treated sample coincided with higher invertase activity (Fig. 5.3C) which probably indicates that this mRNA is translated into active enzyme. However, it has been shown previously that the accumulation of hexoses in the vacuole does not appear to be controlled by the activity of invertases

(Hawker 1969; Davies and Robinson 1996). BTOA-treatment therefore seems to affect the mechanism of hexose accumulation in another manner, perhaps at the step of sucrose movement from the phloem or the movement of sugars across the plasma membrane.

Auxins have been shown to affect gene expression, both positively and negatively, in different plant tissues and plant species (reviewed by Guilfoyle 1986; Theologis 1986). BTOA-treated berries display both positive and negative effects on the level of expression of different genes. The expression of invertase mRNA was maintained after véraison in the BTOA-treated samples in contrast to the control samples (Fig. 5.3E) and, although GRIP4 expression was delayed by two weeks in the BTOA-treated samples, expression was maintained later in development when it was not detected in the control samples (Fig. 5.5). Negative regulation of UFGT and Vv*Chi*4 mRNA expression was observed, with the BTOA-treated samples showing induction two weeks later than the control samples. If the northern blots are compared, we can see that the changes in gene expression in the BTOA-treated samples were not due to changes in the mRNA profile due to non-specific degradation or an overall decrease in mRNA levels. For example, CHS and UFGT were not detected in the ten and 12 week postflowering BTOA-treated samples, yet invertase was readily detectable in both.

In contrast to climacteric fruit, ethylene does not seem to play a pivotal role in grape berry ripening. The level of ethylene is low in ripening grape berries (Coombe and Hale 1973; Alleweldt and Koch 1977; Weaver and Singh 1978). Depending on the time of application, exogenous ethylene or the ethylene releasing compound ethephon can either advance or retard the onset of ripening (Hale *et al.* 1970; Coombe and Hale 1973). Therefore, it seems that ethylene may be able to affect grape berry ripening but only as a secondary factor in

conjunction with other growth regulating hormones. It has been suggested that the increase in ABA levels which begin at véraison may be the trigger for ripening and that these levels of endogenous ABA may influence the effect of the exogenous application of a growth regulatory compound (Coombe and Hale 1973; Hale and Coombe 1974; Coombe 1976; Scienza *et al.* 1978; Cawthon and Morris 1982; Kataoka *et al.* 1982). Coombe (1976) went on to suggest that the decrease in ABA levels prior to its increase after véraison may be a part of this control process, although there is no direct evidence for this.

The studies described in this chapter support the role of plant growth hormones in the control of the expression of genes involved in grape berry ripening. The hormonal control appeared to be mediated via changes in the transcription patterns of these genes, although changes in the rate of mRNA turnover and posttranscriptional control may also be involved. The influence of the auxin-like compound BTOA on both ripening and developmentally regulated gene expression suggests that auxins may play a role in the development and ripening of grape berries. The decrease in IAA levels prior to véraison observed by Cawthon and Morris (1982) is also consistent with this suggestion. Perhaps auxins act as an inhibitor of ripening which is therefore not initiated until the auxin levels pass below a certain threshold. The application of BTOA to the berries may increase the levels of auxins and thus delay ripening. A similar scenario has been described for strawberry fruit where auxin levels decrease during development to low levels during ripening and where ripening can be delayed by the application of exogenous auxins (Given *et al.* 1988). However, the picture is complicated by the fact that BTOA treatment delays the rise in ABA levels normally associated with ripening. It is possible that BTOA treatment results in a delay in the decline of auxin levels which is somehow linked to the control of ABA levels. Therefore, grape berry ripening may be initiated by a combination of a decline in auxin

levels coupled with an increase in ABA levels. The increase in ABA levels may not be a cause of ripening but a result of the stress imposed on the berry during the ripening process, since ABA is known to be involved in the mediation of plant stress responses. The dramatic changes that occur at véraison, for example the rapid influx of sugars, may cause the berry considerable stress resulting in the increase in ABA levels and thus an ABA-mediated stress response.

The exact nature of the processes leading to the induction of ripening in grapes is still to be elucidated. However, the tools have been set in place for a further dissection of the factors which influence grape berry ripening. Treatments which decrease the levels of auxin early in berry development may reveal more about the role of auxin inhibition in ripening. However, the use of anti-auxins or auxin transport inhibitors would perhaps be complicated by the pleiotropic effects of these chemicals. It has been suggested that auxin signal transduction is mediated via phospholipase A (Scherer and Arnold 1997) which may offer another target for the manipulation of the effects of auxins on grape berries. The application of ABA to berries may also reveal aspects of the coordination of the various ripening processes. If this application could 'uncouple' the expression of various stress related mRNAs from the general ripening process, then it would suggest that ABA accumulation is a result and not a cause of ripening. Ultimately, a mutagenesis experiment from which ripening mutants could be obtained and characterised, would perhaps provide most valuable information about the control of ripening in grape berries.

Chapter 6

Grape myb-*like genes*

6.1 Introduction

In order to understand the control of anthocyanin biosynthesis in grape berries, the characterisation of grape anthocyanin regulatory genes is necessary. These clones may then be used to manipulate anthocyanin biosynthesis *in vivo* by using transgenic technology. The structural genes of the anthocyanin pathway are controlled by regulatory genes and these have been identified in some plant species (Section 1.7.3). These regulatory genes show homology to either *myc*- or *myb*-like transcription factors and have been shown to control the anthocyanin pathway at different points in different species. Transgenic studies have shown that some of the regulatory genes can induce anthocyanin biosynthesis in heterologous plant species, although the effect depends on both the source of the regulatory gene(s) and the target plant species (e.g. Lloyd *et al.* 1992; Quattrocchio *et al.* 1993; Mooney *et al.* 1995). However, the *myb* gene families in plants appear to have roles in other processes (Martin and Paz-Ares 1997) and so any homologue isolated may not necessarily be a transcription factor that regulates the expression of structural genes from the anthocyanin pathway. This is also true for *myc*-like genes, as *myc* homologues have been isolated from *Arabidopsis* that do not map to loci involved in anthocyanin synthesis (Urao *et al.* 1996; de Pater *et al.* 1997).

Several approaches were taken to clone anthocyanin regulatory genes from grape, one of which lead to the cloning of grape *myb*-like cDNAs. This chapter describes the isolation of

two *myb*-like cDNA clones from post-véraison grape berries and their partial characterisation.

6.2 Materials and methods

6.2.1 Plaque screening of grape cDNA library

6.2.1.1 Plating of library and lysates

XL1-Blue MRF' cells were grown in 50 mL of L broth (1% NaCl, 1% bacto-tryptone, 0.5% yeast extract [pH 7.0]) + 0.2% maltose + 10 mM $MgSO_4$ until they reached an OD_{600} of 0.5. These cells were centrifuged at 2000 rpm for 10 min and gently resuspended in 10 mM $MgSO_4$ to an OD_{600} of 0.5. Phage stocks were diluted to an appropriate level and added to either 600 µL (for 150 mm diameter plates) or 200 µL (for 85 mm diameter plates) of the prepared XL1-Blue cells. The mixture was incubated for 15 min at 37°C in a slowly shaking incubator to attach the phage to the bacteria. Top agar (0.5% NaCl, 0.2% $MgSO_4$, 0.5% yeast extract, 1% NZ amine, 0.7% agar [pH 7.5]), which had been equilibrated at 48°C, was added to the infected cultures and then poured onto NZY plates (0.5% NaCl, 0.2% $MgSO_4$, 0.5% yeast extract, 1% NZ amine, 1.5% agar [pH 7.5]) which were then incubated at 37°C until plaques of an appropriate size had formed.

6.2.1.2 Plaque lifts

Plaque lifts were performed as described in the Amersham instruction manual supplied with the Hybond N nylon membrane with slight modifications. Membrane discs of the

appropriate size were laid on the plates for 2 min and if duplicates were made the second filter was transferred for 4 min. The membrane was removed and placed DNA side up on filter paper soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 2 min. Following this, the membrane was placed DNA side up on filter paper soaked in neutralisation solution (1.5 M NaCl, 0.5 M Tris-HCl [pH 8.0]) for 5 min. After a 2 min wash in 2 × SSC, the membrane was air dried, UV-crosslinked and stored at 4°C pending hybridisation and detection.

6.2.1.3 Hybridisation and washing of membranes

Hybridisations were effected in a Hybaid mini hybridisation oven using rotating glass cylinders. The membranes were prehybridised in 0.1 mL cm^{-2} of prehybridisation solution (0.25 M sodium phosphate [pH 7.0], 1 mM EDTA [pH 8.0], 7% [w/v] SDS) at 65°C for at least 1 h. Radioactive probe was prepared (Section 2.2.6) and denatured, added to the prehybridisation solution and hybridised for 15 h at 65°C. Non-specifically bound probe was removed by washing the membrane as follows: two 10 min washes in 2 × SSC, 0.1% SDS at room temperature; and a 15 min wash in 1 × SSC, 0.1% SDS at 65°C. This treatment is equivalent to a hybridisation stringency of ~75% (i.e. allowing approximately 25% mismatch between pairing nucleotides). The probe was detected by exposing the membrane to Kodak XAR film at -80°C with intensifying screens.

6.2.1.4 Isolation of single plaques from agar plates

During the early rounds of screening agar plugs containing several plaques were removed using sterile pasteur pipettes. These plugs were vortexed briefly in 1 mL of SM buffer

(100 mM NaCl, 8 mM $MgSO_4$, 50 mM Tris-HCl [pH 7.5], 0.01% [w/v] gelatin) and stored at 4°C following the addition of 20 µL of chloroform. Following three rounds of screening, single plaques were isolated as agar plugs and treated as above.

6.2.1.5 In vivo *excision of pBluescript*

The ExAssist/SOLR system (Stratagene) was used to excise pBluescript from the λZAP II vector. The plaque of interest was isolated as described above (Section 6.2.1.4). In a 50 mL tube, 100 µL of the phage stock was mixed with 200 µL of XL-1 Blue cells (OD_{600} = 1.0) and 1 µL of ExAssist helper phage, and the mixture incubated at 37°C for 15 min to allow phage adsorption. This was incubated for a further 2 h at 37°C, following the addition of 3 mL of 2 × YT media (1% NaCl, 1.6% bacto-tryptone, 1% yeast extract [pH 7.0]). The tube was heated at 70°C for 20 min and spun for 15 min at 4000*g* to pellet the cells. The supernatant, which contains both the helper phage and excised phagemid, was collected and 1 µL used to infect 200 µL of SOLR host cells (OD_{600} = 1.0). The helper phage contains an amber mutation which prevents its replication in non-suppressing host strains such as SOLR cells. The infected SOLR cells were incubated at 37°C for 15 min and aliquots plated on media containing ampicillin (100 µg mL^{-1}) to select for bacteria harbouring pBluescript.

6.2.2 General DNA manipulations

During the cloning and subcloning of the *myb*-like cDNA clones a number of general techniques were employed. The techniques were as described in Sambrook *et al.* (1989).

6.2.3 Genomic DNA extraction and Southern analysis

DNA was extracted from a Shiraz young leaf sample as described by Thomas *et al.* (1993) and outlined in Section 3.2.4. This DNA was used for Southern analysis following the protocol in Section 3.2.5.

6.2.4 Total RNA extraction and northern analysis

Total RNA was extracted from the various grapevine tissue samples using the method described in Section 2.2.5. Radiolabelled probes were prepared and northern analysis carried out as described in sections 2.2.6 and 2.2.7.

6.3 Results

6.3.1 Cloning and characterisation of *myb*-like transcription factors

A cDNA library had been constructed from mRNA isolated from post-véraison (ten weeks postflowering) Shiraz grape berries by Dr. C. Davies (CSIRO, Adelaide). This library had been constructed in λZAP II vector, and packaged into bacteriophage. Approximately 800,000 plaques were screened for both *myb* and *myc* homologues. The probe used for the *myb* screening was a partial cDNA clone coding for *C1* (Dr. C. Martin, John Innes Institute, Norwich, UK), a maize *myb*-like gene known to regulate anthocyanin genes (Paz-Ares *et al.* 1987). Two probes were used for the *myc* screening. One probe encoded *Delila*, a *myc*-like gene isolated from snapdragon (Dr. C. Martin, John Innes Institute, Norwich, UK; Goodrich *et al.* 1992) and the other was a homologue from maize, *B-peru* (J. de Majnik,

CSIRO, Canberra: Chandler *et al.* 1989). Both of these genes are involved in the regulation of structural genes from the anthocyanin biosynthesis pathway.

No positive clones were obtained with either of the *myc*-like probes. Four plaques which hybridised on the initial *myb* screen tested positive through two more rounds of screening. Plasmids were rescued from the four plaques allowing further manipulations to be carried out in *E. coli*. The plasmid DNA was digested with *Sal* I to excise the cDNA inserts and these digests showed that the clone Vv*myb*1 had an insert size of ~1.3 kb, Vv*myb*2 and Vv*myb*4 were ~ 1.4 kb in length and Vv*myb*3 was ~2 kb.

Vv*myb*1 and Vv*myb*2 were chosen for full sequence analysis after primary sequencing of both ends of all the clones showed that both had homology to *myb*-like genes but were different from each other. Vv*myb*4 was 100% homologous to Vv*myb*2 and Vv*myb*3 was a chimaeric clone containing Vv*myb*1 and a fragment of another unknown gene. Vv*myb*1 and Vv*myb*2 were subcloned (see Appendix A) and fully sequenced across both DNA strands (Figs. 6.1 & 6.2). When the amino acid sequence of Vv*myb*2 was derived from the nucleic acid sequence there was no single open reading frame which went beyond 360 base pairs into the sequence. When the sequence was compared to other *myb*-like genes, it was found that there was an insertion in the cDNA that interrupted the highly conserved DNA-binding region common to these genes. The insert had a 5'-splice site, branch site and 3'-splice site structure common to plant introns (see Fig. 6.2) and it is therefore assumed that this cDNA was generated from an mRNA species that had not been completely processed. The clone Vv*myb*4 was sequenced over this region and also found to possess the intron. It is likely that Vv*myb*2 and Vv*myb*4 represent amplified copies of the same cDNA clone.

Figure 6.1 The sequence of V*vmyb*1. Nucleic acid sequence and derived amino acid sequence of the V*vmyb*1 cDNA.

```
   1 GGAAAAGAGAAGACGAGAGAGAAAGAAGAAAGAAAGATGAGGAATGCATCTTCAGCATCA  60
                                             M  R  N  A  S  S  A  S

  61 GCTCCACCTTCATCTTCTTCAAAAACACCATGCTGCATCAAGGTTGGATTGAAGAGGGGG  120
     A  P  P  S  S  S  S  K  T  P  C  C  I  K  V  G  L  K  R  G

 121 CCATGGACGCCGGAGGAAGACGAGGTTCTGGCCAATTACATCAAGAAAGAAGGAGAAGGC  180
     P  W  T  P  E  E  D  E  V  L  A  N  Y  I  K  K  E  G  E  G

 181 CGGTGGCGCACCCTCCCGAAGCGCGCCGGTCTCCTCCGCTGCGGCAAGAGCTGTCGCCTC  240
     R  W  R  T  L  P  K  R  A  G  L  L  R  C  G  K  S  C  R  L

 241 CGCTGGATGAACTACCTCCGCCCTTCCGTCAAGCGCGGCCAGATCGCCCCCGATGAAGAG  300
     R  W  M  N  Y  L  R  P  S  V  K  R  G  Q  I  A  P  D  E  E

 301 GATCTCATTCTCCGCCTCCACCGACTTCTCGGCAACAGGTGGGCTTTGATTGCCGGAAGA  360
     D  L  I  L  R  L  H  R  L  L  G  N  R  W  A  L  I  A  G  R

 361 ATTCCGGGCCGGACGGATAATGAGATAAAAAACTACTGGAACACACACCTGAGCAAGAAG  420
     I  P  G  R  T  D  N  E  I  K  N  Y  W  N  T  H  L  S  K  K

 421 CTGATCAGCCAGGGAATAGATCCGAGAACCCATAAGCCATTGAACCCTAATTCATCTGTT  480
     L  I  S  Q  G  I  D  P  R  T  H  K  P  L  N  P  N  S  S  V

 481 GATGTGAAAGCTTCTTCTTCAAAGGCAAAAGCTGTTATGAACCCTAACCCTAACCCTAAC  540
     D  V  K  A  S  S  S  K  A  K  A  V  M  N  P  N  P  N  P  N

 541 CCTAATCCTTCTCCTTCAGAAAAAGCAGCAGCCAACAAGGAAGCGGGGAACTTCAAGAGT  600
     P  N  P  S  P  S  E  K  A  A  A  N  K  E  A  G  N  F  K  S

 601 GACAATCAGTATCAGATTGGGGCAGCTGGCAATGATGGCAGTGCCAATATCCAGAATTCG  660
     D  N  Q  Y  Q  I  G  A  A  G  N  D  G  S  A  N  I  Q  N  S

 661 GATGGTTCCGGGACCGGATTGAGGAGCAGCAACAACGAAGAAGACGATGACCTTAACTGT  720
     D  G  S  G  T  G  L  R  S  S  N  N  E  E  D  D  D  L  N  C

 721 GGCACCGATGATGTCTTCTCTTCATTTTTGAACTCATTGATCAATGAGGATGTGTTCCCT  780
     G  T  D  D  V  F  S  S  F  L  N  S  L  I  N  E  D  V  F  P

 781 GGACAACACCATCTCCAACAACAGCACCATGGTGGTCTCATTGCACCGGGCTCCGATGCT  840
     G  Q  H  H  L  Q  Q  Q  H  H  G  G  L  I  A  P  G  S  D  A

 841 TTGATCTCTACTTCTTCAGTCCAGTCGTTCGGGTTCGGTACCAGCTGGGAAGCTGCAGTC  900
     L  I  S  T  S  S  V  Q  S  F  G  F  G  T  S  W  E  A  A  V

 901 ATGTCTTCCACGTCTGCTTTTAGCCAAATCGATCACTCCAAGAGTTTAACGATCAACCTG  960
     M  S  S  T  S  A  F  S  Q  I  D  H  S  K  S  L  T  I  N  L

 961 ATAAGCGGTTCTGACAGCCGGTGTTCTTCAATTTGGCTTCAACACCTGCAATGAGATATA 1020
     I  S  G  S  D  S  R  C  S  S  I  W  L  Q  H  L  Q  *

1021 TAGTTAATAAGAATTCTAGGGTTTGGGTTGTTAATTTATGTATGTGGTTGTGTTGTTGTG 1080

1081 TTAGTATGCTTTATTTGTATTGTGAACGAATCATGGATTCAGAAAAACGATATTGTGTTT 1140

1141 CGCTTCCGATCAAGAATTTGAATTTAGCACGGTTGTTCATCACAATATTTATGCAAATTC 1200

1201 CGAAAATTCCTTCAAAAAAAAAAAAAAA 1228
```

