The influence of viticultural treatments on the accumulation of flavonoid compounds in grapes and their contribution to wine quality

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CHAPTER 1

General introduction

1.1 Introduction

There is an ancient association between man, the grapevine and the wine produced from its fruit where wine has both religious and cultural significance and there is now evidence that moderate wine consumption can also have significant health benefits (Lairon and Amiot 1999; Plumb et al. 1998). One group of plant secondary metabolites known as flavonoids contribute to the visual character, taste and health benefits of wine, particularly the classes; anthocyanins, flavonols and tannins. While the initial focus grape flavonoid research was on the development of anthocyanins and their contribution to red wine colour, the accumulation of other flavonoids such as tannins and flavonols has been investigated recently due to their influence on grape and wine quality (Boulton 2001; Brossaud et al. 2001; Downey et al. 2003a; Downey et al. 2003b; Harbertson et al. 2002; McDonald et al. 1998; Price et al. 1995a; Spayd et al. 2002).

1.2 Wine production

1.2.1 Winemaking

Winemaking involves the extraction of juice from ripe grapes and fermentation of that juice with a cultured yeast. While extraction and fermentation processes are extremely variable, wine quality is largely determined by the composition of the grapes in respect of colour, acid, sugar and phenolic content as well as the management of these parameters during vinification and maturation (Boulton 2001; Boulton et al. 1998; Rankine 1998; Sacchi et al. 2005).

1.2.2 The wine industry

The Australian Bureau of Statistics (2003) reports global wine production is about 30 billion litres annually. While Europe continues to dominate wine production, Australia's wine and grape industry is steadily increasing, with wine production now around one billion litres annually. Australia is one of the largest exporters of wine and in 2002, approximately 500 million litres of wine was exported, with a wholesale revenue of $2 billion. While the greatest export market for

Australia is the United Kingdom, which accounts for 50% of wine exports, the continued success of the Australian wine industry is dependent upon ongoing production of quality, rather than quantity wines (Bureau et al. 2000; Osmond and Anderson 1998).

1.2.3 The health benefits of wine

The health benefits of wine have recently been investigated in relation to the class of secondary metabolites known as flavonoids (prominently the flavonols and flavan-3-ols) and their role as antioxidants (Baldi et al. 1995; Plumb et al. 1998; Sato et al. 1996). The antioxidant properties of these phenolic components are thought to aid in the prevention of free radical associated diseases such as coronary heart disease, atherosclerosis, stroke and some cancers (Hertog and Hollman 1996; Lairon and Amiot 1999; McDonald et al. 1998; Plumb et al. 1998).

1.3 Grape development

The European grape species, *Vitis vinifera* L., produces the majority of wine grapes, table grapes and raisins in Australia, as in many other countries (Antcliff 1988; Coombe 1988). Grapes are a non-climactaric fruit that exhibit a double sigmoid pattern of development with two distinct phases of growth separated by a lag phase (Coombe 1992; Robinson and Davies 2000) **(Figure 1.1)**. The initial phase of growth (fruit set) mainly results from cell division while the second phase, at the onset of berry ripening (veraison) is characterised by cell enlargement and significant changes in metabolism including, accumulation of sugar, softening of berries, synthesis of flavonoids, metabolism of organic acids and synthesis of flavour compounds (Coombe 1992; Pirie and Mullins 1980; Robinson and Davies 2000).

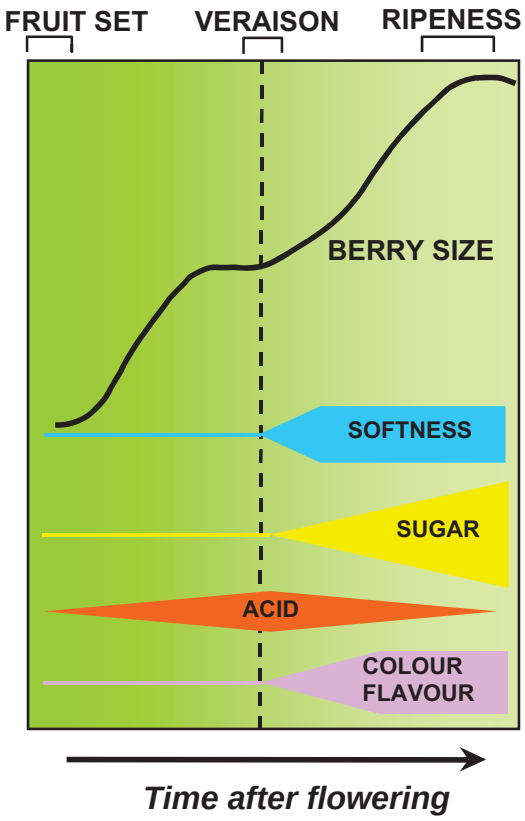


Figure 1.1 Schematic representation of grape berry development showing the bimodal pattern of berry growth & the pattern of secondary metabolite accumulation (Coombe 1992;Coombe 1995;Coombe & Bishop 1980).