Figure 6.2 The sequence of V*vmyb2*. Nucleic acid sequence and derived amino acid sequence of the V*vmyb2* cDNA. The putative intron is represented by the lower case letters, and the 5'-splice site, branch site and 3'-splice sites are represented in bold.

```
1 ATTGATCCACGCAGAGAAGCAAGCCAGAGGGATGAGAAATCCGGCATCTGCGTCTACGAG  60
                                  M  R  N  P  A  S  A  S  T  S

61 TAAGACTCCGTGCTGTACCAAGGTTGGGTTGAAAAGAGGACCATGGACGCCTGAGGAAGA 120
    K  T  P  C  C  T  K  V  G  L  K  R  G  P  W  T  P  E  E  D

121 TGAGCTTCTAGCTAATTATGTGAAGAGAGAAGGTGAAGGGAGGTGGAGGACGCTGCCGAA 180
    E  L  L  A  N  Y  V  K  R  E  G  E  G  R  W  R  T  L  P  K

181 GCGAGCTGGGTTGCTGCGGTGCGGCAAGAGCTGTCGCCTCCGGTGGATGAATTATCTTCG 240
    R  A  G  L  L  R  C  G  K  S  C  R  L  R  W  M  N  Y  L  R

241 GCCGTCAGTGAAGCGCGGCCAGATAGCTCCCGATGAGGAAGATCTCATTCTTCGCCTCCA 300
    P  S  V  K  R  G  Q  I  A  P  D  E  E  D  L  I  L  R  L  H

301 TCGCCTGCTCGGTAACAGgtgctgtttgtatcatttttttcctggtctgaaatttgcctca 360
    R  L  L  G  N  R

361 gatcctaacatattgatgcatttctctttttcttaaatttttgagacatttctttgattt  420

421 tcagGTGGTCTCTGATTGCCGGAAGGATCCCGGGGCGTACAGACAATGAGATCAAGAACT 480
        W  S  L  I  A  G  R  I  P  G  R  T  D  N  E  I  K  N  Y

481 ACTGGAACACCCATCTCAGCAAGAAACTCATCAGCCAAGGAATAGATCCCAGAACCCACA 540
    W  N  T  H  L  S  K  K  L  I  S  Q  G  I  D  P  R  T  H  K

541 AGCCACTAAACCCTAAACCTAATCCATCACCAGATGTTAATGCTCCTGTCTCAAAATCAA 600
    P  L  N  P  K  P  N  P  S  P  D  V  N  A  P  V  S  K  S  I

601 TTCCAAATGCAAACCCTAACCCTAGTTCTTCCCGGGTGGGAGAAATTGGAAGCAACCATG 660
    P  N  A  N  P  N  P  S  S  S  R  V  G  E  I  G  S  N  H  E

661 AGGTCAAGGAGATTGAAAGTAATGAAAATCACAAGGAGCCGCCTAACCTGGATCAGTATC 720
    V  K  E  I  E  S  N  E  N  H  K  E  P  P  N  L  D  Q  Y  H

721 ACAGTCCACTTGCGGCCGATAGCAATGAGAATTGGCAAAGCGCAGATGGGTTGGTAACGG 780
    S  P  L  A  A  D  S  N  E  N  W  Q  S  A  D  G  L  V  T  G

781 GACTACAAAGCACCCATGGTACCAGCAACGATGACGAAGACGATATCGGGTTCTGCAACG 840
    L  Q  S  T  H  G  T  S  N  D  D  E  D  D  I  G  F  C  N  D

841 ACGATACATTCTCTTCATTTTTGAATTCTTTGATTAACGAGGATGTGTTTGGAAATCATA 900
    D  T  F  S  S  F  L  N  S  L  I  N  E  D  V  F  G  N  H  N

901 ATCATCATCATCAGCAGCAGCAGCAACAGCAGCTGCAGCAGCTGCAGCAGCCATCTAATG 960
    H  H  H  Q  Q  Q  Q  Q  Q  Q  L  Q  Q  L  Q  Q  P  S  N  V

961 TGATTGCACCATTGCCCCACCCAGCAATTTCTGTGCAGGCCACCTTCAGTAGTAGCCCTA 1020
    I  A  P  L  P  H  P  A  I  S  V  Q  A  T  F  S  S  S  P  R

1021 GAACTGTCTGGGAACCTGCTGCACTAACATCTACATCGGCTCCTTTAGTCCACGATCAAA 1080
     T  V  W  E  P  A  A  L  T  S  T  S  A  P  L  V  H  D  Q  K

1081 AAGACTCCATGTCTCCCTGAAAATGATGAATCAAGCTTATATGAGTTTGATTAAAATCTG 1140
     D  S  M  S  P  *

1141 TGTGATGTAATTTGTTGTTTCCCTTTTTGTGTTTTGATGTTGTATTAGCTTCCACTCTTG 1200

1201 GTTATTTAATTAGGATGTTAATTAAAGCTTTATGTGTGAATATCGTATGTGAAATGGATC 1260

1261 CTTGCAACTAAGTTGTTTTTCCCCAAAAAAAAAAAAAAA 1300
```

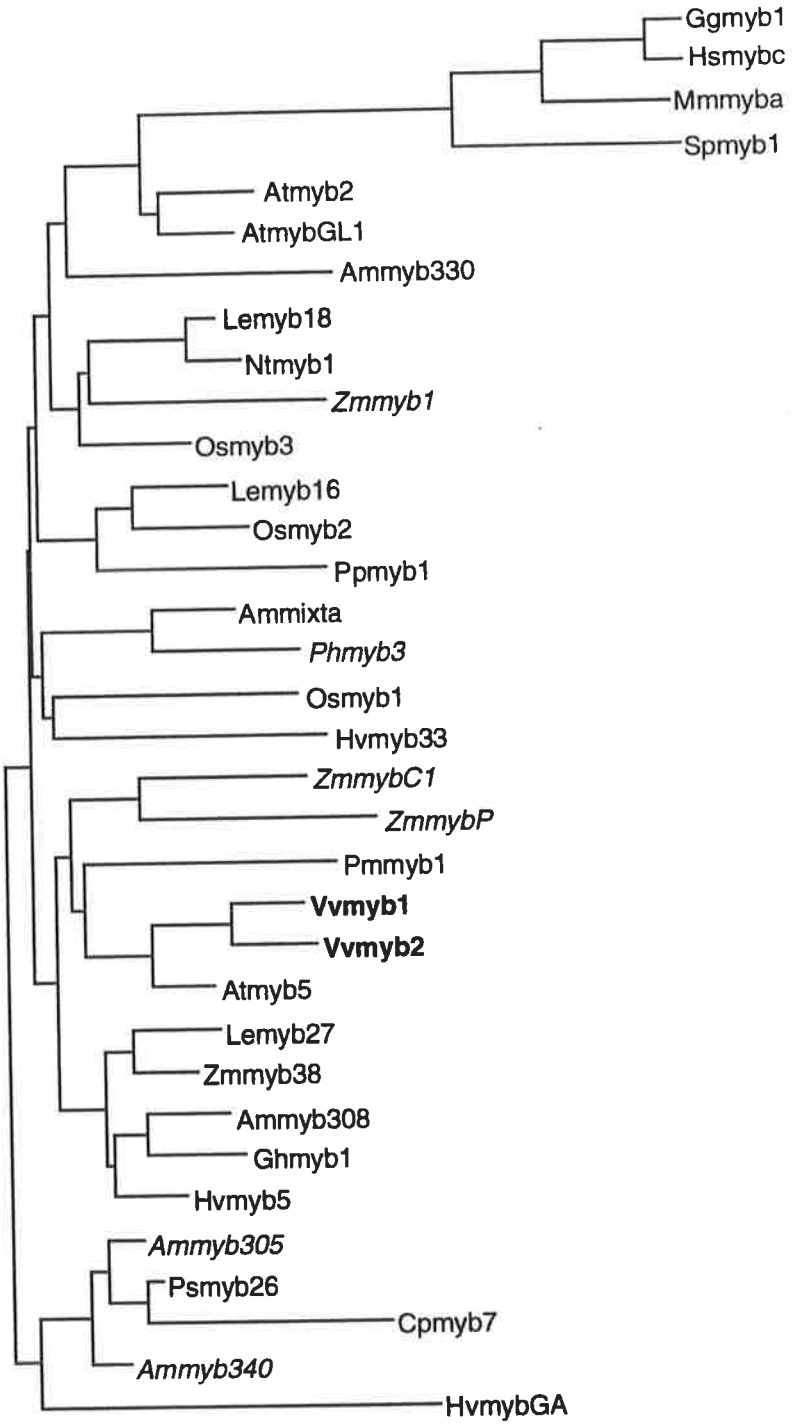
Vv*myb*1 is 67% homologous to Vv*myb*2 at the amino acid level, and most of this identity occurs in the amino terminal regions of the proteins. This region of the protein coincides with the DNA-binding domain and is highly conserved among plant *myb*-like proteins (Martin and Paz-Ares 1997). This homology with other plant *myb* genes provides strong evidence that the two genes cloned are also *myb* transcription factors. An alignment of the DNA-binding domains from a selection of plant *myb* proteins and Vv*myb*1 and Vv*myb*2 is shown in Figure 6.3. The names of the *myb*-like genes isolated from other species have been standardised to make it easier to identify the species from which they were obtained. These DNA-binding domains usually consist of two imperfect repeats (termed R2 and R3) which distinguish the plant *myb* genes from the animal *myb* genes which have three imperfect repeats (R1, R2 and R3). These proteins also have regularly spaced tryptophan residues (arrowed in Fig. 6.3) which are implicated in DNA binding (Anton and Frampton 1988). The first tryptophan residue of the R3 repeat is often replaced by another hydrophobic residue in plant *myb* proteins, and this is also the case for the grape *myb*-like cDNAs cloned which have isoleucine at this position. There are also additional regions of plant *myb* proteins which have features of transcriptional activator domains, but the function of these regions has only been proven in a few cases (Goff *et al.* 1991; Baranowskij *et al.* 1994). A dendogram which represents the similarity between the complete amino-acid sequence of *myb*-like genes from a number of plant species and other organisms is shown in Figure 6.4. The non-plant species form a separate group as can be seen at the top of the figure, and this is presumably due to the presence of the additional 'R1' repeat in these sequences. The degree of similarity amongst the plant sequences shows that in many cases *myb*-like genes from one species are more homologous to those from another species than from their own species. For example, three tomato *myb* homologues, Lemyb16, 18 and 27, show less homology to each other than to *myb* genes from other species. However, the

Figure 6.3 Homology between the DNA-binding domains of plant *myb* genes and Vv*myb*1 and Vv*myb*2. Sequence alignment of the two repeats of the DNA-binding regions of a selection of known plant *myb* gene sequences using the CLUSTALW program (Thompson *et al.* 1994). The sequences were obtained either from the GenBank sequence database under the accession numbers U26935 (*Arabidopsis*; Atmyb5), L04497 (Cotton; Ghmyb1), X95296 (Tomato; Lemyb27), Y11415 (Rice; Osmyb1), Z13996 (Petunia; Phmyb3), U39448 (Black spruce; Pmmyb1), X67050 (Moss; Ppmyb2), X06333 & U57002 (Maize; ZmmybC1 & ZmmybP) or the PIR sequence database under the accession numbers JQ0957 (Snapdragon; Ammyb330) and S35729 (Barley; Hvmyb2). The grape *myb* sequences were deduced from the cDNA clones Vv*myb*1 and Vv*myb*2. The consensus line indicates where residues are completely conserved by an asterisk (*) and where residues are identical in at least 70% of the sequences by a period (.). The arrows represent the positions where conserved tryptophan residues are found in the R2 and R3 repeats of animal *myb* proteins, the first one of the R3 repeat being replaced by another hydrophobic residue in plants.

```
                    ↓                   ↓                   ↓
Ammyb330    TNKGAWTKEEDQRLINYIRAHGEGCWRSLPKAAGLLRCGKSCRLRWINYLRPD
Atmyb5      MKRGPWTVEEDEILVSFIKKEGEGRWRSLPKRAGLLRCGKSCRLRWMNYLRPS
Ghmyb1      TNKGAWTKEEDQRLINYIRVHGEGCWRSLPKAAGLLRCGKSCRLRWINYLRPD
Hvmyb2      TNKGAWTKEEDQRLIAYIRANGEGCWRSLPKAAGLLRCGKSCRLRWMNYLRPD
Lemyb27     TNKGAWTKEEDERLISYIRAHGEGCWRSLPKAAGLLRCGKSCRLRWINYLRPD
Osmyb1      LKKGPWTPEEDEKLIAYIKEHGQGNWRTLPKNAGLSRCGKSCRLRWTNYLRPD
Phmyb3      LKKGPWTPEEDQKLLAYIEEHGHGSWRALPAKAGLQRCGKSCRLRWTNYLRPD
Pmmyb1      LNKGAWSAEEDSLLGKYIQTHGEGNWRSLPKKAGLRRCGKSCRLRWLNYLRPC
Ppmyb2      LRRGPWTSEEDQKLVSHITNNGLSCWRAIPKLAGLLRCGKSCRLRWTNYLRPD
ZmmybC1     VKRGAWTSKEDDALAAYVKAHGEGKWREVPQKAGLRRCGKSCRLRWLNYLRPN
ZmmybP      LKRGRWTAEEDQLLANYIAEHGEGSWRSLPKNAGLLRCGKSCRLRWINYLRAD
Vvmyb1      LKRGPWTPEEDEVLANYIKKEGEGRWRTLPKRAGLLRCGKSCRLRWMNYLRPS
Vvmyb2      LKRGPWTPEEDELLANYVKREGEGRWRTLPKRAGLLRCGKSCRLRWMNYLRPS
Consensus     * *. .**  *  ..   *.. ** .*. ***.********** ****.

                    ↓                   ↓                   ↓
Ammyb330    LKRGNFTEEEDEIIIKLHSLLG NKWSLIAGALP GRTDNEIKNYWNTHIKRK
Atmyb5      VKRGGITSDEEDLILRLHRLLG NRWSLIAGRIP GRTDNEIKNYWNTHLRKK
Ghmyb1      LKRGNFTEEEDELIIKLHSLLG NKWSLIAGRLP GRTDNEIKNYWNTHIKRK
Hvmyb2      LKRGNFTDDEDELIIRLHSLLG NKWSLIAGQLP GRTDNEIKNYWNTHIKRK
Lemyb27     LKRGNFTEEEDELIIKLHSLLG NKWSLIAGRLP GRTDNEIKNYWNTHIRRK
Osmyb1      IKRGRFSFEEEEAIIQLHSILG NKWSAIAARLP GRTDNEIKNYWNTHIRKR
Phmyb3      IKRGKFTLQEEQTIIQLHALLG NRWSAIATHLP KRTDNEIKNYWNTHLKKR
Pmmyb1      IKRGNITADEEELIIRMHALLG NRWSIIAGRVP GRTDNEIKNYWNTNLSKK
Ppmyb2      LKRGIFSEAEENLILDLHATLG NRWSRIAAQLP GRTDNEIKNYWNTRLKKR
ZmmybC1     IRRGNISYDEEDLIIRLHRLLG NRWSLIAGRLP GRTDNEIKNYWNSTLGRR
ZmmybP      VKRGNISKEEEDIIIKLHATLG NRWSLIASHLP GRTDNEIKNYWNSHLSRQ
Vvmyb1      VKRGQIAPDEEDLILRLHRLLG NRWALIAGRIP GRTDNEIKNYWNTHLSKK
Vvmyb2      VKRGQIAPDEEDLILRLHRLLG NRWSLIAGRIP GRTDNEIKNYWNTHLSKK
Consensus   .**     *.  *. .* .** * *. **.  * .***********...
```

Figure 6.4 (Overleaf) Amino acid sequence comparison of the two grape *myb*-like cDNA clones with those from other species. The dendogram was calculated using the DISTANCES and GROWTREE programs of the University of Wisconsin GCG software package (Devereux *et al.* 1984). The sequences were obtained either from the GenBank sequence database under the accession numbers X79108 (Snapdragon; Ammixta), U26935 & L22786 (*Arabidopsis*; Atmyb5 & AtmybGl1), U33917 (Resurrection plant; Cpmyb7), X03477 (Chicken; Ggmyb1), L04497 (Cotton; Ghmyb1), M13666 (Human; Hsmybc), X87690 & X70881 (Barley; HvmybGA & Hvmyb33), X99210, X98308 & X95296 (Tomato; Lemyb16, Lemyb18 & Lemyb27), L35261 (Mouse; Mmmyba), U72762 (Tobacco; Ntmyb1), Y11415, Y11351 & Y11414 (Rice; Osmyb1, Osmyb2 & Osmyb3), Z13996 (Petunia; Phmyb3), U39448 (Black spruce; Pmmyb1), X67050 (Moss; Ppmyb2), Y11105 (Pea; Psmyb26), U96090 (Sea urchin; Spmyb1), X06333 & U57002 (Maize; ZmmybC1 & ZmmybP) or the PIR sequence database under the accession numbers JQ0958, JQ0960, JQ0957 & JQ0959 (Snapdragon; Ammyb305, Ammyb308, Ammyb330 & Ammyb340), JQ2390 (*Arabidopsis*; Atmyb2), S35729 (Barley; Hvmyb2), S04898 & S04899 (Maize; Zmmyb1 & Zmmyb38). The grape sequences were deduced from the cDNA clones Vv*myb*1 and Vv*myb*2 and are shown in bold, and the *myb*-like genes known to activate flavonoid genes are written in italics.

grape clones show greatest homology to one another and, amongst the other plant clones, their next nearest neighbour is the Atmyb5 gene cloned from Arabidopsis (Li *et al.* 1996; Fig. 6.4). The subset of *myb*-like genes that have been shown to activate and/or bind to the promoters of flavonoid structural genes does not form a distinctive group, which means that this type of *myb* homologue can not be identified through sequence analysis.


Ggmyb1
Hsmybc
Mmmyba
Spmyb1
Atmyb2
AtmybGL1
Ammyb330
Lemyb18
Ntmyb1
Zmmyb1
Osmyb3
Lemyb16
Osmyb2
Ppmyb1
Ammixta
Phmyb3
Osmyb1
Hvmyb33
ZmmybC1
ZmmybP
Pmmyb1
Vvmyb1
Vvmyb2
Atmyb5
Lemyb27
Zmmyb38
Ammyb308
Ghmyb1
Hvmyb5
Ammyb305
Psmyb26
Cpmyb7
Ammyb340
HvmybGA

6.3.2 Southern analysis of the grape *myb*-like cDNA clones

The genomic organisation of Vv*myb*1 and Vv*myb*2 was studied by using both the full-length cDNA clones and specific 3'-end subclones (EPR5 and DER3; Appendix A) as probes.◆ The homology between the 3'-regions of these cDNA clones was only 45% and so no cross-hybridisation was expected considering the washing conditions used. Shiraz genomic DNA was digested with the restriction enzymes *Dra* I, *Eco* RV and *Hind* II which are insensitive to ^{m5}CG and ^{m5}CNG methylation (McClelland and Nelson 1988). The blots were washed at a stringency that allowed for a mismatch of approximately 25% between pairing nucleic acids.

The cDNA clone of Vv*myb*1 has no restriction sites for any of these three enzymes, but Vv*myb*2 possesses an *Eco* RV site approximately 480 bp from the 3'-end of the cDNA clone and there were two major bands hybridising in the lane containing the products of *Eco* RV digestion when probed with Vv*myb*2 (Fig. 6.5A). Vv*myb*1 and Vv*myb*2 detected multiple copies of related sequences, some with higher intensity than others suggesting that they have varying degrees of homology to the respective probes (Fig. 6.5A). When the 3'-end probes were used, multiple bands were still detected in each lane for both probes suggesting the presence of *myb*-like gene subfamilies (Fig. 6.5B). However, the size of the hybridising bands for each probe was different, indicating that the probes used for northern analysis (Section 6.3.3) were specific for each *myb*-like cDNA subfamily, but did not appear to be specific for a single gene.

◆see addendum

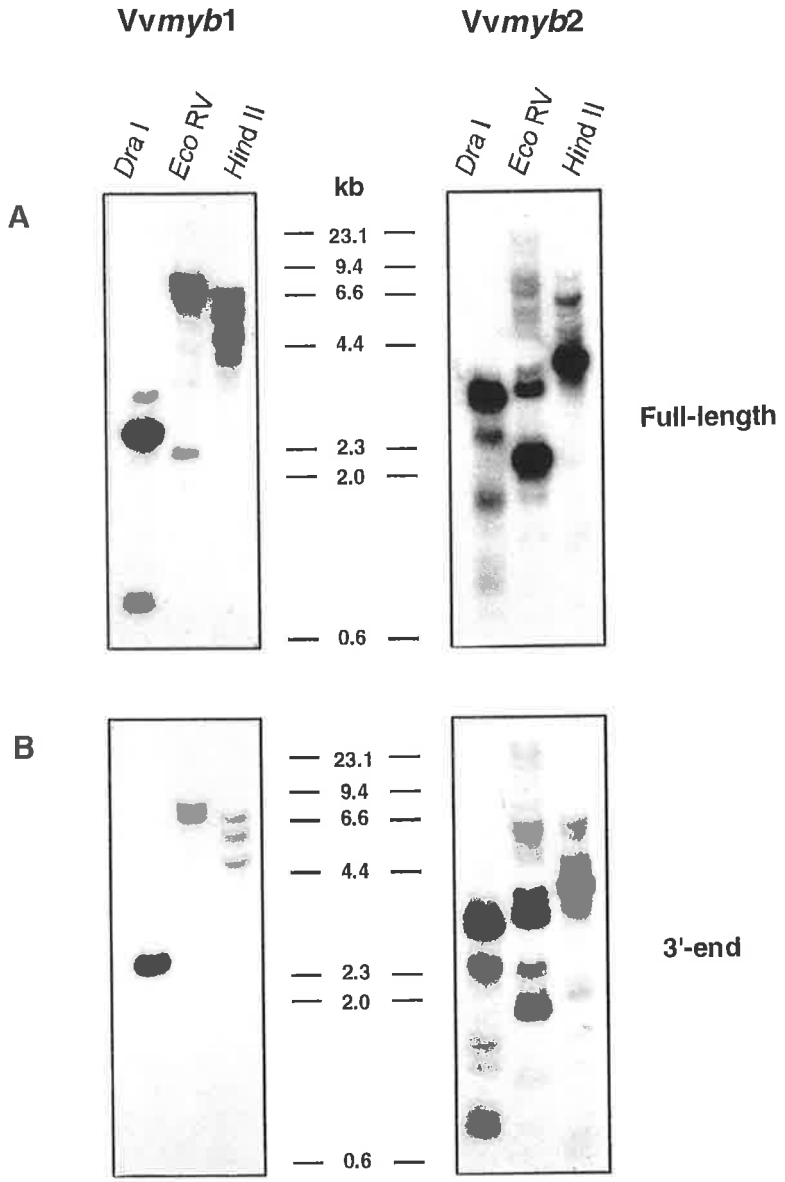


Figure 6.5 Hybridisation of full-length and 3'-end Vv*myb*1 and Vv*myb*2 probes to grape genomic DNA. Southern analysis of Shiraz genomic DNA probed with ^{32}P-labelled probes of the grape *myb*-like cDNAs. The DNA was digested with restriction enzymes as indicated above each lane. The top figure (A) is the filters after being probed with full-length Vv*myb*1 or Vv*myb*2 and the bottom figure (B) is the same filters stripped and probed with 3'-end probes of the same clones. DNA size standards are indicated between each set of blots.