During the ripening phase, it is these physiological and biochemical changes that determine the quality of the fruit at harvest (Robinson and Davies 2000). The relative proportions of each of these components in the grape, and subsequent wine, can be influenced considerably by site, season and viticultural practice (Mullins et al. 1992).

1.4 Flavonoids

The flavonoids, found only in plants, comprise a group of polyphenolic compounds based on a flavan skeleton which is variously substituted with glycosyl, hydroxyl, methyl and acyl moieties (Koes et al. 1994; Shirley 1996) **(Figure 1.2)**. Flavonoids occur mainly as conjugated compounds, commonly glycosides, and can form extensive polymers with other flavonoids, metal ions and other molecules (Haslam 1998). The flavan ring structure of each flavonoid has a distinct absorbance spectrum, allowing specific absorption of light in both the ultraviolet and visible spectra (Harborne and Williams 2000; Jordan 2004; Shirley 1996).

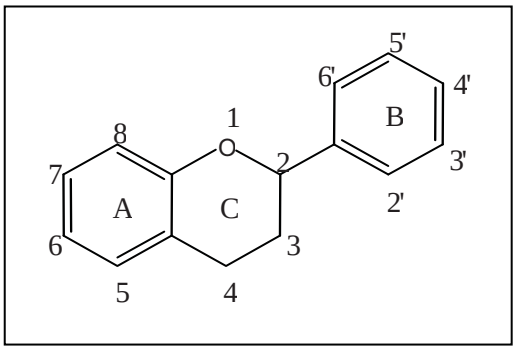


Figure 1.2 The flavan skeleton of the plant secondary metabolites, flavonoids, showing the numbering of carbon atoms & ring labels (Harborne 1967; Haslam 1998).

There are over 4000 known flavonoids in plants, each with a diverse physiological function (Koes et al. 1994; Shirley 1996; Stafford 1991). The physiological roles of flavonoids include;

- Protection from UV irradiation due to the chromophoric nature of the flavonoid ring structure (Jordan 2004; Markham et al. 1998; Smith and Markham 1998).
- Producing intensely pigmented compounds, forming bright red, blue and purple colours (Grotewold 2006; Schwinn and Davies 2004). These colours act as bird and insect attractants for pollination and seed dispersal (Koes et al. 2005; Markham et al. 1998; Saito and Harborne 1992; Shirley 1996).
- Acting as an antioxidant, scavenging free radicals suggested to have medicinal and therapeutic benefits (Bagchi et al. 2000; Cos et al. 2004; Hertog and Hollman 1996; Lairon and Amiot 1999; Plumb et al. 1998)
- Acting as signalling molecules in both a plant defence mechanism and in establishing plant-microbe interactions (Debeaujon et al. 2000; Koes et al. 1994; Peters and Constabel 2002; Shirley 1996).

Anthocyanins, a subclass of flavonoids, are prominent floral pigments and various colour mutants have been used to elucidate the steps of the flavonoid biosynthetic pathway (Harborne 1967; Stafford 1990). Studies of these compounds in numerous plant species, including grapes, have been instrumental in developing our understanding of the genetic and molecular basis of pigmentation (Boss et al. 1996b; Downey et al. 2006; Holton and Cornish 1995; Sparvoli et al. 1994).

1.5 Flavonoids in grapes & wine

Of the myriad of flavonoid compounds detected in plants, the three main classes of flavonoid compounds commonly detected in grapes and wines include the anthocyanins, flavonols and tannins. Of these three classes of flavonoids, tannins are present in greatest proportion in red grapes and wine, followed by anthocyanins, with flavonols being present in relatively low amounts (Downey et al. 2003b; Kennedy et al. 2006a; Souquet et al. 1996). Much of the visual character and taste of red wines is due to the presence of these flavonoid compounds derived from the seed and skin of the grapes (Glories 1988).