6.3.3 Northern analysis of the grape *myb*-like cDNA clones

The expression patterns of the two grape *myb*-like clones were investigated to determine whether one had a similar expression pattern to UFGT (Figs. 2.7, 3.1 and 3.2), which might suggest that it has a role in the regulation of the UFGT gene. Subclones from the 3'-ends of each of the grape *myb*-like cDNA clones were radiolabelled and used to probe northern blots. Total RNA samples isolated from a berry skin developmental series, which were used for the studies in Chapter 2, were probed with the *myb*-like clones and these are presented in Figure 6.6.

Vv*myb*1 showed greatest expression in the flower and two weeks postflowering samples with a reduction in expression four weeks postflowering. No Vv*myb*1 mRNA was detectable in the six and eight weeks postflowering samples, and then expression was again detected in all the samples from ten weeks postflowering through to 16 weeks postflowering. This pattern of expression is very similar to that seen for all of the anthocyanin pathway genes except UFGT (Fig. 2.7). The expression of Vv*myb*2 was slightly different with maximum expression at two weeks postflowering and the lowest level of Vv*myb*2 mRNA expression was detected at six weeks postflowering (Fig. 6.6). Although it is difficult to compare expression levels in different northern blots, the expression levels of both genes after véraison (eight weeks postflowering) are both similar and very low, whereas Vv*myb*2 seems to be expressed at a greater level in the samples taken early in berry development (Fig. 6.6). This probe also hybridised to another transcript of greater length in the flower and two weeks postflowering samples. The detection of multiple transcripts on northern blots probed with *myb*-like cDNA clones has been reported

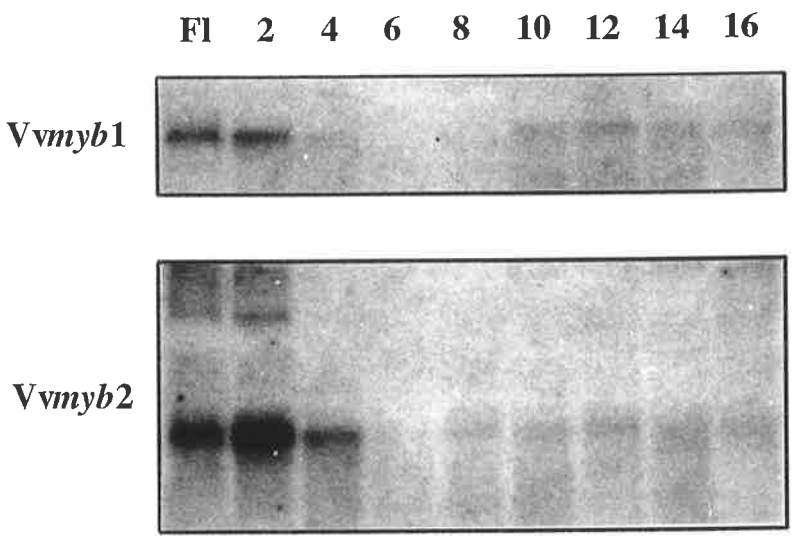


Figure 6.6 Temporal expression of Vv*myb*1 and Vv*myb*2 in grape berry skin during berry development. Northern blots are of total RNA from grape flowers (FL) and grape berry skin samples taken at eight different stages during development (the numbers indicate weeks postflowering), probed with grape cDNA clones which code for *myb*-like proteins.

for some other *myb*-like genes cloned from plants (Jackson *et al.* 1991; Avila *et al.* 1993; Grotewold *et al.* 1991; Lin *et al.* 1996).

Total RNA samples extracted from different tissues of the grape cultivar Shiraz were also analysed for the expression of the grape *myb*-like genes (Fig. 6.7). Vv*myb*1 mRNA was detected in all of the samples, although at a very low level in the green cane and root samples. Low levels of expression were detected in all the other tissue samples with the highest levels seen in the mid leaf and flower samples. Vv*myb*2 was more readily detected than Vv*myb*1 in all of the samples. The highest level of expression was found in the flower and mid leaf samples, and significant levels of Vv*myb*2 mRNA were also detected in the other leaf samples, tendrils, green cane and seeds. Low levels of expression were seen in root and in berry skin and flesh samples taken 14 weeks postflowering. The Vv*myb*2 probe

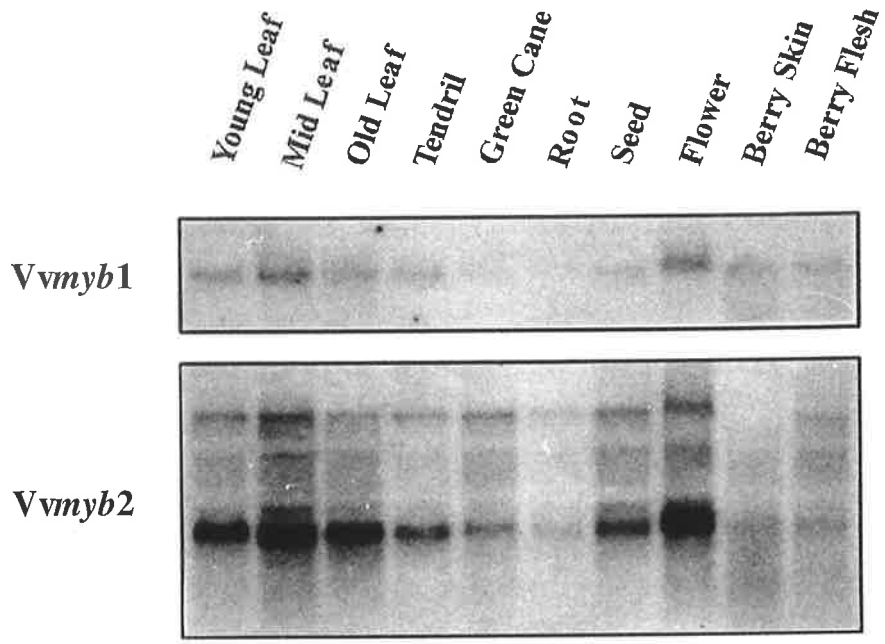


Figure 6.7 Expression of grape *myb*-like genes in various grapevine tissues. Northern blots are of total RNA from grapevine tissue samples probed with the grape cDNA clones as indicated on the left side of the figure.

also hybridised to a larger transcript in all of these samples, except the 14 weeks postflowering berry skin sample.

Grape cultivars with white-skinned berries and those with black-skinned berries were also analysed for *myb*-like gene expression (Fig. 6.8). Total RNA was isolated from berry skin samples, which are described in Chapter 3, and used to produce northern blots. These blots were probed with the 3'-end subclones of Vv*myb*1 and Vv*myb*2. Vv*myb*1 expression was detected in all samples except Muscat Gordo and the highest levels were detected in Pinot Noir (Fig. 6.8). Vv*myb*2 was highly expressed in both Pinot Noir and Semillon with lower levels of expression detected in the other species. Again, Muscat Gordo had no detectable

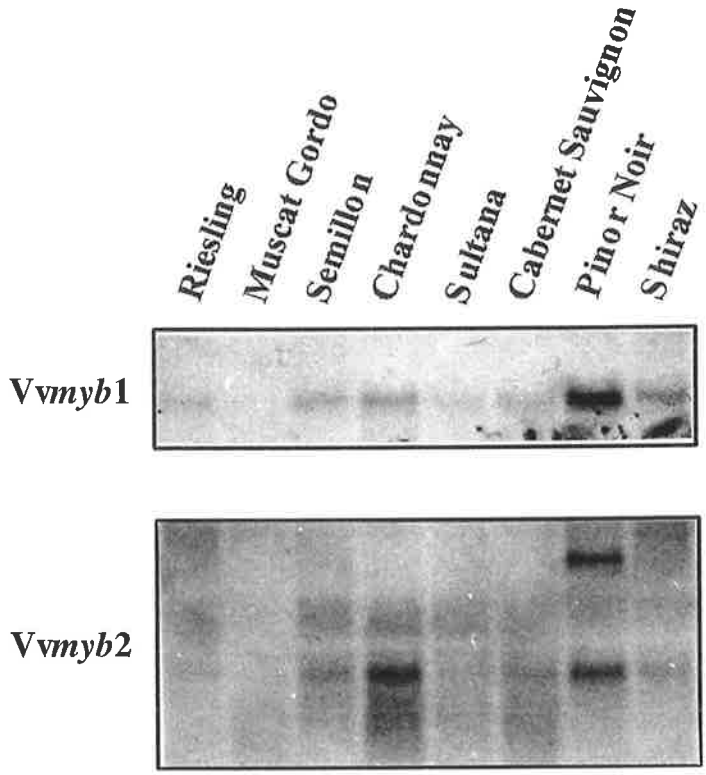


Figure 6.8 Expression of grape *myb*-like genes in the skins of white and black grape cultivars. Northern blots are of total RNA from grape berry skin samples probed with grape cDNA clones as indicated on the left side of the figure.

mRNAs homologous to Vv*myb*2. Pinot Noir was the only sample that displayed the presence of a larger transcript homologous to Vv*myb*2.

6.4 Discussion

This chapter describes the isolation of two *myb*-related cDNA clones from ripening grape berries. The predicted polypeptide products of these genes have structural features that are common to *myb*-like transcriptional activators. Both contain a *myb* DNA-binding domain which is characterised by two imperfect repeats (Fig. 6.3). These repeats contain conserved

tryptophan residues (arrowed in Fig. 6.3) which are predicted to be important for DNA-binding activity (Anton and Frampton 1988). The first tryptophan of the second repeat is usually replaced by another aromatic/hydrophobic amino acid in plants, and this is the case for the grape *myb*-like cDNAs as well. When compared to other plant *myb*-related clones (Fig. 6.4), Vv*myb*1 and Vv*myb*2 show the greatest similarity to each other and a homologue isolated from *Arabidopsis*, Atmyb5 (Li *et al.* 1996). Unfortunately, the *myb* sequences do not fall into groups related to their functions, and so the functions of the grape clones can not be determined by sequence analysis alone.

Southern analysis revealed that both *myb*-like clones were members of small gene families (Fig. 6.5). This is not surprising as 14 different *myb*-related cDNAs have been isolated from tomato (Lin *et al.* 1996) and Jackson *et al.* (1991) reported cloning six different *myb*-like cDNA clones from snapdragon floral tissue. Use of only the DNA-binding region of one of the grape clones and washing at lower stringency may have revealed an even larger *myb*-like gene family in grapes. Probes from the 3'-ends of the clones showed that more than one gene homologous to each of those isolated were present in the genome (Fig. 6.5B).◆ It appears that these *myb*-like genes are members of small subfamilies. This makes the northern analyses difficult to interpret as multiple genes may be contributing to the signal seen in any tissue at any stage of development.

Vv*myb*1 and Vv*myb*2 were expressed in a broad range of grape tissues (Figs. 6.6, 6.7 & 6.8). During berry skin development Vv*myb*1 mRNA was detected early in development, decreased to undetectable levels in the six and eight weeks postflowering samples and was then expressed again after véraison. This pattern of expression is very similar to that seen for several of the 'early' structural genes from the anthocyanin biosynthesis pathway

◆see addendum

(Fig. 2.7). Vv*myb*2 mRNA was detectable in berry skin throughout development (Fig. 6.5). Vv*myb*1 and Vv*myb*2 were also expressed in all other grape tissues analysed, although Vv*myb*2 mRNA was more readily detectable (Fig. 6.6). However, the differences in signal intensity between the two probes could be due to artefacts of northern analysis. Muscat Gordo berry skin lacked detectable expression of both grape *myb*-like genes, but expression was detected for both genes in all other varieties analysed (Fig. 6.7). The expression patterns of either Vv*myb*1 or Vv*myb*2 were not similar to that of UFGT and thus it is unlikely that they have a role in regulating anthocyanin biosynthesis in grapes. As both genes appear to be expressed in most tissues, this suggests that they perhaps play a role in regulating genes necessary for the viability of all cell types or in specific cell types found in most plant tissue. However, the fact that both probes hybridised to multiple genes on a Southern blot means that the mRNA detected in the various samples may have been transcribed from different genes.

The inability to isolate cDNA clones from the grape berry cDNA library homologous to the *myc*-like genes *Delila* and *B-peru* could be due to several reasons. Perhaps the stringency of the hybridisation and washing conditions was too high to allow the detection of *myc* homologues. The *myc* genes isolated from other plant species show homology only in certain regions found at the N-terminus and the helix-loop-helix DNA-binding domains (Purugganan and Wessler 1994). There is considerably more homology amongst the monocot genes than between the monocot and dicot genes isolated. Nevertheless, Southern analysis was carried out on Shiraz genomic DNA using the *Delila* and *B-peru* probes, and in both cases cross-hybridising bands were detected and the same pattern was seen with both probes (data not shown). The ability to detect grape DNA fragments homologous to *Delila* and *B-peru* was comparable with the ability to detect grape DNA fragments

homologous to the *myb*-like gene *C1* (data not shown). The results of the Southern analysis demonstrated that there are *myc*-like genes in the grape genome, but the inability to clone these from the post-véraison berry cDNA suggests that they are not highly expressed in this tissue at this stage of development. Degenerate oligonucleotide primers were also designed to conserved regions of *myc*-like genes and the PCR technique used to try and amplify *myc*-like fragments from cDNA prepared from ten weeks postflowering grape berry mRNA. None of the PCR fragments isolated showed sequence homology to *myc*-like transcription factors (data not shown). Post-véraison cDNA was chosen because of the desire to isolate genes that regulate anthocyanin biosynthesis genes. However, it may be a better strategy to attempt to isolate *myc* homologues from a grape genomic library.

Multiple transcripts hybridised to the Vv*myb*2 probe in most grapevine tissues, in Shiraz berry skin two weeks postflowering, and in the Pinot Noir skin sample (Figs. 6.6, 6.7 & 6.8). Only one transcript was detected by the Vv*myb*1 probe. The detection of multiple transcripts by *myb* clones has been described in other plant species (Jackson *et al.* 1991; Avila *et al.* 1993; Grotewold *et al.* 1991; Lin *et al.* 1996). In tomato and petunia, the presence of multiple transcripts was limited to certain *myb* genes, and in the tomato study, different multiple transcript sizes were detected in different tissues (Avila *et al.* 1993; Lin *et al.* 1996). This was not due to the presence of many closely related genes as the probe which hybridised to the most mRNA transcripts in tomato tissues was present as a single gene copy, whereas another gene that had multiple copies in the genome, hybridised to only one sized mRNA transcript (Lin *et al.* 1996). Nevertheless, Southern analysis revealed the presence of small gene subfamilies for each of the grape *myb*-like genes, so it is possible that the different sized transcripts originate from different members of this family. It is possible is that the multiple transcripts are the result of alternative splicing or incomplete processing

of RNA, or the utilisation of different promoter sites. The *P* gene from maize was found to produce different sized transcripts due to both unprocessed or incompletely processed RNA and alternative splicing at the 3'-end of the gene (Grotewold *et al.* 1991). Mis-splicing has also been observed in a maize *myc* homologue, called *intensifier-1*, that is involved in the negative regulation of the flavonoid pathway (Burr *et al.* 1996). The mis-splicing lead to the premature translation termination suggesting that this might play a role in regulating the levels of the functional protein (Burr *et al.* 1996). Alternative gene splicing as a means to regulate gene action seems to be common in eukaryotes (Bingham *et al.* 1988), and so it is possible that this process is involved in regulating Vv*myb*2 activity.

It is difficult to speculate on the functions of Vv*myb*1 and Vv*myb*2 from the limited data available. Where the *myb* genes of a particular species have been well studied they are always found to be members of a large gene family, for example, *Arabidopsis* is estimated to contain more than 100 *myb* genes (Martin and Paz-Ares 1997) and 14 distinct *myb* genes have been isolated from tomato (Lin *et al.* 1996). Only a fraction of those *myb* genes isolated have had their functions deduced and the functions are distinct for different *myb* genes. In maize there are several *myb* genes involved in the regulation of the flavonoid pathway. The *C1* gene activates transcription of structural genes from the anthocyanin pathway resulting in anthocyanin accumulation in the aleurone (Cone *et al.* 1986; Paz-Ares *et al.* 1986; Paz-Ares *et al.* 1987) and another *myb* homologue, *Pl*, plays the same role in the rest of the maize plant (Cone *et al.* 1993). *Zm 1* can activate the promoter of the maize DFR (*A1*) gene, but not the entire pathway, and another *myb* homologue, *Zm 38*, acts as an inhibitor of *C1* on at least the DFR promoter (Franken *et al.* 1994). The maize *P* gene product regulates phlopabene accumulation by activating a specific subset of genes from the flavonoid pathway (Grotewold *et al.* 1994). So in maize, many *myb* genes are involved in

the regulation of the flavonoid structural genes and each has distinct but overlapping targets. Cell shape can also be determined by the activity of *myb* genes. The *Mixta* gene in *Antirrhinum* encodes a *myb* homologue and is involved in the development of the conical shape of petal epidermal cells (Noda *et al.* 1994), and the development of trichomes in *Arabidopsis* is dependent on the *myb*-like gene *GL1* (Oppenheimer *et al.* 1991). Some other *myb* genes have been shown to be expressed in response to plant growth regulators and stresses. A *myb*-related cDNA was isolated from barley aleurone (*Gamyb*) and found to be regulated by gibberellic acid (GA). Furthermore, *Gamyb* could activate transcription from a high-pI α-amylase gene promoter, suggesting a role for *Gamyb* in the GA-regulated change in gene expression in barley aleurone (Gubler *et al.* 1995). Water stress or ABA can induce the expression of *myb* genes from *Arabidopsis* (*Atmyb2*; Urao *et al.* 1993), *Craterostigma plantagineum* (*cpm7* and *cpm10*; Iturriaga *et al.* 1996) and maize (*C1*; Hattori *et al.* 1992).

The ability of *myb* genes to activate the transcription of genes is further complicated by the role of *myc*-like regulators in this process. Some *myb* genes like *C1*, *Pl* and *GL1* require a *myc*-related protein for their activity (Ludwig *et al.* 1989; Cone *et al.* 1993; Larkin *et al.* 1994), whereas others like *P*, *Zm1*, *myb305* and *myb340* can activate transcription from a promoter independently (Franken *et al.* 1994; Grotewold *et al.* 1994; Moyano *et al.* 1996). Therefore, there are diverse roles played by those *myb*-like genes for which functions have been assigned and there are many more *myb* genes which have unknown functions in the plant. Each plant species seems to possess large *myb*-like gene families which regulate specialised physiological functions (Martin and Paz-Ares 1997). Grapevines appear to possess a number of *myb* genes, two of which are described in this chapter. Their functions

have yet to be elucidated and this will probably require transgenic studies (see Chapter 8 for further discussion).

Chapter 7

Grape anthocyanin gene promoters

7.1 Introduction

The previous chapter described the cloning of *myb*-like genes from grape berry cDNA. However, it could not be determined that the cDNAs isolated were involved in the regulation of anthocyanin structural genes. Nevertheless, the expression patterns of these cDNAs suggested that they were not involved in the regulation of UFGT, which appears to be a controlling step in anthocyanin biosynthesis in grapevine. Another approach to cloning anthocyanin regulatory genes involving a functionally broad screen was thought desirable to overcome limitations of heterologous screening. The one-hybrid screening system has been used successfully to isolate cDNA clones coding for specific DNA-binding proteins from both animals and plants (e.g. Li and Herskowitz 1993; Wang and Reed 1993; Kim *et al.* 1997; Ulmasov *et al.* 1997). This chapter describes initial work in developing this system to be used for the isolation of grape anthocyanin regulatory genes.

7.2 Materials and methods

7.2.1 Plaque screening of grape cDNA and genomic libraries

The methods used for cDNA library screening are outlined in the previous chapter (Section 6.2.1). The process of screening the Shiraz genomic DNA library (Dr. R. van Heeswijck, University of Adelaide) is the same as that for the cDNA library (Section 6.2.1) except that the host cells used were XL1-blue MRA. The genomic library does not have the *in vivo*

excision capacity and so phage DNA was purified by the method outlined below (Section 7.2.2). Subsequent DNA manipulations were carried out as described in Sambrook *et al.* (1989).

7.2.2 DNA preparation from λFIX II phage

Phage which had tested positive during screening of the grape genomic DNA library (Dr. R. van Heeswijck, University of Adelaide) were amplified by harvesting from completely lysed 85 mm NZY plates that had been overlaid with 2.5 mL of SM buffer and shaken overnight at 4°C. This phage was plated out again at high density on five 150 mm NZY plates and harvested by overlaying with 5 mL of SM buffer and shaking at 4°C overnight. The bulk phage preparation was then vortexed briefly and spun at 4000*g* for 10 min before collecting the supernatant and adding 0.02 volumes of chloroform.

DE52 resin was prepared by placing it in several volumes of 0.05 N HCl and then slowly adjusting the pH back to 6.8 with concentrated NaOH. The resin was then washed several times with L broth until equilibrated and finally made to 75% resin and 25% L broth. The resin was then stored at 4°C following the addition of 0.1% sodium azide.

The phage DNA preparation was begun by adding 0.8 mL of phage lysate to 0.8 mL of DE52 resin which had been pelleted in a centrifuge and the supernatant removed. This lysate and resin was mixed constantly for 10 min then centrifuged for 5 min at 14,000*g* and the supernatant recovered. Centrifugation was repeated to remove the rest of the resin from the supernatant. The DNA was precipitated by the addition of 100 µL of 5 M NaCl and 540 µL of isopropanol and incubating at -20°C for 30 min. DNA was pelleted by

centrifugation at 14,000*g* for 10 min and the pellet washed with 70% ethanol. Following resuspension in 200 µL TE, contaminating proteins were removed with two phenol:chloroform:*iso*-amylalcohol (25:24:1) extractions and one chloroform:*iso*-amylalcohol (24:1) extraction. The DNA was again precipitated by the addition of 0.1 volume of 5 M NaCl and 2 volumes of ethanol and incubating at -70°C for 30 min. Nucleic acids were pelleted by centrifugation at 14,000*g* for 15 min, the pellet washed with 70% ethanol and then dried and resuspended in 20 µL of water.

7.2.3 Promoter walking

Libraries for promoter walking were constructed by Dr. M. Thomas (CSIRO, Adelaide) following the method of Siebert *et al.* (1995).◆ Four of the libraries, being those constructed from *Dra* I, *Eco* RV, *Hpa* I and *Sca* I restriction digests of Shiraz genomic DNA, were used to attempt the PCR amplification of DNA upstream of the grape DFR and LDOX genes. The nested adaptor primers used were O1 (5'-GGATCCTAATACGACTCACTATAGGG-3') for the first reaction and O2 (5'-AATAGGGCTCGAGCGGC-3') for the second reaction. The gene specific primers for DFR were VVDFR1 (5'-GGCAACATGGAAGACGCCGGTGCAG-3') for the first reaction and VVDFR2 (5'-ATGCGTCTCCGCTTTGGGCAAGTCC-3') for the second reaction. The gene specific primers for LDOX were VVLDOX1 (5'-GCTTTCTTCAACTCCTCCCGGCATC-3') for the first reaction and VVLDOX2 (5'-GCTGGTGAGCTCTTCTTGGGGGCGG-3') for the second reaction. Primary PCR reactions were conducted in 25 µL volumes containing 1 µL of the library DNA, 60 mM Tris-SO_4 (pH 9.1), 18 mM $(NH_4)_2SO_4$, 1.1 mM $MgSO_4$, 200 µM dNTPs, 0.4 µM adaptor primer O1 and gene specific primer, and 0.5 µL of ELONGASE enzyme mix (Gibco BRL). The cycle parameters were as follows: initial

◆see addendum

denaturation step at 94°C for 1 min. followed by 35 cycles of denaturation at 94°C for 30 sec and annealing/extension at 68°C for 6 min. and a final annealing/extension time of 15 min. Secondary PCR reactions were conducted in 40 µL volumes using 0.8 µL of a 1/100 dilution of the first round reaction and the adaptor specific primer O2 and the appropriate nested gene-specific primer. The same cycle parameters were used. PCR products were examined on a 1% agarose gel and PCR products isolated and cloned. General DNA manipulations were performed as described in Sambrook *et al.* (1989).

7.2.4 Nuclear protein extracts

Several methods slightly modified from that described by Sablowski *et al.* (1994) were used to attempt to isolated proteins from grape berry skin nuclei. The basic method involved grinding 5 g of tissue in liquid nitrogen in a mortar and pestle until powdered. This powder was transferred to 15 mL of nuclei extraction buffer which contained 1 M hexylene glycol, 10 mM Pipes-KOH (pH 7.0), 10 mM $MgCl_2$, 1% β-mercaptoethanol, 1 mM PMSF, 5 µg mL^{-1} leupeptin. This slurry was passed through two layers of miracloth and two 7.5 mL aliquots were carefully layered onto two 2.5 mL 30% Percoll cushions in 15 mL centrifuge tubes. The nuclei were pelleted by centrifugation at 700*g* for 10 min at room temperature with a soft start and stop. The pelleted nuclei were resuspended in 250 µL of a buffer containing 20% glycerol, 40 mM KCl, 25 mM Hepes (pH 7.4), 0.1 mM EDTA, 5 mM $MgCl_2$, 0.5 mM DTT, 1 mM PMSF, 5 µg mL^{-1} leupeptin. At this stage an aliquot was usually taken to be viewed by microscopy to determine the success of the nuclei preparation. Proteins were released from the nuclei by adding 5 M NaCl to a final concentration of 0.4 M and shaking the slurry for 1 h. Nuclear debris was pelleted by

centrifugation at 14,000*g* for 15 min and the supernatant assayed for protein concentration using a BioRad protein assay kit.

7.2.5 One-hybrid screening for UFGT promoter binding proteins

The one-hybrid screening system used was based on a kit obtained from Clontech called the MATCHMAKER one-hybrid system.◆ Protocols were based on information received from Clontech, but the descriptions below relate to the specific development of the system to screen for grape UFGT promoter binding proteins.

7.2.5.1 Preparation of target-reporter constructs

Target reporter constructs were prepared using a 166 bp fragment immediately upstream from the translation start point of the UFGT genomic clone GUG14 (see Fig. 7.2). This region of the genomic clone was amplified using specific primers containing restriction sites for insertion into the reporter plasmids pHISi, pHISi-1 and pLacZi. The forward primer UTPP1 (5'-GGAATTCGGGCCGCCATGCAGAATG-3') contained an *Eco* RI restriction enzyme site, and the reverse primer UTPP2 contained a *Sma* I site (5'-TCCCCCGGGTTGGAATGGGGGATGTT-3'). The 20 µL PCR mix contained 1 ng of GUG14 DNA, 1 unit of *Taq* DNA polymerase, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 0.5 µM UTPP1 and UTPP2 primers, 200 µM of dGTP, dCTP, dTTP and dATP. Following an initial denaturation cycle of 3 min at 94°C, 35 cycles of PCR were performed (denaturation 40 s at 94°C; annealing 30 s at 50°C; extension 120 s at 72°C) followed by a 15 min elongation step. The small DNA fragment was precipitated from the PCR mix and then digested with *Eco* RI and *Sma* I.

◆see addendum

Aliquots of 0.1 μg of each reporter plasmid were completely digested with *Eco* RI and *Sma* I in a 20 μL double digest. This allowed the directional cloning of the UFGT promoter fragment upstream of the reporter gene in each case. Ligation reactions were set up containing 5 μL of the digested reporter plasmid, 1 μL of the amplified and digested promoter DNA and 1 unit of T4 DNA ligase. These reactions were allowed to proceed at room temperature overnight. T4 ligase was heat denatured, 1 μL of the ligation reaction used to transform competent DH5α cells by electroporation and transformations plated on L + Amp (100 μg mL^{-1}) plates. Colonies were then screened for inserts of the correct size and sequenced to confirm the UFGT promoter region was present and in the correct orientation. Primers used for the sequencing were: for pLacZi based plasmids, LACSP1 (5'-GCTACAAAGGACCTAATG-3'); for pHISi based plasmids, HISP1 (5'-TTCCCAGTCACGACGTTG-3'); for pHISi-1 based plasmids, HI1SP1 (5'-ATTATCATGACATTAACC-3'). Final target-reporter constructs containing the 166 bp UFGT promoter fragment upstream of the reporter gene were named pULacZi26, pUHISi10 and pUHIS-123.

7.2.5.2 Preparation of yeast strains carrying the target-reporter constructs

The yeast strain used in the one-hybrid system (YM4271) has mutated *ura* and *his* genes and so recombination with the functional *URA3* or *HIS3* on the reporter vectors enables the new yeast strains to grow on media lacking uracil or histidine. The target reporter vectors are first linearised to increase the efficiency of homologous recombination. The plasmids pUHISi10 and pUHISi-123 were digested at the unique *Xho* I site and the pULacZi26

plasmid was digested at the unique *Nco* I site using 1 μg of each plasmid in a total volume of 20 μL.

Competent YM4271 cells were prepared from a 50 mL overnight culture. Enough cells to produce an OD_{600} of 0.2-0.3 were transferred into 300 mL of YPD media (2% bacto-peptone, 1% yeast extract, 2% dextrose, pH 5.8) and incubated at 230 rpm for 3 h at 30°C. The yeast cells were pelleted by centrifugation at 1000*g* for 5 min at room temperature and resuspended in 25 mL of TE buffer. The cells were pelleted again at 1000*g* for 5 min at room temperature and resuspended in 1 mL of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M lithium acetate for a final volume of approximately 1.5 mL.

These competent cells were transformed with the three linearised vectors. Carrier DNA (100 μg of sheared and denatured herring sperm DNA) was mixed with 1 μg of digested reporter plasmid and, as controls, 100 μg of carrier DNA was also mixed with 1 μg of the same uncut reporter plasmid. Aliquots of 100 μL of the yeast competent cells were added to each tube and mixed well followed by the addition of 600 μL of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.1 M lithium acetate, 40% (w/v) PEG 4000. The tubes were mixed well by vortexing and incubated by shaking at 200 rpm for 30 min at 30°C. Each tube then had 70 μL of DMSO added. The cells were heat shocked at 42°C for 15 min, chilled on ice for 2 min, pelleted at 12,000*g* for 10 sec and then resuspended in 150 μL of TE. The pUHISi10 and pUHISi-123 transformants plated on synthetic dropout medium (0.67% yeast nitrogen base without amino acids, 2% dextrose, 1 × dropout solution; SD)/-His plates and pULacZi26 transformants plated on SD/-Ura plates. Colonies appeared in 3-4 days and were then restreaked on the same selection media.

It was necessary to test the 'new' yeast reporter strains for the level of background expression. A yeast strain containing the integrated pULacZi26 construct was tested using a β-galactosidase filter assay as described in Section 7.2.5.5 and was found to have low levels of background. This stain was set aside to be used to construct a dual reporter vector as described later. A yeast strain containing the integrated pUHISi10 construct was also tested for background expression. The promoter driving *HIS3* expression is inhibited by 3-aminotriazole (3AT) and so 5 µL aliquots of a 1 mL suspension of a single yeast colony were plated onto SD/-His media containing 0, 15, 30, 45 and 60 mM 3AT. The yeast only grew on SD/-His and <45 mM 3AT which indicated that background expression was low. To create a dual reporter strain, the yeast with the integrated pULacZi26 construct was transformed again (as described above) with linearised pUHISi10 construct. Thus, the final yeast strain which was used for the library screening (Section 7.2.5.4) had both the *LacZ* and *HIS3* genes under the control of the 166 bp grapevine UFGT promoter fragment.

7.2.5.3 Preparation of an activation domain fusion cDNA library

A fusion library was prepared in order to screen for genes encoding proteins which bind to the UFGT promoter DNA using the reporter yeast strain prepared as described above. Approximately 2 mg of total RNA was isolated from a Shiraz berry skin sample taken 11 weeks postflowering during the 1996-97 growing season. Anthocyanin accumulation is at its greatest rate during this stage of development (data not shown). Intact and pure polyA$^+$ RNA was isolated from the total RNA using a PolyATract mRNA isolation system (Promega) according to the manufacturer's instructions. A cDNA library was then constructed in the pGAD10 vector using a Two-Hybrid cDNA Library Construction Kit

(Clontech) following the protocols supplied. The final library contained approximately 1.5×10^6 recombinant clones and was subsequently amplified.

7.2.5.4 Screening the fusion library for DNA-binding proteins

Screening the fusion library involved transforming the reporter yeast strain described in Section 7.2.5.2. Competent cells were produced and transformation scaled up to allow for screening $>1 \times 10^6$ independent clones as described in the Clontech manual. The transformed cells were plated on SD/-Leu/-His/+45 mM 3AT media, grown for 5 or 6 days and the largest colonies picked and restreaked on the same selective medium. The colonies which grew after being restreaked were picked again and streaked on more of the same selective media in order to be assayed for β-galactosidase activity.

7.2.5.5 Assaying for β-galactosidase activity

The colonies which were isolated from the library screen were assayed for β-galactosidase activity in order to confirm the DNA-binding activity of the fusion clone. This is important as the test for β-galactosidase activity is not as 'leaky' as the test for *HIS* growth selection. Restreaked colonies were transferred to Whatmann #5 filters by laying a dry filter onto the plate. The cells were then permeabilised by submerging the filter in liquid nitrogen for 10 sec. The filter was allowed to thaw at room temperature and placed colony side up on a filter presoaked in Z buffer/X-gal solution (10 mM KCl, 1 mM $MgSO_4$, 150 mM sodium phosphate [pH 7.0], 0.27% β-mercaptoethanol, 0.33 g mL^{-1} X-gal). These filters were then incubated at 30°C for a maximum of 8 h to allow the β-galactosidase activity to turn the colonies blue.

7.2.5.6 Plasmid DNA preparations from yeast cultures

Yeast colonies which had passed both the selective medium and blue/white screening systems were used to inoculate 10 mL aliquots of SD/-Leu liquid medium. The cultures were incubated at 30°C with shaking until the culture was saturated. The yeast were pelleted by centrifugation at 1000*g* for 5 min at room temperature and then resuspended in 200 µL of yeast lysis buffer (2% [v/v] Triton X-100, 1% [w/v] SDS, 100 mM NaCl, 10 mM Tris-HCl [pH 8.0], 1 mM EDTA). The resuspended yeast were transferred to tubes containing 200 µL of acid-washed glass beads and 200 µL of phenol:chloroform:*iso*-amylalcohol (25:24:1). This mixture was vortexed for 2 min to disrupt the yeast cells, and centrifuged at 12,000*g* for 10 min at room temperature. The aqueous phase was collected and the DNA precipitated by the addition of 0.1 volumes of 3M sodium acetate, 1 µL glycogen and 2.5 volumes of ethanol. Following a 30 min incubation at -70°C, the DNA was pelleted by centrifugation at 12,000*g* for 10 min. The pellet was washed with 70% ethanol, dried under vacuum and resuspended in 20 µL of water. This plasmid preparation was used to transform *E. coli* and the rest of the DNA manipulations were carried out using standard techniques (Sambrook *et al.* 1989).

7.3 Results

7.3.1 Cloning of grape UFGT cDNAs and genomic clones

The UFGT cDNA isolated by Sparvoli *et al.* (1994) was a partial clone covering only 532 bp of the 3'-end of the gene. To increase the chance that any genomic clone isolated contained the 5'-end of the coding region and also the promoter, a full-length grape UFGT

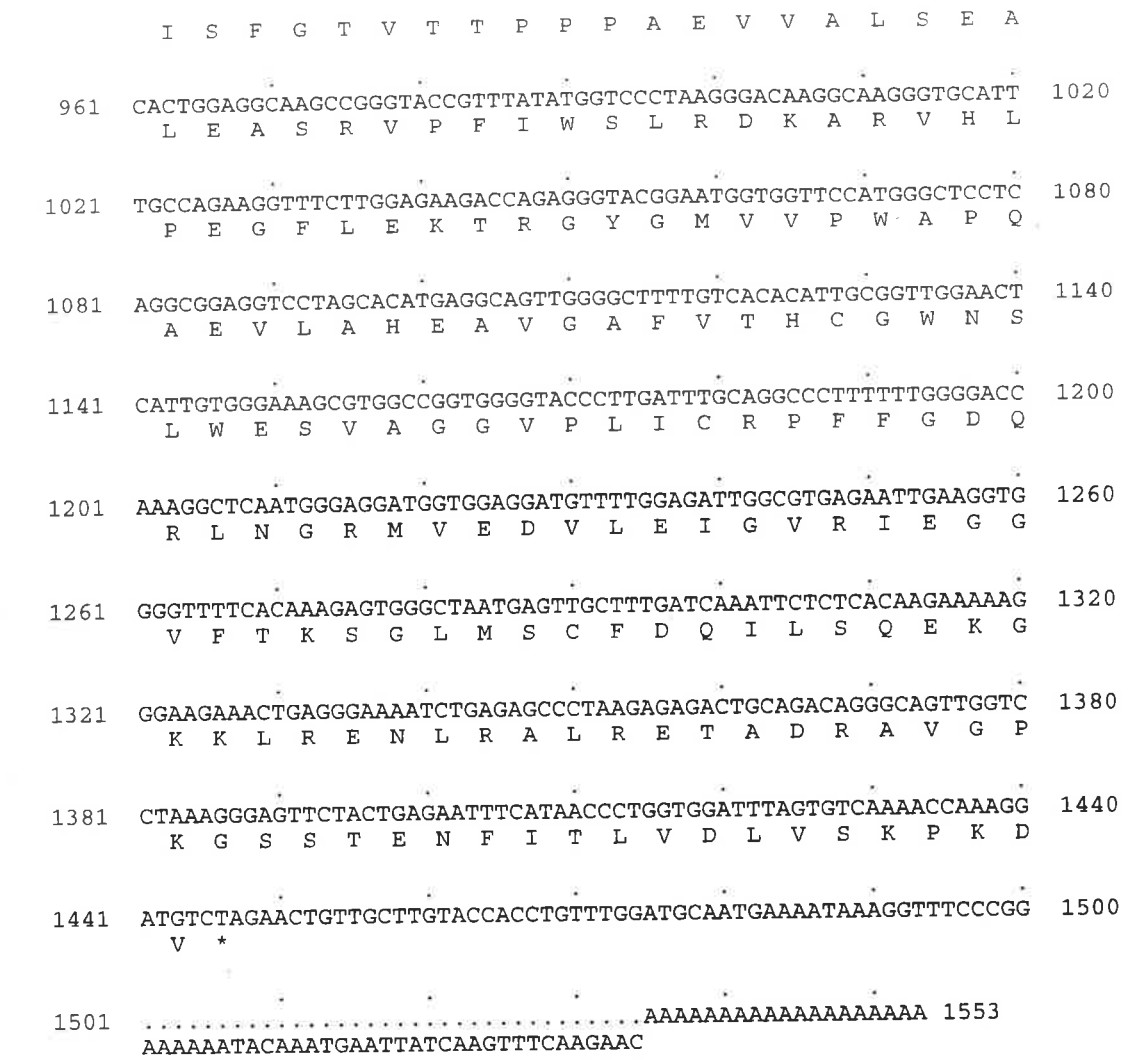
cDNA was cloned. This would allow a probe to be made from the 5'-end of the full-length clone. An aliquot of the cDNA library made from postvéraison berries (Dr. C. Davies, CSIRO, Adelaide) was screened using the clone isolated by Sparvoli *et al.* (1994). Two positive plaques came through three rounds of screening from the original 200,000 plaque aliquot of the library. This low frequency of positives supports the northern analysis (Fig. 2.7) which indicated that it was difficult to detect expression of this gene after véraison. Both of the positive plaques were rescued, subcloned and sequenced over both DNA strands (see Appendix B). One of the clones, Vv*ufgt*2, did not have an ATG start, whereas the other clone, Vv*ufgt*3, did appear to be full-length, but also had an intron (Fig. 7.1). Where the clones overlapped, Vv*ufgt*2 was identical to Vv*ufgt*3.