1.5.1 Anthocyanins

Anthocyanins are the most studied phenolic component of red grapes on account of their contribution to the colour of red wines (Harborne 1967; Mazza 1995; Ribereau-Gayon and Glories 1986). Anthocyanins are located in the skins of grapes, predominantly in the vacuoles of hypodermal layers, but are also present in the pulp of red fleshed grape cultivars (Adams 2006; Mullins et al. 1992; Sparvoli et al. 1994). Anthocyanins found in grapes and wines include; petunidin, peonidin, malvidin, cyanidin and delphinidin and their derivatives, the mono-glucosides, acetyl-glucosides and coumaroyl-glucosides (Boss et al. 1996b). Malvidin-glycosides are the major anthocyanins in red grapes, accounting for around 60% of total pigments (Crippen and Morrison 1986a; Mazza 1995; Mazza and Miniati 1993). The function of anthocyanins has been suggested to be to absorb UV and visible light and to also act as a colour attractant for pollination and seed dispersal (Koes et al. 1994; Saito and Harborne 1992; Smith and Markham 1998).

Anthocyanins extracted from red grape skins during fermentation are fundamentally responsible for the colour observed in resultant wines (Boulton 2001; Mateus et al. 2002; Mazza 1995; Revilla et al. 1999; Ribereau-Gayon and Glories 1986). In young wines, the majority of the colour is due to free anthocyanins, with co-pigment associations occurring to approximately 30-50% of these anthocyanins (Boulton 2001; Revilla et al. 1999; Somers 1971; Somers and Evans 1977). As the wine matures pigment composition becomes progressively more complex, forming stable associations between anthocyanins and other flavonoid compounds to produce polymeric pigments (Boulton 2001; Brouillard et al. 1997; Cheynier et al. 2006; Haslam 1998; Mayen et al. 1995; Mazza 1995). Thus, a critical factor in determining the extent of colour in mature wine is the concentration of anthocyanins and potential co-pigment compounds at harvest.

1.5.2 Flavonols

There are only limited studies on flavonols in grapes, most of which have focused on the induction of flavonols by UV-light and the apparent correlation between this phenomenon and the quality of grapes and subsequent wines (Cheynier and Rigaud 1986; Downey et al. 2004; Haselgrove et al. 2000; Morrison and Noble 1990; Price et al. 1995a; Spayd et al. 2002). The most common flavonol found in grapes is quercetin comprising mainly of quercetin-3-glucoside and quercetin-3-glucuronide (Cheynier and Rigaud 1986; Downey et al. 2003b; Price et al. 1995a). Apart from quercetin, other forms of flavonols have been detected including the glycosides of kaempferol, myricetin and isorhamnetin (Cheynier and Rigaud 1986). Flavonols occur in the upper epidermal layer of the berry skin, consistent with a role in UV screening (Downey et al. 2003b; Flint et al. 1985; Haselgrove et al. 2000; Price et al. 1995a; Price et al. 1995b; Smith and Markham 1998). Flavonols have also been detected in grapevine leaves and stems (Downey et al. 2003b; Hmamouchi et al. 1996; Souquet et al. 1996).

In wines, the same flavonol glycosides that are present in the grapes are found, however these can also be hydrolysed by the acidic conditions of wines, resulting in the evolution of the flavonol aglycones (Blanco et al. 1998; Goldberg et al. 1998; Mayen et al. 1995; McDonald et al. 1998; Price et al. 1995b). Although colourless, flavonols are thought to contribute to wine colour by acting as a co-pigments for anthocyanins (Asen et al. 1972; Baranac et al. 1997; Boulton 2001; Downey et al. 2003b; Lambert 2002; Mateus et al. 2002; Scheffeldt and Hrazdina 1978). This observation suggests that treatments that increase the anthocyanin or flavonol content in grapes could be a valuable contribution to wine quality.

1.5.3 Tannins

There are numerous studies of tannin (flavan-3-ol & proanthocyanidin) content and composition in grapes because of their contribution to wine quality and more recently their potential health benefits (Hertog and Hollman 1996; Lairon and Amiot 1999; Plumb et al. 1998). There are a wide range of tannins readily identified in grapes and red wines including the small oligomeric (flavan-3-ol) monomers; catechin, epicatechin and epicatechin-gallate and the large proanthocyanidin polymers also known as condensed tannins, of which over 20 different types have been identified (Cheynier et al. 1997; Fulcrand et al. 1999; Fuleki and da Silva 1997; Peng et al. 2001; Prieur et al. 1994; Romeyer et al. 1986). In red wines, in addition to condensed tannins, there are also hydrolyzable tannins, which are derived from wood, such as oak barrels or chips or can be added to wine in powdered form (Ribereau-Gayon 1972). Hydrolyzable tannins will not be considered in this investigation. The role of tannins in grapes is uncertain but their

bitterness and astringency are thought to act as feeding deterrents to herbivorous animals and insects (Downey et al. 2006; Feeny 1976; Harborne and Grayer 1993).

Tannins have been detected in both the seeds and skins of grapes, with total tannins being higher in the seed than in skins (Amrani-Joutei et al. 1994; de Freitas and Glories 1999; Downey et al. 2003a; Harbertson et al. 2002; Kennedy et al. 2000a; Prieur et al. 1994; Souquet et al. 1996; Thorngate and Singleton 1994). In the skins, tannins are located in the hypodermal layers, while in seeds they are located in the soft parenchyma, between the cuticle and hard seed coat (Adams 2006). Seed tannins comprise of the procyanidins; catechin, epicatechin and epicatechin-gallate (Escribano-Bailon et al. 1995; Prieur et al. 1994), whereas skin tannins also include epigallocatechin (Souquet et al. 1996). Most of the tannins detected in skins and seeds are present as proanthocyanidin polymers, with longer polymers detected in skins compared to seeds (Brossaud et al. 1999; Cheynier et al. 1997; Downey et al. 2003a; Kennedy et al. 2001; Souquet et al. 1996). Tannins are not detected in the pulp of grape berries, although trace amounts have been detected in the vasculature (Coombe 1987; Ricardo da Silva et al. 1991; Souquet et al. 1996) and considerable levels have also been detected in grapevine stems and leaves (Bogs et al. 2005; Darne 1993; Ricardo da Silva et al. 1991). Whilst tannin composition in skin and seeds is similar in red and white grapes, there is considerable variation in the relative proportions between varieties (Amrani-Joutei et al. 1994; Cheynier et al. 1997; Ricardo da Silva et al. 1991; Romeyer et al. 1986; Seddon 2006).

During red winemaking, tannins are extracted from the skin and seeds of grapes and from wood barrels or chips used during ageing of wine (Kovac et al. 1995; Peyrot des Gachons and Kennedy 2003; Revilla et al. 1999; Ribereau-Gayon and Glories 1986; Thorngate and Singleton 1994). Approximately, 65% of the phenolics in wines come from the skins and the remaining 35% from the seeds, due to difficulty in extracting tannins from seeds, which are lignified (Revilla et al. 1997; Ribereau-Gayon and Glories 1986; Thorngate and Singleton 1994). Tannins are responsible for the astringent and bitter properties in red wines that contribute to mouth feel (Gawel et al. 2000; Glories 1988; Thorngate 1997). Astringency is a common feature of young wines and is usually correlated with tannin content (Czochanska et al. 1979; Fulcrand et al. 1996; Kennedy et al. 2006b). However, as wine matures, the bitterness and astringency decreases where tannin polymers polymerise to form large complexes with other compounds (Brossaud et al. 2001; Brouillard et al. 1997; Cheynier et al. 1997; Mateus et al. 2001; Noble 1994; Vidal et al. 2003). When this polymerisation reaction occurs with proanthocyanidin oligomers, the polymeric compounds formed are less soluble and can precipitate from the wine (Fulcrand et al. 1996; Haslam 1998; Somers et al. 1987; Thorngate 1997; Timberlake and Bridle 1976).

However, when polymerisation reactions occur with anthocyanins, it can increase the solubility of the large polymeric compounds whilst decreasing susceptibility of anthocyanins to degradation by low pH's and bleaching (Bakker 1998; Baranowski and Nagel 1983; Boulton 2001; Cheynier et al. 2006; Cheynier et al. 1997; Cheynier et al. 2000; Dallas et al. 1996; Gawel et al. 1998; Kennedy et al. 2001; Malien-Aubert et al. 2001; Mateus et al. 2002; Schwartz et al. 2005; Somers 1971; Timberlake and Bridle 1976; Vidal et al. 2002).