These clones isolated from the Shiraz cDNA library differed slightly in sequence from that isolated by Sparvoli *et al.* (1994) being 97% homologous over the length of the truncated clone. This may reflect genetic differences between the two different *V. vinifera* varieties from which the clones were isolated - Shiraz and Lambrusco.

A subclone obtained from the 5'-end of Vv*ufgt*3 was used to probe a 400,000 plaque aliquot of a Shiraz genomic library (Dr. R. van Heeswijck, University of Adelaide). Two plaques tested positive through three rounds of screening, and were then amplified and the DNA purified. The phage preparations were cut with the restriction endonucleases *Eco* RI, *Not* I and *Sac* I and were shown to have different restriction patterns (data not shown). The DNAs from these digests were Southern blotted and probed with the 5'-end probe used to screen the library. The *Sac* I digests revealed bands of approximately 3 kb that hybridised

Figure 7.1 The sequence of Vv*ufgt*3. Nucleic acid sequence and derived amino acid sequence of the Vv*ufgt*3 cDNA. The putative intron is represented by the lower case letters, and the 5'-splice site, branch site and 3'-splice sites are represented in bold. The sequence of Vv*ufgt*2 is identical to that of Vv*ufgt*3 except it lacks the first 6 bases and the putative intron, and it contains an extra 34 bp before the poly-A tail as indicated at the bottom of the figure.

```
   1  ATGTCTCAAACCACCACCAACCCCCATGTGGCCGTCCTGGCCTTCCCCTTCTCCACCCAT   60
      M  S  Q  T  T  T  N  P  H  V  A  V  L  A  F  P  F  S  T  H

  61  GCAGCCCCCCTCCTTGCCGTCGTTCGCCGGCTTGCTGCCGCTGCCCCTCATGCAGTCTTC  120
      A  A  P  L  L  A  V  V  R  R  L  A  A  A  A  P  H  A  V  F

 121  TCCTTCTTCAGCACCAGCCAATCCAACGCCTCCATCTTCCACGACTCCATGCATACCATG  180
      S  F  F  S  T  S  Q  S  N  A  S  I  F  H  D  S  M  H  T  M

 181  CAATGTAATATCAAGTCCTATGATATCTCCGACGGTGTGCCTGAGGGGTATGTGTTCGCC  240
      Q  C  N  I  K  S  Y  D  I  S  D  G  V  P  E  G  Y  V  F  A

 241  GGGCGGCCCCAGGAGGATATTGAGCTGTTCACGAGGGCTGCGCCGGAGAGCTTTAGGCAG  300
      G  R  P  Q  E  D  I  E  L  F  T  R  A  A  P  E  S  F  R  Q

 301  GGGATGGTAATGGCTGTGGCCGAGACAGGGCGGCCAGTGAGCTGCCTGGTGGCTGACGCA  360
      G  M  V  M  A  V  A  E  T  G  R  P  V  S  C  L  V  A  D  A

 361  TTCATTTGGTTTGCCGCAGATATGGCAGCAGAGATGGGGTtGGCTTGGCTGCCGTTTtGG  420
      F  I  W  F  A  A  D  M  A  A  E  M  G  L  A  W  L  P  F  W

 421  ACTGCAGGGCCTAACTCACTCTCCACCCATGTTTACATTGATGAAATCAGAGAAAAGATT  480
      T  A  G  P  N  S  L  S  T  H  V  Y  I  D  E  I  R  E  K  I

 481  GGAGTTTCAGgtggttttacctgctaatttgttttctaagatttttgatgctttaaaaga  540
      G  V  S

 541  ttgattgttcaatcccttacgttacagGCATTCAAGGCCGTGAAGACGAGCTGCTCAATT  600
                              G  I  Q  G  R  E  D  E  L  L  N  F

 601  TCATTCCCGGAATGTCTAAAGTACGTTTTCGCGACCTGCAGGAAGGCATCGTGTTCGGAA  660
        I  P  G  M  S  K  V  R  F  R  D  L  Q  E  G  I  V  F  G  N

 661  ACCTAAACTCGCTCTTCTCACGCATGCTCCATCGGATGGGCCAAGTGCTACCTAAGGCGA  720
        L  N  S  L  F  S  R  M  L  H  R  M  G  Q  V  L  P  K  A  T

 721  CTGCAGTTTTCATAAACTCCTTCGAGGAGCTCGACGATTCCCTAACCAATGATCTCAAAT  780
        A  V  F  I  N  S  F  E  E  L  D  D  S  L  T  N  D  L  K  S

 781  CCAAGCTCAAGACGTACCTCAATATCGGTCCATTCAACCTAATAACCCCACCGCCGGTTG  840
        K  L  K  T  Y  L  N  I  G  P  F  N  L  I  T  P  P  P  V  V

 841  TACCCAACACAACCGGCTGCCTCCAATGGCTCAAAGAAAGAAAACCCACCTCGGTCGTGT  900
        P  N  T  T  G  C  L  Q  W  L  K  E  R  K  P  T  S  V  V  Y

 901  ACATTAGCTTTGGCACCGTCACGACACCACCCCCAGCCGAGGTTGTAGCCCTATCTGAGG  960
```

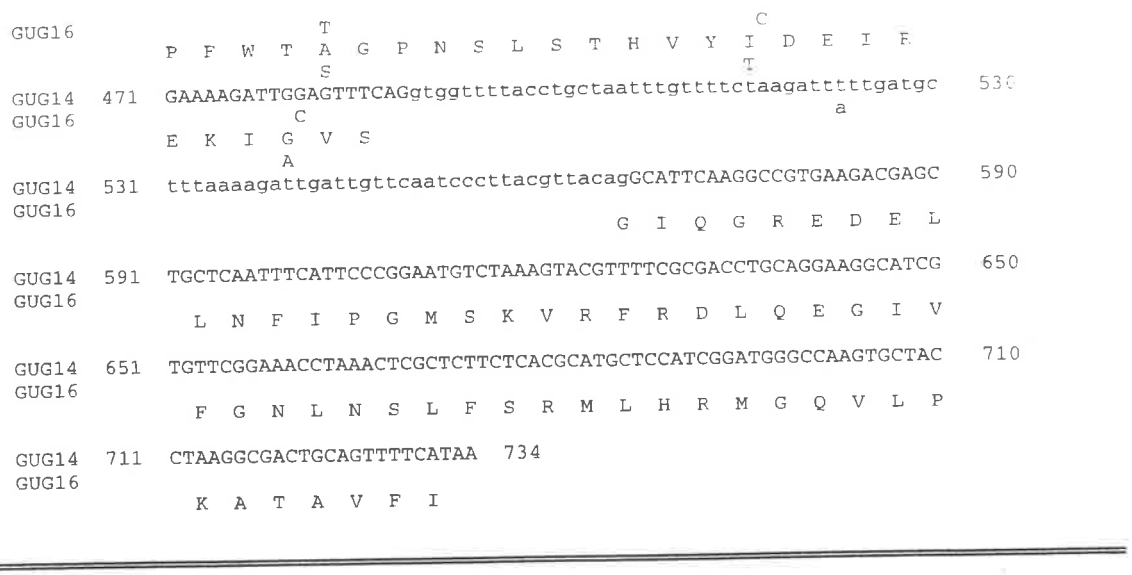


to the 5'-end probe in both positive plaques. These were cloned into pBluescript and sequence obtained for the 5'-end of the coding region and about 600 bp upstream of the ATG start (Fig. 7.2). Interestingly, the clones were slightly different in both the promoter and coding regions. GUG14 was 100% homologous to the cDNA clones and was thus used for the one-hybrid screening (Section 7.3.4). GUG16 was 99% homologous to GUG14 in the coding region and 97% homologous in the promoter region.

Certain sequences within the promoters show homology to motifs recognised by flavonoid regulatory genes. The *myc* recognition sites are characterised by the nucleotide sequence CANNTG which is common for basic/helix-loop-helix (bHLH) type transcription factors

Figure 7.2 Grape UFGT genomic sequences and putative regulatory motifs. The sequences of GUG14 and GUG16 are given from -609 as related to the expected translation start site as the transcription start is unknown. Dashes indicate 'gaps' in either sequence when they are aligned, and differences in the sequences are indicated in both the nucleotide and deduced amino acid sequences. A putative TATA-box and ATG start are represented in bold and an intron is denoted by lower case letters. Double underlining appears under proposed *myc*-binding sites, bold arrows indicated proposed *myb*-binding sites and the boxed regions contain a 19 bp repeat.

```
GUG14 -609  TCTTTAAAATATTTTTCAATTGTTTTTTGGTAACAAAATTCTATCTAATAACCAAAATAT   -550
GUG16

GUG14 -549  AAAAAATATTTTTTTTAGTTC....TCTTCATAAAAGTAATATATATCATGTGGAATACA   -490
GUG16              A             TCTT              C

GUG14 -489  AAAACTTTTAAAACATTCTCTACCTTTTCAATATTACT..AAAAAAAACAATTTTAAATT   -430
GUG16                                             AA

GUG14 -429  ATTCTTAA.AACAATGGGGTTTTAATCAAATTAATTTTGAAAACATTAATTTATTTCAAA   -370
GUG16              C      A

GUG14 -369  AAATTTATTGAATCATATTTTTCAAATTAGAAAAC.AATTTTATGTTGTTTAGAATAGAA   -310
GUG16                                          A       T   C       G

GUG14 -309  AATTATTTTTGAAGTAAAATTAACAAATATGCTCTTGCTACTACTCGCCAAGTATATCCC   -250
GUG16         A      T                C                    A

GUG14 -249  ACCAATGGCAAAGTAAAAGCTCACACAGAGCTTTCACTTGCCCCTGGTTTTGTT...TTT   -190
GUG16                                                              TTG

GUG14 -189  TTTTTCCCATTTTTTCTTTCTTTGGCCGCCATGCAGAATGGTGGTTTGGTTTTGGGTTGG   -130
GUG16                                              ...

GUG14 -129  TTTGTTAGGAGGGTGGGAATTGGGATGACAACCCCCATGCAGTTGCCACTCTCACAACCC   -70
GUG16

GUG14  -69  CCATGCAGTTGCTCTCATTTATAATCTTCAACAGCCAAAACCCAAATTGTAACATCCCCC   -10
GUG16

GUG14   -9  ATTCCAACCATGTCTCAAACCACCACCAACCCCCATGTGGCCGTCCTGGCCTTCCCCTTC    50
GUG16                                          A
                  M  S  Q  T  T  T  N  P  H  V  A  V  L  A  F  P  F
                                    K
GUG14   51  TCCACCCATGCAGCCCCCCTCCTTGCCGTCGTTCGCCGGCTTGCTGCCGCTGCCCCTCAT   110
GUG16
            S  T  H  A  A  P  L  L  A  V  V  R  R  L  A  A  A  A  P  H

GUG14  111  GCAGTCTTCTCCTTCTTCAGCACCAGCCAATCCAACGCCTCCATCTTCCACGACTCCATG   170
GUG16
            A  V  F  S  F  F  S  T  S  Q  S  N  A  S  I  F  H  D  S  M

GUG14  171  CATACCATGCAATGTAATATCAAGTCCTATGATATCTCCGACGGTGTGCCTGAGGGGTAT   230
GUG16                                          G G           G
            H  T  M  Q  C  N  I  K  S  Y  D  I  S  D  G  V  P  E  G  Y
                                             V              A
GUG14  231  GTGTTCGCCGGGCGGCCCCAGGAGGATATTGAGCTGTTCACGAGGGCTGCGCCGGAGAGC   290
GUG16                                                T
            V  F  A  G  R  P  Q  E  D  I  E  L  F  T  R  A  A  P  E  S
                                                   M
GUG14  291  TTTAGGCAGGGGATGGTAATGGCTGTGGCCGAGACAGGGCGGCCAGTGAGCTGCCTGGTG   350
GUG16
            F  R  Q  G  M  V  M  A  V  A  E  T  G  R  P  V  S  C  L  V

GUG14  351  GCTGACGCATTCATTTGGTTTGCCGCAGATATGGCAGCAGAGATGGGGGTGGCTTGGCTG   410
GUG16
            A  D  A  F  I  W  F  A  A  D  M  A  A  E  M  G  V  A  W  L

GUG14  411  CCGTTTTGGACTGCAGGGCCTAACTCACTCTCCACCCATGTTTACATTGATGAAATCAGA   470
```



(Roth *et al.* 1991; Tuerck and Fromm 1994; Littlewood and Evan 1995). Similar sequences are present in the grape UFGT promoter and are indicated by the double underlining in Figure 7.2. The motifs that plant *myb*-like proteins are able to bind are reported to differ considerably from mammalian *myb* proteins and also amongst themselves (Martin and Paz-Ares 1997). Nevertheless, the grape UFGT promoter has regions which show homology to *myb* binding sites. The same regions which show homology to the bHLH binding regions could also be *myb* binding regions following the consensus of [C/T]AAC[G/T]G which show the complementary sequence C[C/A]GTT[G/A] as they are located on the complementary DNA strand (Biedenkapp *et al.* 1988; Urao *et al.* 1993; Tuerck and Fromm 1994), and these are indicated by the bold arrow beneath the sequence. There is also a region homologous to the *P* protein recognition site [C/A]TCC[T/A]ACC (Grotewold *et* al. 1994) which again is on the complementary DNA strand (thus reads GGT[A/T]GGT[G/T] in Figure 7.2) and is indicated by the bold underlining arrow. Two copies of a tandem repeat of 19 bp are also present just upstream of the putative TATA box and these are boxed in Figure 7.2.

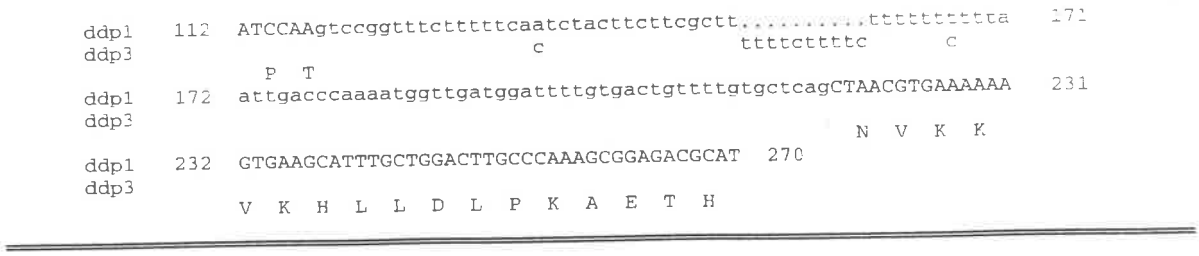
7.3.2 Promoter walking of grape DFR and LDOX

It was planned to use the UFGT promoter in gel retardation studies to compare the presence of interacting proteins in different tissues. The DFR and LDOX promoters were also isolated to act as controls, since they are expressed in pigmented and non-pigmented tissues (see Chapters 2, 3 and 4). These genes were also reported to be present in single copies in the grape genome (Sparvoli *et al.* 1994) and so it was thought that results from gel retardation studies would be easier to interpret if only one gene was involved. The promoter walking technique (Siebert *et al.* 1995) was used to clone the LDOX and DFR promoters. This technique is less labour intensive than genomic screening and overcomes the problems associated with the purification of phage DNA. Also, the subsequent identification and cloning of those regions of a genomic clone containing sequence immediately upstream of the coding region of the genes is not necessary.

Following the two rounds of PCR (see Section 7.2.3) discrete products were amplified from the *Dra* I and *Hpa* I libraries using the DFR primers and from the *Hpa* I library using the LDOX primers. These bands were excised from an agarose gel and cloned into pBluescript. Sequence from the coding regions of all three clones and the region immediately upstream of the translation start was obtained. Two different sized clones (ddp1 and ddp3) were obtained from the DFR primers and the *Dra* I library and upon sequencing were shown to be amplified from different grape DFR genes (Fig. 7.3). The clone obtained from the DFR primers and the *Hpa* I library (dhp1) was identical to ddp3, but contained less sequence upstream of the translation start.

Figure 7.3 Grape DFR genomic sequences and putative regulatory motifs. The sequences of ddp1 and ddp3 are given from -968 as related to the expected translation start site as the transcription start is unknown. Dashes indicate 'gaps' in either sequence when they are aligned, and differences in the sequences are indicated in the nucleotide sequences. A putative TATA-box and ATG start are represented in bold and an intron is denoted by lower case letters. Double underlining appears under proposed *myc*-binding sites and bold arrows indicated proposed *myb*-binding sites.

```
ddp1  -968  AAATAAAGGTTAAAATTTTCATCATAAAGCTTCGTCAAGAGCTTTGGGCATCCACTAAGG   -909
ddp3                       C

ddp1  -908  AGCAGAAAGCAGTGCCACGAAAAAATTGTGGAAAAAA....ATAG.TGTAGCTGTAGGTC   -849
ddp3                                             TACTG   C           C

ddp1  -848  GTGGGTTCAGTTGTCACTTTCTCACAACTAGTTTCAATCAGCCAACTGGGGACCATGAAT   -789
ddp3                                                 T     A

ddp1  -788  GGGGGAAAGCTTATTAGT..TTATTTCATCACTTATCATTTTTCCTTTATTTCTTCATGC   -729
ddp3                       C  CA   A A T G ......

ddp1  -728  CTTTTCCAGTAGCGTTCCTACGAAACATATTGGACTCCTGATCTTCTGATTGCGTAATAG   -669
ddp3                   C          . G                           G

ddp1  -668  GGAGGCCATCACGATCTTCCATATTCTGATGATTTTGCTTCGTTGCATAAATGGGAAAGA   -609
ddp3                                                    C

ddp1  -608  AGTATATCCCAAGTTGTCATTAGCGGATCAGATAAGAAATTAATCGTGTAAAGATTGTTC   -549
ddp3                               A

ddp1  -548  CTAATGCCATTCTTTTTTTGAACTCTTTTCGACAATTACACGAGTTCCC...........   -489
ddp3                           T                             ATCGCAAACGA

ddp1  -488  ...................ATTTGGTGGCTTGCTTCTTGCTATAGAAGATATTTCAAAGG   -429
ddp3        TATATATATTCATTGGAAG              A

ddp1  -428  GCTCACGCTGTGTTGAAGTGCATTTTCCCCCGAATCATGCGAGAATTTATTATGATGGTG   -369
ddp3

ddp1  -368  AATAAACTATTGGACCCCGAGAGCGATGGGCGCCGCCCGCATGACCAGCTAACCTTCTTC   -309
ddp3                            A

ddp1  -308  CTTCCTACAATTCGTGAAATTGGATTTCTAGAAGTTGTGACGCTCTTGGGAGACTTCTTA   -249
ddp3                 G

ddp1  -248  ACTTGCAAATTCGAAACTACAATCCTTAGAAAGAGTGAGCAGGTAGGAGCTACCATCCAC   -189
ddp3

ddp1  -188  GTGCTACAACCGTAGAGGCCCACACCACCAACCCGTCTAAACGAGGCCAAAACAGGGTGT   -129
ddp3                T        A

ddp1  -128  TAGATATGGGTTCATGAAGTCGAGTGCATATATAATCCTGGAATCGGCGAGAAGGCAACA   -69
ddp3                          A          TATAT                 G A

ddp1   -68  AAACAATTTGGGCCTTTCAATCTTTTCTTGTTGCTTTCTTTTTCTTCCTCGGATTATTTT   -9
ddp3                        C     .               A         C     C

ddp1    -8  TGAGAAGTATGGGTTCACAAAGTGAAACCGTGTGCGTCACCGGTGCCTCCGGTTTCATCG   51
ddp3
                    M  G  S  Q  S  E  T  V  C  V  T  G  A  S  G  F  I  G

ddp1    52  GTTCATGGCTGGTCATGAGGCTCCTGGAGCGCGGCTACACTGTTCGGGCCACCGTTCGCG   111
ddp3
              S  W  L  V  M  R  L  L  E  R  G  Y  T  V  R  A  T  V  R  D
```



The DFR genomic clones were significantly different in the promoter region and the intron, but they were identical in the coding region of the gene. The promoters have no features which stand out, except some regions of homology to *myc*-binding sites and one region with homology to a *myb*-binding site on the complementary DNA strand. The coding regions of the genomic clones are 97% homologous to the same region of the cDNA clone isolated by Sparvoli *et al.* (1994).