1.6 Biosynthesis of flavonoids

The biosynthesis of flavonoids is the culmination of two secondary metabolic pathways, the shikimate pathway and the phenylpropanoid pathway. The phenylpropanoid pathway synthesises flavonoids from carboxylated acetyl-CoA (malonyl-CoA) and the amino acid phenylalanine, which is produced in the shikimate pathway (Heller and Forkmann 1993). The final products of the phenylpropanoid pathway are generally considered to be anthocyanins, as shown in (**Figure 1.3**) (Boss et al. 1996b; Dooner et al. 1991). Together with anthocyanins, numerous other diverse end products are formed including lignin, aurones, flavones, isoflavonoids, flavonols and proanthocyanidins (tannins) (Harborne 1967; Haslam 1998).

Most of the known phenylpropanoid pathway genes and cDNAs encoding the biosynthetic enzymes have been cloned in a variety of different species (Burbulis and Winkel-Shirley 1999; Charrier et al. 1995; Dixon et al. 2005; Forkmann 1993; Gong et al. 1997; Tanaka et al. 1996; Tanner et al. 2003; Winkel-Shirley 2001; Winkel-Shirley 2002; Xie et al. 2003). In grapevine, the *Vitis vinifera* genes for most of the biosynthetic enzymes have been cloned, including: PAL, CHS, CHI, F3H, F3'H, F3'5'H, DFR, LDOX, LAR, ANR, FLS, UFGT; and recently the synthesis of three major classes of flavonoids (anthocyanins, tannins and flavonols) during berry development has been determined in red grapes (Bogs et al. 2005; Bogs et al. 2006a; Downey et al. 2003b; Ford et al. 1998a; Robinson and Davies 2000; Sparvoli et al. 1994).

1.6.1 Anthocyanins

Anthocyanins are located in berry skins and accumulation in Shiraz berries occurs after veraison, coinciding with expression of UDP-glucose:flavonoid 3-O-glucosyl transferase gene (*VvUFGT*), which catalyses the conversion of anthocyanidins to the more stable anthocyanins (Boss et al. 1996a; Boss et al. 1996b; Boss et al. 1996c). White grapes do not produce anthocyanins and have mutations that result in their failure to express the gene encoding the biosynthetic enzyme UGFT, one of the final steps in anthocyanin synthesis (Boss et al. 1996a; Boss et al. 1996c; Sparvoli et al. 1994).

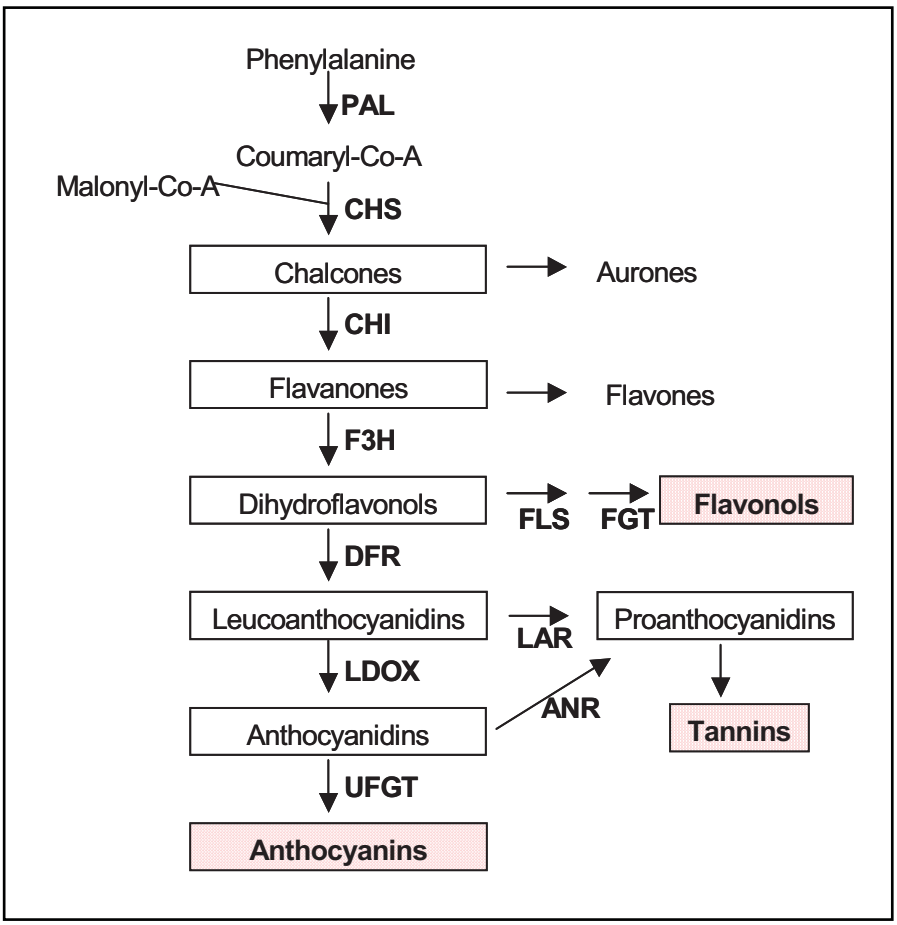


Figure 1.3 Stylised phenylpropanoid pathway showing major products, intermediates & biosynthetic enzymes.

PAL- phenylalanine ammonia lyase
CHI- chalcone isomerase
FLS- flavonol synthase
DFR- dihydroflavonols-4-reductase
ANR- anthocyanidin reductase
UFGT-UDP glucose flavonoid-3-O-glucosyltransferase
CHS- chalcone synthase
F3H- flavanone-3ß-hydroxylase
FGT- flavonol glycosyl-transferase (putative)
LDOX- leucoanthocyanidin dioxygenase
LAR- leucoanthocyanidin reductase

1.6.2 Flavonols

Flavonols are located in berry skins, developing flowers and leaves (Downey et al. 2003b). There are two phases of flavonol synthesis and accumulation during grape berry development, the first, around flowering and the second starting 1-2 weeks after veraison continuing throughout ripening (Downey et al. 2003b; Haselgrove et al. 2000). Accumulation of flavonols coincides with expression of flavonol synthase gene (*VvFLS1*) (Downey et al. 2003b) which catalyses the conversion of dihydroflavonols to the corresponding flavonol aglycones. Two genes encoding FLS have been isolated from *Vitis vinifera* L. cv. Shiraz, *VvFLS1* and *VvFLS2* (Downey et al. 2003b). It was shown that *VvFLS1* was more highly expressed than *VvFLS2* (Downey et al. 2003b) and subsequently, expression of *VvFLS1* was attributed to flavonol accumulation during grape berry development and low levels observed in shaded fruit (Downey et al. 2003b; Downey et al. 2004).

In grapevines the flavonols detected are in the glycosylated form, and as such it is postulated that there must be a glycosylation enzyme responsible for flavonol glycosylation (i.e. a flavonol specific glycosyl-transferase (FGT))(Ford et al. 1998a). To date, the only FGT, which has been identified and functionally tested, is in *Petunia*, (Miller et al. 2002; Miller et al. 1999; Vogt and Taylor 1995). While the *Arabidopsis thaliana* genome sequencing project has putatively identified further FGTs, no sequence exists for any flavonol specific glycosyl-transferase in grapevine.

1.6.3 Tannins

Tannins are located in both seed and skin of berries and high levels have also been detected in developing flowers (Bogs et al. 2005; Coombe 1987; Downey et al. 2003a; Souquet et al. 1996). It is now clear that there are two important phases of tannin development in grapes; a) tannin synthesis and accumulation and b) tannin maturation.

Tannin synthesis and accumulation starts very early in berry development, around flowering, and continues until veraison in skins, and for 1-2 weeks after veraison in seeds (de Freitas and Glories 1999; Downey et al. 2003a; Kennedy et al. 2001; Kennedy et al. 2000a; Kennedy et al. 2000b). This period of tannin biosynthesis has recently been shown to coincide with the expression of leucoanthocyanidin reductase (*VvLAR*) and anthocyanidin reductase (*VvANR*) genes for formation of the tannin monomers catechin and epicatechin, respectively (Bogs et al. 2005). It was found that in grape a single gene codes the ANR enzyme, whereas two closely related genes encode LAR, yet it was unclear as to whether this represented two genes of LAR with differing properties (Bogs et al. 2005). Despite this recent publication identifying the genes responsible for synthesis of the flavan-3-ols, there are at least two unknowns in the area of tannin biosynthesis. The first is how tannin polymers are formed in the cell and the second relates to the compartment of the cell this reaction takes place (Adams 2006).