All the clones obtained from the band amplified from the *Hpa* I library using the LDOX primers were identical, and one, lhp5, was chosen for further sequencing. The sequence of lhp5 is presented in Figure 7.4.

The LDOX genomic clone described above and the LDOX cDNA isolated by Sparvoli *et al.* (1994) are identical over the region where they overlap. The promoter region of the lhp5 has many interesting features including a large palindrome, a microsatellite sequence just upstream of a putative TATA box and three copies of a 7 bp tandem repeat at approximately -450. There are also several regions which show homology to *myb*- and *myc*-binding sites as indicated in Figure 7.4.

Figure 7.4 Grape LDOX genomic sequence and putative regulatory motifs. The sequence of lhp5 is given from -679 as related to the expected translation start site as the transcription start is unknown. A putative TATA-box and ATG start are represented in bold. a tandem repeat is separated by vertical lines and a microsatellite is denoted by italics. Double underlining appears under proposed *myc*-binding sites. bold arrows indicated proposed *myb*-binding sites and a large palindrome is boxed.

```
-679  ACATAGAGAGAGGACATCAACCAATTTCAATGTTAGGGTTTATCAGAAATTGACATACAA   -620
-619  ATTAAAGTTTTTATGCATTAAAACTTCACCCAATTTAATTAAATATTAACATCCCCACAG   -560
-559  AATCATCTGCATTTGTCAGCTAGCTAAAGGTCAAAGTCAAAAGTCTGGGAAAGCTATCCT   -500
-499  GGGAGGAGGGGTAGCTGAAACGTGTATGGCTTAGA|TGGTAGA|TGGTAGA|TGGTAGA|TGGT   -440
-439  AAGGAATCTTTAGAGAAGTTTACGGTCATGAGGTGATTGGCCAAGGGCCAAGAGGTTCGT   -380
-379  GTTGCCCGTTGATTTCTCCGGTCTAAATCTCACAAGGGTTAGAAGGCCCCGTGCCTGAAA   -320
-319  CCTTCCAGTCAGCGCACCTTCCTTCTTGGTTCTCTTCGGCTGTTGAGTGTTGAATCTGGT   -260
-259  GCGGAGGTCATCACGTGAGTAACTGACACAATCTCTCTCTCTCTCTGATTTGGTGGGTTG   -200
-199  GTAGCTTGGTAGCGCTACCCCATTTCTCATGTTTCTAGAGTAACACAGTATCTTTAAGCA   -140
-139  TTTGGCTTAAGGTTTAGTTGAAGAGACGATCTCTATAAATTAAAGCTTCAAAAATGACCA   -80
 -79  ATGCAGCGTGCTGATCATTCAGACTGCAAGCAAGAGAGAAAGGGAAGGGAAAACAAGTAG   -20
 -19  ATCAGTGATATTTACTAGAATGGTGACTTCAGTGGCTCCTAGAGTTGAGAGCTTGTCCAG   40
                        M  V  T  S  V  A  P  R  V  E  S  L  S  S

  41  CAGTGGGATCCAGTCAATCCCCAAAGAGTACATCCGCCCCCAAGAAGAGCTCACCAGC   98
       S  G  I  Q  S  I  P  K  E  Y  I  R  P  Q  E  E  L  T  S
```

7.3.3 Nuclear protein isolation from grape berry skins

Having isolated the grape UFGT, DFR and LDOX promoters, the aim was then to identify specific regions of the UFGT promoter which regulate its expression after véraison. This required gel retardation studies to demonstrate interaction of a region of the promoter with nuclear proteins from postvéraison berry skins. Thus, it was necessary to have a reliable protocol for the extraction of useable amounts of nuclear proteins from grape berry skins. A method based on that of Sablowski *et al.* (1994) was followed and modified in an attempt to release native proteins from a crude grape skin nuclei preparation. Tobacco leaves were used as a control to check the success of the nuclei preparation method.

Analysis of the crude nuclei preparations under the microscope revealed that the major problem in gaining workable amounts of nuclear protein was in the initial homogenisation step. The number of nuclei obtained from grape skins was considerably lower than the number obtained from tobacco leaf (see Table 7.1). The inclusion of abrasives such as sand and detergents such as Triton X-100 had little affect on the recovery of nuclei from the grape skins. The Percoll gradient did not seem to be the cause of the low yield as extracts viewed by microscopy before the gradient step showed that the release of nuclei was much less in the grape skins than in the tobacco leaves. The amount of protein released per nuclei from the nuclei preparations was similar for grape skins and tobacco leaves (Table 7.1). Thus, it seems that the inability to obtain sufficient nuclear protein from grape berry skins was due to the difficulty associated with releasing nuclei from this tissue.

Table 7.1 *Typical results from nuclear protein extracts from tobacco leaf and grape berry skin.*

	Tobacco leaf	Grape berry skin
Grams fresh weight used	5	5
Approximate number of nuclei	2×10^8	3×10^6
µg protein	1636	11
µg protein/g fresh weight	327	2.3
µg protein/nucleus	2×10^{-6}	8×10^{-7}

7.3.4 One-hybrid screen using a portion of the grape UFGT promoter

The one-hybrid screening system allows the identification of cDNA clones whose protein products bind to a specific DNA sequence or promoter. The inability to prepare nuclear protein extracts from grape skins meant that the binding region for regulators of UFGT transcription could not be identified. Studies into the promoters of anthocyanin biosynthesis genes in both snapdragon (Almeida *et al.* 1989; Martin *et al.* 1991; Sablowski *et al.* 1994) and maize (Roth *et al.* 1991; Grotewold *et al.* 1994; Tuerck and Fromm 1994) have shown that controlling elements are usually present within a few hundred base pairs upstream of the transcription start site. Although the transcription start site for grape UFGT is not known, the presence of the tandem repeats containing *myc* recognition sequences just upstream of the putative TATA box (Fig. 7.2) suggested that this region may be a suitable target to use for the one-hybrid system. The region of the genomic clone GUG14 from -166 to -1 as numbered in Figure 7.2 was amplified by PCR and cloned upstream of the *HIS3* and *LacZ* genes in their respective one-hybrid reporter vectors. These vectors were then linearised, transformed into yeast and the desired transformants selected. Expression of both genes was at a low background level indicating that these vectors were suitable for screening, and so a dual yeast reporter strain containing both HIS and LacZ selection was prepared. A cDNA library made from mRNA isolated from grape berry skins harvested 11 weeks postflowering was constructed in the GAL4 activation domain fusion vector pGAD10. This stage of development of the berries coincides with the initial stage of anthocyanin accumulation in the berry skins.

Three large scale transformations of the dual yeast reporter strain with aliquots of the grape skin cDNA library were carried out. In each case the number of yeast transformants

obtained was approximately 5×10^5 colony forming units. The largest colonies were restreaked on selective media to eliminate 'escapes' from the first round of screening. Colonies which grew after the second round of screening were restreaked on selective media and assayed for β-galactosidase activity. This screen is intended to eliminate false positives as the expression of *LacZ* is less 'leaky' than the expression of the *HIS3* gene. The three rounds of screening identified nine yeast transformants for further study. Plasmids were isolated from the yeast cells and transformed into *E. coli*. The plasmids were purified from the bacteria and sequenced using a primer specific to the pGAD10 fusion vector. The inserts from three of the plasmids (UPBL4, UPBAT42 and UPBAT43) were cloned into pBluescript for sequencing. The sequences were compared to those on the various databases and putatively identified as indicated in Table 7.2.

Table 7.2 *Size and identity of cDNA clones isolated using the yeast one-hybrid screen for UFGT promoter binding proteins.*

Clone	Size (~bp)	Putative Identity	Closest Match (species & accession number)
UPBL1	300	26s rRNA	*Medicago sativa* Z11498
UPBL2	550	26s rRNA	*Nigella damascena* U52635
UPBL4	500	18s rRNA	*Scorodocarpus borneensis* G2138105
UPBL5	600	inorganic pyrophosphatase	*Arabidopsis thaliana* X57545
UPBL6	750	18s rRNA	*Daphniphyllum sp.* U42531
UPBS5	600	18s rRNA	*Ceanothus sanguineus* U42799
UPBS25	900	18s rRNA	*Casuarina equisetifolia* U42515
UPBAT42	850	18s rRNA	*Cercidiphyllum japonicum* CJD783
UPBAT43	400	collagen-like protein	*Herpes saimiri* G331028

7.4 Discussion

Two cDNA clones encoding grape UFGT were isolated from an aliquot of 400.000 plaques of the postvéraison library. These low numbers were not surprising as the detection of UFGT by northern analysis was difficult and UFGT had the lowest levels of expression of all the anthocyanin biosynthesis genes tested (Chapters 2, 3 and 4). Both clones were identical in the coding region, but Vv*ufgt*2 possessed 34 bp more in the 3'-untranslated region immediately prior to the poly-A tail, and the Vv*ufgt*3 clone possessed an intron. The identification of the additional sequence in Vv*ufgt*3 as an intron is supported by the presence of sequences homologous to intron splice sites and branch sites, the presence of the same sequence in the genomic clone GUG14 and the absence of the intron from the Vv*ufgt*2 cDNA clone . The reason for its presence is unknown. The simplest explanation is that the clone originates from the isolation of unprocessed nuclear RNA when the original cDNA library was made. The accumulation of unspliced message in plant and animal tissues has been observed in several systems. The *hsp82* mRNA of *Drosophila* and yeast actin mRNA have been shown to be unprocessed when cells from these species are exposed to heat shock (Yost and Lindquist 1988; 1991). The same phenomenon has not been observed in plants as petunia *hsp70* mRNA and soybean *Gshsp26-A* mRNA are still spliced correctly under heat shock conditions (Czarnecka *et al.* 1988; Winter *et al.* 1988). Nevertheless, the splicing of both of these transcripts was found to be disrupted following exposure to high levels of heavy metals (Czarnecka *et al.* 1988; Winter *et al.* 1988), and hypoxia has been shown to affect the splicing of *Adh1* RNA in maize plants (Ortiz and Strommer 1990). There are also reports of cDNAs with introns being isolated from plants that have not been exposed to stress (Paz-Ares *et al.* 1990; Arondel *et al.* 1991; Grotewold *et al.* 1991). The *Bronze2* gene in maize has been shown to produce unspliced transcripts in

response to cadmium stress (Marrs and Walbot 1997), but one line of maize accumulated more unspliced message than another line under both field and glasshouse conditions, suggesting a genetic factor could be involved as well (Nash and Walbot 1992). Splicing failure could be seen as a reduction of the efficiency of the splicing process or a regulated event. Both the Vv*ufgt*3 and Vv*myb*2 (Chapter 6) cDNAs isolated from the ripening berry library had introns, but a number of other cDNAs have been isolated from the library and none seem to contain introns (Dr. C. Davies, CSIRO, Adelaide, pers. comm.). Marrs and Walbot (1997) have observed that the lack of splicing of the *Bronze2* mRNA in maize under cadmium stress is not a general phenomenon as many other test genes were correctly spliced, suggesting that only a subset of genes are affected by non-splicing. If splicing is a regulatory process, then at which level does it work? It may play a role in posttranscriptional gene regulation. However, Nash and Walbot (1992) report that the spliced and unspliced *Bronze2* mRNAs have identical half-lives. Unspliced messages have also been shown to be exported into the cytoplasm and translated (Yost and Lindquist 1988; Frey *et al.* 1990). Thus, it seems that the unspliced messages are capable of being translated into alternative (and usually truncated) proteins when compared to the spliced messages. Whether this is simply a means of controlling the levels of the normally produced protein or a process to specifically produce the alterative protein is unknown.

The UFGT clones described in this chapter and the cDNA clone isolated by Sparvoli *et al.* (1994) show 97% similarity and the DFR clones isolated by promoter walking also show 97% identity in the coding region to the DFR cDNA clone isolated by Sparvoli *et al.* (1994). The differences could be due to genetic variation between the two varieties from which the clones were isolated. Thomas *et al.* (1993) have observed that there is much genetic variation amongst *V. vinifera* cultivars. Grapes are also highly polymorphic

(Thomas and Scott 1993) which may explain the differences between the two Shiraz UFGT genomic clones isolated (Fig. 7.2) and between the two Shiraz DFR clones (Fig. 7.3). Sparvoli *et al.* (1994) suggested that both UFGT and DFR were present as single gene copies in the grape genome, so perhaps the close similarity between the sequences suggests that the clones isolated are alleles. Nevertheless, we can not discern whether the two UFGT clones or the two DFR clones are alleles or different genes. It is interesting that both the UFGT cDNA clones isolated are homologous to only one (GUG14) of the two genomic clones isolated. There are slight differences in the promoter regions of both genes, but nothing to suggest that one should not be transcribed. It is possible that there are more controlling elements upstream or downstream of the region sequenced, or that a larger cDNA screening program may result in the isolation of cDNAs homologous to the GUG16 genomic clone.

The identification of regulatory motifs in promoters by sequence homology alone is fraught with danger. Martin and Paz-Ares (1997) state that the DNA-binding specificities of plant *myb*-like proteins differ considerably among themselves. It could be expected that as more *myb*-like proteins (and perhaps any class of transcription factor) have their binding specificities elucidated that the consensus motifs may become more degenerate. The *myc*-like protein consensus for example is very ambiguous, being CANNTG, and some of these sites are also recognised as being able to bind also G-box binding factors and *myb*-like proteins. There is little sequence similarity between anthocyanin gene promoters believed to be controlled by the same regulatory genes. Tuerck and Fromm (1994) report a loose consensus sequence found in maize anthocyanin promoters, but Martin *et al.* (1991) found that comparing the promoter sequences from the early and late pathway genes in snapdragon revealed little about the nature of their control. It is apparent that a promoter

must be dissected in detail (e.g. Roth *et al.* 1991) to determine the exact location of motifs bound by regulatory genes. However, there is then the complication that *in vivo* there may be multiple factors interacting in the regulation of these genes (Franken *et al.* 1994; Tuerck and Fromm 1994; Moyano *et al.* 1996). Therefore, it would be highly speculative to suggest that *myc*- and *myb*-like transcription factors play a role in regulating the DFR, LDOX and UFGT genes in grape berry skins by simply looking at promoter sequences. The lack of any consensus in the promoters sequenced raises the possibilities that either these genes are under the control of different regulators or maybe the same regulatory proteins have the ability to bind to many similar but not identical motifs. These questions await the isolation of grape anthocyanin regulatory genes and/or the development of gel retardation assays for use with grapevine tissues.

The inability to isolate nuclei proteins proved a hurdle in the functional dissection of the promoters. The problem did not seem to be related to the extraction of protein from the crude nuclei preparation, but rather, the low yield of nuclei from grape berry skins. This is probably due to the nature of the skin cells. The size of the cell walls of the cells of the exocarp or skin have been shown to be much thicker than those from the mesocarp and endocarp of grape berries (Hardie *et al.* 1996). The exocarp cells also remain much smaller throughout berry development than the mesocarp and endocarp cells (Hardie *et al.* 1996). Therefore, the inability to homogenise the grape berry skin tissue and then release the nuclei from these small, thick-walled cells may be the reason for the low nuclear protein yield from this tissue. Perhaps a combined enzymatic and mechanical maceration is required to release the nuclei from this tissue. Alternatively, it may be easier to use another grape tissue to investigate regulators of the anthocyanin genes. The anthocyanin biosynthesis pathway genes were originally cloned from grape seedlings which had been induced to accumulate

anthocyanins by exposing them to continuous light (Sparvoli *et al.* 1994). It would probably be easier to isolate nuclear proteins from grape seedlings and thus use this system to learn more about the control of the anthocyanin structural genes in grapes. However, the control of anthocyanin production in grape seedlings may be different from the control of anthocyanin production in the berry skins, as has been seen in different maize tissues (for review see Mol *et al.* 1996). It is also possible that the light induction of anthocyanin genes involves different promoter regions and transcription factors than does the induction caused by the onset of berry ripening.

The results obtained with the one-hybrid system were disappointing. The high incidence of false positives suggested that the conditions for the use of the system must be optimal to isolate clones that are actually interacting with the promoter. The one-hybrid system has been used successfully to clone DNA-binding proteins (e.g. Li and Herskowitz 1993; Wang and Reed 1993; Ulmasov *et al.* 1997) but in these cases tandem copies of a known DNA-binding motif were used in the target-reporter constructs. Kim *et al.* (1997) used an 82 bp fragment of the carrot *Dc3* gene promoter to clone a bZIP factor. They reported difficulties with the system which resulted in the isolation of many false positives, and were forced to modify the system by using a centromeric plasmid containing the *HIS3* reporter gene and an episomal plasmid containing the *LacZ* reporter gene (Kim *et al.* 1997). These modifications reduced the incidence of false positives. It seems clear that changes are required to enable the system to be used for the purpose of cloning grape DNA-binding factors.◆ It may be necessary to develop gel retardation assays to identify regulatory regions of the UFGT promoter. This should make the target-reporter gene constructs more specific to the regulatory protein of interest and reduce the amount of 'non-specific' promoter DNA available for other proteins to bind and perhaps induce reporter gene expression.

◆ see addendum

Alternatively, it may be possible to use the same vectors that Kim *et al.* (1997) used as they appeared to reduce the number of false positives escaping the screening process. The manufacturers promote the use of random primers to increase the chance of cloning the DNA-binding region of a protein if it is located at the 5'-end of an mRNA species. However, the predominance of ribosomal clones isolated using the one-hybrid system suggests that it is perhaps unwise to use random primers when constructing the fusion library.

The reasons behind the appearance of false positives during the one-hybrid screening are unknown, but there are several possibilities for their existence. It is possible that the activation domain fusion constructs can indeed code for a protein that has the ability to specifically or non-specifically bind to the promoter region inserted upstream of the reporter genes. This may still be the case even if the cDNA itself does not encode a transcription factor as the reading frame may by chance encode a DNA-binding protein. Nevertheless, the chance of this happening is extremely rare, and examination of the sequences of the false positives revealed that the proteins produced by the fusion constructs did not possess any known DNA-binding potential. In fact, all of them had in-frame stop codons very close to the start of the cDNA insert. The size of the promoter fragment used in the reporter vectors may allow transcription factors from the yeast itself to bind and activate the reporter genes. However, initial studies on the background expression of these genes indicated that the induction of these genes by endogenous yeast proteins was low. Perhaps the proteins formed by the fusion construct in the transformants in which we saw background expression had induced the yeast to produce factors which could bind and activate the reporter genes. However, when the activation domain-cDNA fusion clones were retransformed into yeast they were no longer able to induce expression of the reporter gene. This suggests that the

background was due to the original transformed yeast and not the plasmid harbouring the activation domain-cDNA fusion. It was also possible that some of the activation domain fusion library plasmids had been nicked during the purification process, and thus could integrate into the yeast genome. Thus, the induction of the reporter genes may be due to the presence of a cDNA integrated into the yeast genome with DNA-binding potential, and not due to another cDNA in an episomal plasmid. Nevertheless, PCR amplification using primers specific to the pGAD10 vector did not reveal the presence of activation domain-cDNA fusion clones integrated into the yeast genome.

As I have already stated, it seems that the conditions for the yeast one-hybrid system must be optimal in order for desired results to be achieved. Discussions with visiting scientists who have used the two-hybrid system indicate that this similar system also has problems with background which is often related to the reporter vectors used (Dr. S. de Vries, Wageningen Agricultural University, pers. comm.; Dr. M. Vivier, University of Stellenbosch pers. comm.) and a similar problem was reported by Kim *et al.* (1997) with the one-hybrid system. Unfortunately, time did not permit any attempts to optimise the one-hybrid system for use in isolating grape transcription factors. However, the level of background was relatively low, and the basic protocols for one-hybrid screening are now in place, so it should be possible to fine tune the system so that it can be used to isolate the cDNA clones of interest.

Chapter 8

Concluding discussion

The ripening process in grape berries involves a series of subprograms which are coordinated to convert the hard, green, inedible berries into soft, sugar-filled, flavoursome fruit. In black and red grapes the accumulation of anthocyanins in the skins of the grape berries is a major part of the ripening process. The composition and quantity of these anthocyanin compounds are determinants of the quality of the final grape product, be it wine, table fruit, dried fruit, jelly or juice. In order to manipulate the anthocyanin content of the grape berry, it is important to understand the control of their accumulation in the fruit. Studies into anthocyanin accumulation in other plant species have revealed that the anthocyanin biosynthesis pathway is controlled at the transcriptional level by similar regulatory factors but at different stages of the pathway (for review see Martin and Gerats 1993a). In this thesis the molecular approach to studying the expression of anthocyanin pathway genes and their control has been extended to grapevines and in particular grape berry skins.

8.1 Anthocyanin accumulation and pathway control in grape berries

8.1.1 Total anthocyanins

Anthocyanin accumulation in Shiraz berry skins begins at véraison and continues throughout berry ripening (Fig. 2.2E). The anthocyanin pathway appears to be controlled in grapevines in a manner different from that seen for maize, petunia and snapdragon, the species most studied thus far. In grape berry skins during development there were two

phases of anthocyanin pathway gene expression (Fig. 2.7). Early in development all the genes except UFGT were expressed followed by a reduction in the expression of all the genes during the lag phase of grape berry development. Then, after véraison, there was a co-ordinate induction of all the genes from the pathway including UFGT and this coincided with the accumulation of anthocyanins in the skin. These results suggest that UFGT is under a different regulatory regime than the genes from the rest of the anthocyanin pathway. The correlation between anthocyanin synthesis and UFGT gene expression was also seen when flavonoid pathway gene expression was studied in various grapevine tissues (Chapter 3) and the skins of white and coloured berries (Chapters 3 and 4). In most of the unpigmented tissues mRNAs homologous to all the genes from the flavonoid pathway except UFGT were detected. It was also demonstrated that when ripening is delayed by the application of a synthetic auxin (BTOA), anthocyanin accumulation occurs later in development and the induction of UFGT gene expression is delayed (Chapter 5). Thus, it appears that UFGT plays an important role in the accumulation of anthocyanins in grapevines. However, the gene encoding the putative dehydratase which is thought to catalyse the reaction between LDOX and UFGT (Heller and Forkmann 1988) has not been cloned and could be controlled in a manner similar to UFGT. Genes involved in transporting the anthocyanins into the vacuole, whose functions are required after UFGT in the anthocyanin pathway, may also be controlled in the same manner as UFGT. Therefore, it is perhaps more correct to state that the control of anthocyanin biosynthesis occurs beyond the LDOX step of the anthocyanin biosynthesis pathway.

Total colour in the berries is a measure of quality, so any means of increasing the amount of anthocyanins accumulating in the berries would be an advantage to both the grape grower and the winemaker. Viticultural practices have been developed to try and increase the

amount of colour in berries and these are reviewed in Section 1.5.2. Nevertheless, it may be possible to genetically manipulate the grape berry to enable it to accumulate more anthocyanins. As UFGT has been shown to be important in the accumulation of anthocyanins in the berry skins, upregulating its expression may result in the accumulation of more anthocyanins. There are several strategies that could be employed to try and increase UFGT expression. Either the grape UFGT gene itself or grape regulatory factors that induce UFGT gene expression could be over-expressed in the berry skins. Unfortunately, many attempts to increase the expression of an endogenous gene in plants have encountered the phenomenon of co-suppression, where a transgene in the sense orientation can inactivate the expression of the endogenous homologue (for review see Jorgensen 1995) thus having the opposite effect from that desired. Furthermore, if high UFGT expressing transgenic plants are obtained *in vitro* they may be subsequently co-suppressed on transfer to the field as has been reported (Palauqui *et al.* 1996). Another option is to introduce genes into grapevine that encode UFGT or anthocyanin regulatory genes from other plants. Schwinn *et al.* (1997) transformed lisianthus with the UFGT gene from snapdragon and found that new anthocyanin structures were produced (Markham 1996). Nevertheless, the total amount of anthocyanins produced was not altered (Schwinn *et al.* 1997). The use of regulatory genes from one plant species to increase anthocyanin accumulation in another has been reported (Lloyd *et al.* 1992; Mooney *et al.* 1995) and perhaps would be the most promising approach. The effect on anthocyanin synthesis depends on both the source of the regulatory genes and the target species. For example, the *Delila* gene had no effect on *Arabidopsis* pigmentation (Mooney *et al.* 1995) whereas the *R* (*Lc*) gene induced the production of more anthocyanins in normally pigmented *Arabidopsis* tissues (Lloyd *et al.* 1992). However, the *Delila* gene increased anthocyanin synthesis in tomato flowers and vegetative tissues when introduced into this species, but in tobacco only

increased anthocyanin accumulation in the flowers (Mooney *et al.* 1995). The *myb*-like *C1* gene alone had no effect on anthocyanin accumulation in tobacco or *Arabidopsis*, but when *C1* and *R* expressing *Arabidopsis* were crossed, tissues that were not normally pigmented accumulated anthocyanins (Lloyd *et al.* 1992). It appears that the *C1* gene (*myb*-like) plays a role in the tissue specificity of anthocyanin synthesis whereas the *R* gene (*myc*-like) influences the amount of pigmentation. There are three possible strategies for the use of regulatory genes to increase anthocyanins in grape berries. One is to express a *myc*-like regulatory gene (e.g. *Lc* or *Delila*) in the berry skin after véraison, as these genes seem to increase anthocyanin synthesis in normally pigmented tissues. Another approach would be to express both *myc*- and *myb*-like genes in the berry skins before véraison in an attempt to induce anthocyanin synthesis earlier than usual and increase the total amount of anthocyanins accumulated before harvest. The berry flesh may also be a target for these genes, thus increasing overall anthocyanin levels by inducing the whole of the grape berry to produce anthocyanins. Grape berry flesh appears to have the capacity to produce anthocyanins as several red fleshed varieties are known (e.g. Alicante Bouschet, Rubired) suggesting that this approach is feasible.

The branching nature of the anthocyanin/flavonoid biosynthesis pathway means that there is competition between enzymes for the same substrate (Holton and Cornish 1995). For example, DFR, F3'H, F3'5'H and flavonol synthase (FLS) can all use dihydroflavonols as substrates. It may be possible to increase the flux down the anthocyanin pathway by suppressing genes involved in the formation of other flavonoid compounds produced from anthocyanin precursors. Grape berry skins are known to produce flavonols (Cheynier and Rigaud 1986; Price *et al.* 1995) and proanthocyanidins (Escribano-Bailón *et al.* 1990; Chapter 2) both of which are generated from intermediates of anthocyanin biosynthesis.

Flavonols are produced from dihydroflavonols by FLS and proanthocyanidins are made from leucoanthocyanidins by the activity of leucoanthocyanidin reductase (LAR). Holton *et al.* (1993b) have shown that anthocyanin accumulation can be increased in tobacco flowers by co-suppressing the FLS gene. Therefore, reducing FLS and/or LAR expression in grape berry skins may increase anthocyanin accumulation by increasing the flux down the anthocyanin pathway. However, the importance of tannins and other phenolics to the colour of red wine (Section 1.5.4) may mean that increasing anthocyanins at the expense of these compounds will not enhance wine colour. Perhaps strategies to improve colour should aim to increase the activity of all the enzymes of the pathway or the flux down the pathway by increasing the concentration of early intermediates to keep a balance between anthocyanins and other flavonoids, both of which are reported to contribute to wine colour.

The transport of anthocyanins into the vacuole will also influence the amount of anthocyanins that accumulate in the berry skins. Anthocyanins are secondary metabolites that can be toxic to the plant cells that produce them and thus are recognised and transported to the vacuole in a similar way to herbicides and other xenobiotics (Marrs 1996). This process is believed to involve the 'tagging' of the anthocyanin with the tripeptide glutathione by glutathione S-transferase (GST) and the recognition and entry of these conjugates into the vacuole by way of a glutathione-conjugate pump (Martinoia *et al.* 1993; Marrs *et al.* 1995). Marrs *et al.* (1995) suggest that in maize the anthocyanin-glutathione conjugate may then be metabolised and acylated in the vacuole in a similar fashion to herbicide-glutathione conjugates. The sequestering of anthocyanins in the vacuole is an important process for the survival of those cells producing anthocyanins. It could be speculated that the efficiency of the conjugation and conjugate pump will be important in determining the amount of anthocyanins that accumulate in these cells, as the

toxicity of these compounds would suggest that a feedback inhibition would protect these cells if their levels rise in the cytoplasm.

The *Bronze-2* gene in maize is the last genetically defined step in anthocyanin synthesis and has been shown to encode a GST that catalyses the formation of anthocyanin-glutathione conjugates (Marrs *et al.* 1995). The cloning of a GST from petunia which maps to the anthocyanin biosynthesis gene locus *An13* has also been reported (E. Souer and R. Koes pers. comm. cited in Marrs 1996), and the clone is able to complement both *an13* petunia mutants and *bz-2* maize mutants (MR Alfenito pers. comm. cited in Marrs 1996). Some mutant *bz-2* maize ears have bronze coloured kernels as anthocyanins accumulate in the cytoplasm, whereas others have pale pink kernels due to the ability of another GST to use cyanidin-3-glucoside as a substrate, although only poorly (Walbot *et al.* 1994). Thus, it appears that the amount of anthocyanin accumulated in the vacuole is influenced by GSTs. It follows that the efficiency of the glutathione-conjugate pump may also influence the rate and perhaps the amount of anthocyanins that accumulate in the vacuole. A glutathione S-conjugate pump has been isolated from *Arabidopsis* and is a member of the ABC transporter family (Lu *et al.* 1997). The activity of the transporter was demonstrated in isolated membranes from yeast cells expressing the clone and was shown to be competent in transporting glutathione S-conjugates of xenobiotics and endogenous substrates including cyanidin 3-glucoside (Lu *et al.* 1997). An ABC-like transporter that shows a high degree of homology to the *Arabidopsis* clone has been cloned from grape berries but awaits further characterisation (Dr. C. Davies, CSIRO, Adelaide, pers. comm.). The transport of anthocyanins into the vacuoles of grape skin cells is an aspect of anthocyanin accumulation that will need to be investigated if anthocyanin levels in berries are to be increased.

8.1.2 Specific anthocyanins

The type of anthocyanins produced by a grape variety will also influence the colour of the grape product. Grape berry skins produce at least 16 different species of anthocyanins. all of which derive from either the dihydroxylated anthocyanidin cyanidin or the trihydroxylated anthocyanidin delphinidin. The various anthocyanin species have different colour spectra and so the relative amounts of each will contribute to the final colour of the fruit. Furthermore, in the case of wine production, the anthocyanin species have different properties with regard to extractability, stability and the reactions they undergo during winemaking and the subsequent storage of the wine (Section 1.5.4). Also, if the colour of grape berries can be altered this may provide products that can be marketed as being novel, especially in the table grape industry.

The accumulation of the various anthocyanin species during the ripening of Shiraz grape berries is reported in Chapter 2. All the anthocyanins increased in concentration during development and the major anthocyanins were malvidin 3-monoglucoside, malvidin 3-acetylglucoside and malvidin 3-*p*-coumaroylglucoside (Fig. 2.4). There was a general decrease in the percentage of total anthocyanin 3-monoglucosides during development whereas the levels of anthocyanin 3-acetylglucosides and 3-*p*-coumaroylglucosides tended to increase during this time (Fig. 2.6A). This was due to a greater accumulation of malvidin based acylated anthocyanins than any of the monoglucosides (Fig. 2.5). The malvidin derivatives increased as a percentage of total anthocyanins during development as the percentage of the other species decreased slightly (Fig. 2.6B). Nevertheless, the flux down the trihydroxylated and dihydroxylated branches of the pathway remained fairly constant,

although the percentage of trihydroxylated anthocyanins was almost five times more than the percentage of dihydroxylated anthocyanins (Fig. 2.6C).

Malvidin-based anthocyanins are the major anthocyanins that accumulate in the wine grapes Shiraz, Cabernet Sauvignon and Pinot Noir (Fig. 4.1). This suggests that the activity of F3'5'H is important to the colour development in these grape berry skins. A F3'5'H gene was first cloned from petunia by Holton *et al.* (1993a), but has since been cloned from other plant species (e.g. Toguri *et al.* 1993; Tanaka *et al.* 1996; Nielsen and Podivinsky 1997). The F3'5'H gene is involved in producing anthocyanin structures that provide pigment at the blue end of the spectrum which is lacking in important ornamentals such as carnations, chrysanthemums and roses (Holton and Cornish 1995). Given that there are several F3'5'H sequences now published it should be possible to design oligonucleotide primers for the PCR amplification of grape F3'5'H cDNA. F3'5'H is a member of the cytochrome P-450 family of genes so it would be important to ensure that the primers will not simply amplify P-450 sequences in general as they do show similarity in a number of small regions. One would expect that the F3'5'H gene would be expressed in grape berry skins in a similar pattern to those genes early in the anthocyanin biosynthesis pathway (see Fig. 2.7). The presence of prodelphinidins in the young berry skin samples was observed when extracts were hydrolysed with concentrated acid and analysed by HPLC (data not shown). Thus, there is evidence of F3'5'H activity early in berry development. Similarly, cyanidin based proanthocyanidins also accumulate early in berry development (data not shown) and so it would be expected that F3'H gene expression could be detected at this stage of development as well as after véraison. F3'H may be useful to clone if the type of anthocyanins that accumulate in the berry skins is going to be altered. It has been reported that a cDNA clone with F3'H activity has been isolated from petunia (Holton and Cornish

1995). Antisense or sense co-suppression could be used to alter the activity of these enzymes in the grape berry skins and thus force the anthocyanin pathway down one or other of the branches of the pathway, thus altering the anthocyanin species produced.

Methyltransferase activity also appears to be important in determining the types of anthocyanins that accumulate in the skins of wine grapes, as the major species found were malvidin-based, and to a lesser extent peonidin-based, both of which are methylated in the B-ring of the molecule. There is only one report of an anthocyanin methyltransferase being cloned and that was by Quattrocchio *et al.* (1993) from petunia. However, the sequence has not been released on any of the gene databases. It may be possible to use other sequences from non-anthocyanin methyltransferases to develop a strategy for the cloning of grape anthocyanin methyltransferases, but the lack of knowledge about the enzyme and the location of catalytic and substrate binding domains may limit this approach.

The modification of anthocyanins by acyltransferases is not well studied enzymatically and no anthocyanin acyltransferase genes have been cloned. It was thought that the acylation of anthocyanins was important for the transport of anthocyanins into the vacuole and for methylation (see Heller and Forkmann 1988), although this may not be true for all plant species. In most grapes that are known to produce wine with stable colour there are significant amounts of acylated anthocyanins (e.g. Shiraz and Cabernet Sauvignon in Fig. 4.1). However, Pinot Noir does not produce any acylated anthocyanins, but still accumulates high levels of anthocyanins in the vacuole (Merlin *et al.* 1985). The fact that malvidin 3-monoglucosides accumulate in the vacuoles of Pinot Noir (Merlin *et al.* 1985) suggests that acylation is not required for vacuolar transport or methylation. The cloning of acyltransferases using mutants may prove difficult. It is possible that the acyltransferases

may act on many substrates as part of the xenobiotic detoxification pathway (Marrs 1996) which also involves GST and the glutathione pump (see above). Therefore these genes may be regulated independently of the anthocyanin biosynthesis pathway. Perhaps the best approach to isolate acyltransferase genes would be to begin with the purification of the protein, as methods for assaying acyltransferase activity have been developed (e.g. Teusch and Forkmann 1987; Callebaut *et al.* 1996) However, it may be best to use a species other than grape as the high phenolic content of grape tissue makes protein studies difficult. Nevertheless, Pinot Noir could be used as a negative control throughout these studies because of its inability to produce acylated anthocyanins.

Grapes do not produce pelargonidin-based anthocyanins. This phenomenon is also seen in petunia where cyanidin- and delphinidin-derivatives accumulate, but no pelargonidin-derivatives are produced because of the specificity of the DFR enzyme (Forkmann and Ruhnau 1987). Meyer *et al.* (1987) were able to create a transgenic petunia that produced pelargonidin-derivatives by introducing the maize *A1* gene, which codes for DFR, into a petunia mutant which usually accumulates dihydrokaempferol. Thus, a petunia with novel flower colour was produced. It may be possible to engineer grapes that produce pelargonidin-based anthocyanins and therefore with a novel berry colour. The complete lack of any pelargonidin-based anthocyanins in grapes suggests that one of the structural genes is unable to utilise a substrate with only one hydroxyl group in the B-ring. The enzyme with this substrate specificity will need to be identified, perhaps through feeding experiments. Grapevines will then need to be transformed with a gene from another species that codes for the enzyme that blocks pelargonidin production in grapes. However, in order to get significant production of pelargonidin-derivatives, the endogenous pathway will probably need to be altered as well. This would involve the down-regulation of F3'H and

F3'5'H to inhibit the hydroxylation of the B-ring of the anthocyanin and thus block the production of the substrate for the endogenous enzyme. A grape berry with a unique colour resulting from pelargonidin accumulation may be useful for the table grape industry as the product would be marketable as being novel. Also, a rich colour, but with a lighter hue than normally seen in grapes may be more desirable for markets in Asia, which is a major export destination of Australian table grapes.

8.2 Anthocyanin biosynthesis regulatory genes

The coordinate induction of all the anthocyanin biosynthesis genes at véraison in Shiraz grape berry skins during development (Fig. 2.7) suggests that these structural genes are under the control of regulatory genes and that anthocyanin accumulation is controlled at the level of transcription. As outlined in Section 1.7.3, regulatory genes control the expression of anthocyanin biosynthesis genes in other plant species and, although they encode similar *myc*- and *myb*-like transcription factors, these regulatory genes control the pathway at different points in different species. The induction of all the anthocyanin biosynthesis genes in ripening berry skin (Fig. 2.7) and in dark-grown grape seedlings subsequently exposed to light (Sparvoli *et al.* 1994) appears to mirror the control seen in pigmented maize tissues. However, mRNAs homologous to all the genes of the anthocyanin pathway except UFGT were detected early in berry development (Fig. 2.7) and in almost every unpigmented grape tissue tested (Fig. 3.1). Thus, there seemed to be three levels of control of the anthocyanin pathway. One where there is little expression of any of the genes of the pathway, another where all the genes are expressed and anthocyanins accumulate and another where all the genes except UFGT are expressed. There are a multitude of possible scenarios involving regulatory genes to explain the expression patterns seen and these are extensively discussed

at the end of the relevant chapters of this thesis. In summary, the regulation may involve one subset of regulatory genes for the expression of the early genes of the pathway and another subset for regulation of the expression of all the genes of the pathway including UFGT. Alternatively, UFGT expression may require both a regulatory gene which also controls expression of the early genes of the pathway and another transcription factor that is specific for UFGT and only produced when anthocyanins accumulate. The comparison of the patterns of expression seen for the anthocyanin biosynthesis genes in the skins of black and white grape cultivars (Fig. 3.2) suggests there are two levels of control after véraison as the expression of anthocyanin genes in Sultana and Muscat Gordo was very different to the other white cultivars. However, this may reflect the nature of the mutation that has led to loss of anthocyanin accumulation rather than the nature of the control of the pathway. For example, Semillon, Chardonnay and Riesling may have mutations in the regulatory gene that specifically controls UFGT or in regions of the UFGT gene itself which concerns the transcription of this gene. The rest of the pathway is also down-regulated and this may be because either the regulatory gene controlling UFGT expression has a direct qualitative effect on the expression of these early genes, or perhaps there is a feedback inhibition of the UFGT substrate on the early pathway genes. The mutations in Sultana and Muscat Gordo appear to result in a complete down regulation of the pathway, both early genes and UFGT. This implies that the mutation in these varieties involves a regulatory gene that regulates the expression of all the anthocyanin pathway genes, either directly through interaction with the promoters, or indirectly through the induction of the anthocyanin regulatory genes themselves.

The control of the anthocyanin pathway in grapevines will not be fully understood until the genes regulating the expression of the structural genes are cloned and characterised.

Chapters 6 and 7 describe attempts to clone grape homologues to the anthocyanin regulatory genes isolated from other species. *Myb*-like genes were isolated from a post-véraison grape berry library, but the role these transcription factors play in gene regulation awaits further study. Different *myb*-like genes have been shown to have diverse roles in plant development and physiological functions (Paz-Ares *et al.* 1986; Oppenheimer *et al.* 1991; Grotewold *et al.* 1994; Noda *et al* 1994), but unfortunately, the various *myb*-like genes do not fall into groups based on sequence homology and function. The characterisation of *myb*-like genes is difficult due to their presence as large gene families in plant genomes (Lin *et al.* 1996; Martin and Paz-Ares 1997), post-transcriptional control of their activity (Moyano *et al.* 1996), multiple transcript sizes (Avila *et al.* 1993; Lin *et al.* 1996) and the ability of multiple *myb*-like proteins to interact with the same promoter (Franken *et al.* 1994; Moyano *et al.* 1996). Gel retardation studies and the use of transient expression systems would confirm the ability of the *myb*-like gene products to bind promoters and induce expression of specific genes, but will not confirm the role of the transcription factors *in planta*. The elucidation of the functions of the grape *myb*-like homologues will require transgenic studies. Transformation of grape has been achieved in the CSIRO laboratory in Adelaide for the cultivar Sultana and this is being extended to other grape cultivars (Dr. T. Franks and Dr. M. Thomas pers. comm.). So there exist the tools to introduce sense and antisense constructs of the *myb*-like grape genes Vv*myb*1 and Vv*myb*2 into grapevine and observe the effects that over expression and suppression of these genes have on the development of the plants. It may also be possible to use other plant species more amenable to transgenesis for functional studies. The role of *myc*-like and *myb*-like genes in the regulation of anthocyanin biosynthesis has been demonstrated by introducing maize or snapdragon genes into other species (Lloyd *et al.* 1992; Mooney *et al.* 1995), although the phenotype of the transgenic plants depended on the target species as

well as the genes introduced. A *myb*-like gene from snapdragon has also been shown to be involved in phenylpropanoid and lignin production by transgenic studies in tobacco (Dr. C. Martin, John Innes Institute, Norwich, UK, pers. comm.). Therefore, it is possible to use other species to demonstrate *myb*-like gene function, although obviously any conclusions will have to account for the effect the target species has in these studies.

The cloning of more *myb*-like genes from grapes could be achieved using PCR technologies due to the high degree of homology shown between these sequences in the DNA-binding region of the protein. The cloning of *myc*-like genes appears to be more difficult, although the recent reports of *myc* homologues being isolated from *Arabidopsis* (Urao *et al.* 1996; de Pater *et al.* 1997), bean (Kawagoe and Murai 1996) and rice (Hu *et al.* 1996) has expanded the number of sequences that can be used to design oligonucleotides primers for PCR amplification of *myc*-like genes. It may be possible to isolate *myc*-like genes from a genomic library as the problems associated with the abundance of the corresponding mRNA in the sample used for cDNA library making would not be encountered. However, the function of any *myb* or *myc* homologue isolated will then have to be elucidated and this is a difficult process. The one-hybrid system is potentially a useful tool for the isolation of regulatory genes as the clones selected need to interact with a promoter sequence during screening. The isolation of promoters from grape DFR, LDOX and UFGT genes will enable the identification of regulatory genes that are able to interact with most of the genes from the anthocyanin pathway. However, the one-hybrid system will require fine-tuning before it can be applied to this grape system and this will probably require the adaptation of methods for nuclear protein extraction and gel retardation studies so they can be applied to grapes. Alternatively, some of the grape berry colour mutants described in Chapter 4 may be used for the isolation of regulatory genes. The Cabernet Sauvignon and White Cabernet

grapes would be ideal sources for the isolation of differentially expressed genes using either the differential screen or differential display method. The White Cabernet mutant had reduced expression of all of the anthocyanin pathway genes, but only UFGT expression could not be detected. Thus, it is possible that these two varieties will only differ in the expression of UFGT, genes that regulate the expression of UFGT and genes beyond UFGT in the pathway. However, the regulatory genes may be expressed at too low a level to be detected by differential screening, and so it may be necessary to carry out cold-plaque screening (Hodge *et al.* 1992) to increase the probability of isolating genes specifically expressed in Cabernet Sauvignon berries.

There are many possible methods to use for the isolation of regulatory genes of the anthocyanin biosynthesis pathway from grapevine. The path of choice will be determined by the ability to apply techniques to grape berries which are a difficult tissue with which to work. Nevertheless, the isolation and characterisation of grape anthocyanin regulatory genes will advance the knowledge of the control of this pathway in grapes and allow the development of strategies for the genetic manipulation of anthocyanin biosynthesis in grapes.

8.3 Grape berry ripening

The application of an auxin-like compound (BTOA) to grape berries before véraison resulted in a delay in the onset of ripening of the treated fruit. The delay was observed in a number of physiological factors (Figs. 5.2, 5.3 and 5.4), and was similar to previous observations (Weaver 1962; Hale 1968; Hale *et al.* 1970; Coombe and Hale 1973). An increase in ABA concentration, usually associated with ripening in grape berries, was

delayed by the BTOA treatment (Fig. 5.6). It was also shown that the auxin treatment affected the expression of several genes in a positive or negative manner (Figs. 5.3, 5.4 and 5.5).

A common theme to many of the studies involving the use of plant hormones to alter ripening in grape berries is that the berries appear to require 'competence' to respond to the hormone. That is, the effect of the treatment often depends on the time of application (e.g. Hale *et al.* 1970; Coombe and Hale 1973; Hale and Coombe 1974). This suggests that grape berry ripening is controlled by more than one factor and the whole process can not be advanced by the addition of excessive amounts of one of these factors as is the case for climacteric fruit where ethylene is used to induce ripening. Research has shown that the exogenous application of a number of plant growth regulators can affect grape ripening, including; ethylene (Hale *et al.* 1970; Coombe and Hale 1973), ABA (Hale and Coombe 1974; Kataoka *et al.* 1982); auxin (Weaver 1962; Hale 1968; Hale *et al.* 1970; Coombe and Hale 1973; Hale and Coombe 1974; Davies *et al.* 1997) and brassinosteroids (Rujuan *et al.* 1994). Although, the control of ripening in grape berries appears to be complex, it may be possible using molecular probes to investigate the role each growth regulator has on the specific subprograms that are occurring in the ripening berries.

Since the BTOA-treatment experiment was completed, genes involved in grape cell wall metabolism (K. Nunan, University of Adelaide) and a number of genes that are ripening-induced have been isolated (Dr. C. Davies, CSIRO, Adelaide). Some of these clones may be used as probes, along with the anthocyanin cDNA clones, to link the hormone treatments to specific physiological processes that occur during grape berry ripening. The tools required for physiological and molecular studies on the role of plant growth regulators in

grape berry ripening have now been developed and can be used to extend the research on grape berry ripening beyond the effects of BTOA.

8.4 Conclusion

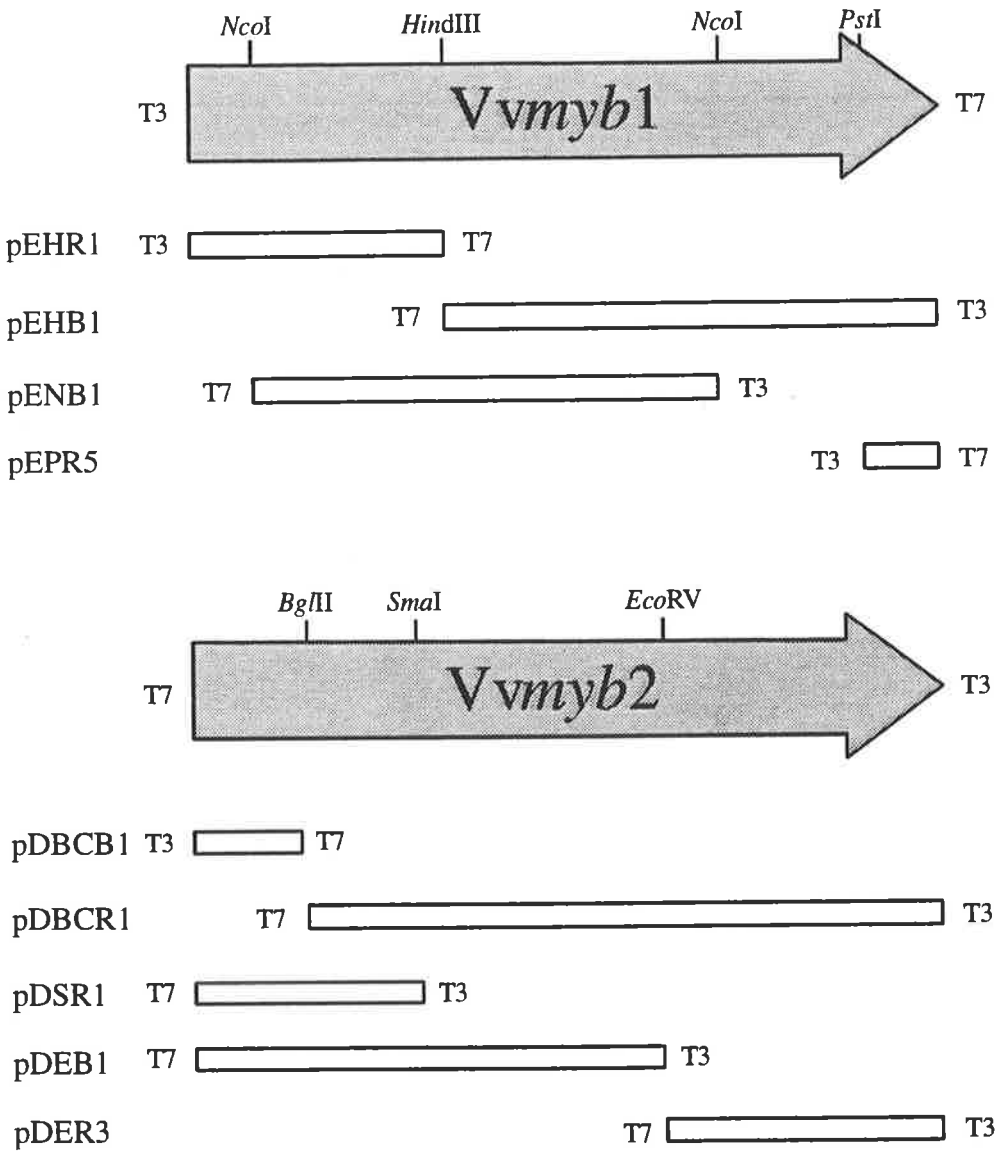
Expression of all of the anthocyanin pathway genes except UFGT was detected in most unpigmented tissues, and UFGT was the only gene that showed an absolute differential expression pattern between white and coloured grape berry skins. These results suggest that UFGT is under a different regulatory regime compared to the other anthocyanin pathway genes in grapevine, and that anthocyanin synthesis is controlled at a later stage than seen in species previously studied. Expression of all of the anthocyanin genes except UFGT early in berry development and in most of the unpigmented tissues tested correlates with the production of condensed tannins in the young berries and may also be responsible for the synthesis of a number of other phenolic compounds. Treatments that delay grape ripening and thus anthocyanin biosynthesis alter the expression of UFGT, but also influence the pattern of CHS gene expression.

White grapes lacked UFGT expression, but also showed decreased expression of other anthocyanin pathway genes suggesting alteration of regulatory genes has occurred. Attempts were made to isolate genes that encode *myb*- and *myc*-like transcription factors, which have been shown to regulate structural genes from the anthocyanin pathway in other plant species. Two *myb* homologues were isolated, but the expression pattern of these genes does not support a role for them in the control of UFGT gene expression and thus anthocyanin accumulation. Further development of techniques, initiated during this study, for the isolation and characterisation of transcription factors will be required to advance our

understanding of the regulation of anthocyanin biosynthesis in grapes. The opportunity also exists to extend our knowledge of the role of growth regulators upon ripening in grape berries and this may be applicable to non-climacteric fruit in general. The result of future studies building on the research presented in this thesis could then be applied to the genetic modification of grape cultivars that will benefit the grower and the consumer.

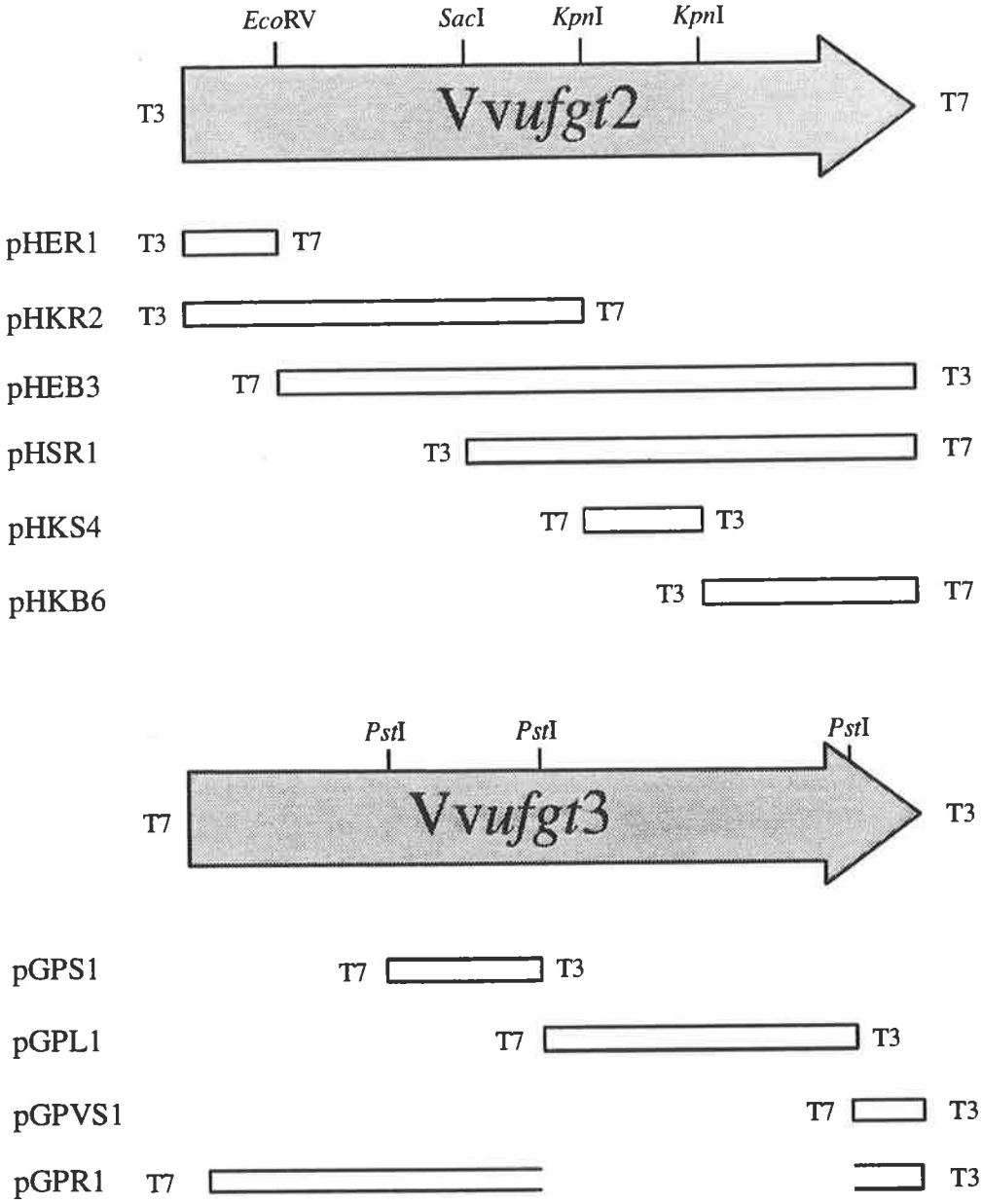
Appendix A

Subclones generated for the sequencing of the grape *myb*-like cDNA clones isolated. The restriction endonuclease sites used to generate the subclones are indicated on the arrows representing the full length clones. All clones were generated using pBluescript SK(+), and their orientation in the plasmid displayed by the location of the T3 and T7 primer sites. The fragments subcloned are represented by the unshaded boxes and the names of the subclones are indicated on the left of the figure.



Appendix B

Subclones generated for the sequencing of the grape UFGT cDNA clones isolated. The restriction endonuclease sites used to generate the subclones are indicated on the arrows representing the full length clones. All clones were generated using pBluescript SK(+), and their orientation in the plasmid displayed by the location of the T3 and T7 primer sites. The fragments subcloned are represented by the unshaded boxes and the names of the subclones are indicated on the left of the figure.



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Addendum

Page 59 It should be pointed out that the proanthocyanidin data is presented per gram fresh weight. Grape cells are taking up a lot of water which makes it difficult to interpret whether the proanthocyanidins are being turned over in ripening berries are whether they are not being synthesised after véraison. It is also interesting that the relationship between anthocyanin and proanthocyanidin production was not reciprocal. This suggests that there is some type of metabolic channelling occurring in the flavonoid pathway in grape berries.

Page 72 The Shiraz UFGT cDNA clones described in Chapter 7 enable a better interpretation of the Southern data. Both cDNA clones possessed an *Eco* RV site close to the 5'-end, whereas a *Dra* I site was only found in the putative intron in the Vv*ufgt*3 clone. Therefore we would expect to see at least two bands in the Shiraz DNA lanes in both Southern blots, as is indeed the case. Also, re-examination of the *Dra* I Southern suggests that there is some evidence for polymorphism between the grapevine varieties tested.

Page 131 Introns can cause major disruption to the predicted restriction fragment lengths seen in Southern blots. *Myb*-like genes tend to possess introns towards the 5'-end in the DA binding regions. Thus, the Southern blots probed with the full-length clones will be influenced by the introns. However, the 3'-end probes use should not span introns found in these *myb*-like genes.

Page 137 Re-examination of the Southern data suggests that there is only a single Vv*myb*1 gene in grapes, not more as is suggested in this discussion.

Page 145 These DNA libraries are made by ligating a special adaptor to the ends of DNA fragments which have been digested separately with restriction enzymes that have six-base recognition sites and generate blunt ends. The adaptor has an amine group on the 3'-end of the lower strand which blocks extension of this strand and thus the generation of the primer binding site unless a distal gene-specific primer extends a DNA strand opposite the upper strand of the adaptor. If, however, PCR products are formed which contain double-stranded adaptor sequences at both ends, the ends of the individual DNA strands will form panhandle structures after denaturation due to the presence of inverted terminal repeats. These structures are more stable than the primer-template hybrid and will thus suppress amplification of these species. However, when the gene specific primer extends through the adaptor, the product will contain the adaptor sequence only at one end and will not form the panhandle structure. PCR amplification can proceed normally in this case allowing the cloning of these products.

Page 147 The Figure overleaf presents an overview of the One-Hybrid System as presented in the Clontech manual.

Page 169 Another problem with the one-hybrid system and its use to isolate primary regulators of anthocyanin production may involve the way in which these transcription factors act to regulate the structural genes. It has been demonstrated that the *myb*-like and *myc*-like proteins may interact and thus operate as heterodimers (Martin and Paz-Ares 1997). Therefore, the one-hybrid system, which relies on a single protein product interacting with its DNA recognition site may not be suitable for isolating transcription factors which operate as heterodimers.

- Prepare competent YM4271 cells.
  (1 day)

- Separately transform competent YM4271 with each target-reporter vector, linearized (integration) and circular (control).
  (1 day)

- Select for recombinants on the appropriate minimal selection medium.
  (4–6 days)

- Restreak large, well-isolated colonies from cells transformed with linearized plasmid on selection medium.
  (3 days)

- For yeast transformed with *HIS3* reporter constructs, determine the optimal [3-AT] for inhibiting leaky *HIS3* expression.
  (3 days)

- If applicable, integrate *lacZ* reporter construct into the *HIS3* reporter strain.
  (3–5 days)

- Transform reporter strain with AD fusion library; select on SD/–His/–Leu/+optimal [3-AT].
  (4–8 days)

- If applicable, test for β-galactosidase activity.
  (1 day)

- His^+, $LacZ^+$ clones are candidates for expressing AD/library proteins that bind to your target element.