During the later stages of ripening, tannin maturation occurs whereby the extractable levels of tannin starts to decline (Downey et al. 2003a; Iland 1998; Kennedy et al. 2001; Ristic et al. 2004). Physiologically, the decreasing extractability of tannins, particularly from grape skins, represents a decrease in the overall bitterness and astringency of tannins in the grape berry and is likely part of the seed dispersal strategy that include sugar accumulation and anthocyanin biosynthesis in the berry (Downey et al. 2006). Ristic et al. (2004) showed that the decline in seed tannins accompanies colour changes that occur in the seeds during ripening and it has been suggested that this seed browning represents oxidation of tannins during ripening (Kennedy et al.

2000a; Kennedy et al. 2000b). However, the chemical structures resulting from these presumed oxidation reactions have yet to be described. The decrease in extractable tannins during ripening has also been suggested to be the result of complexation of the tannin polymers with other cellular components, including polysaccharides, lignins or proteins (Coombe and McCarthy 2000; Downey et al. 2003a; Fournand et al. 2006; Hazak et al. 2005; Kennedy et al. 2001; Kennedy et al. 2000a). However, the actual mechanism of what causes this decrease in extractable tannin has yet to be elucidated (Dixon et al. 2005; Lepiniec et al. 2006; Schwinn and Davies 2004; Tanner 2004).

1.7 Transcriptional regulation of flavonoids

Regulation of the flavonoid pathway occurs primarily at the transcriptional level for the structural genes, which encode the enzymes for each step of synthesis (Davies and Schwinn 2003b; Lepiniec et al. 2006; Weisshaar and Jenkins 1998; Winkel-Shirley 2001). Initiation of transcription involves DNA-binding of different transcription factors to promoter regions of DNA located upstream from an initiation site of a particular gene (Campbell 1996). Transcription occurs only after successful recognition of the appropriate transcription factor(s) to the specific element/motif contained in the promoter sequence. Recently, the transcriptional regulation of the flavonoid pathway genes in different plant species has gained more attention, as there is growing interest in metabolic engineering strategies aimed at developing agronomically important food crops and fruit with optimized levels and composition of flavonoids.

1.7.1 Activation by transcription factors

There are two major families of transcription factor regulators involved in controlling parts of the flavonoid pathway: the basic helix-loop-helix (bHLH) and MYB proteins (Koes et al. 2005). In *Arabidopsis,* the bHLH (also called MYC) proteins (AtTT8, AtGL3 and AtEGL3) have overlapping functions as regulators of trichome development and flavonoid synthesis (Ramsay et al. 2003; Zhang et al. 2003; Zimmermann et al. 2004). The MYB-type transcription factors specific for specific branches of the flavonoid pathway include the MYB proteins, AtPAP1 and AtPAP2 that regulate anthocyanin synthesis and AtTT2 which regulates tannin synthesis (Borevitz et al. 2000; Nesi et al. 2001). The third protein involved in gene regulation is a WD40 protein, which has broader functions in *Arabidopsis* and is constitutively expressed in most tissues (Walker et al. 1999). A stylized diagram representing the action of the three transcription factor proteins that regulate synthesis of the flavonoid biosynthetic genes is shown in **Figure 1.4**.

Figure 1.4 Schematic diagram showing transcriptional regulation of flavonoid pathway genes. Activation of the flavonoid biosynthetic genes requires the combinations of WD40, bHLH & MYB DNA binding proteins.

Only recently has a transcriptional activator of the flavonol pathway in *Arabidopsis* been identified (Mehrtens et al. 2005). The MYB transcription factor, AtMYB12, was found to be a flavonol specific activator of flavonoid biosynthesis, activating *AtFLS1* and *AtCHS* genes as its primary targets. AtMYB12 was shown to interact with a specific cis-acting promoter element a MRE (MYB Recognition Element) part of light responsiveness unit (LRU)(Hartmann et al. 2005), and unlike anthocyanin and tannin regulation, activation of AtMYB12 did not depend on bHLH co activators (Mehrtens et al. 2005). The influence of light on AtMYB12 was not determined, and therefore it remains unclear whether this transcription factor is also light-sensitive.

In grapevines, the MYB transcription factors responsible for activation of anthocyanin and tannin biosynthesis have recently been identified. Two MYB transcription factors proteins, VvMYBA1 and VvMYBA2, have been shown to regulate *VvUFGT* gene expression and anthocyanin accumulation in red berries (Kobayashi et al. 2002; Kobayashi et al. 2005; Walker et al. 2007). The transcriptional regulator of tannin synthesis, VvMYBPA1, was shown to activate the promoters of *VvLAR* and *VvANR*, but not *VvUFGT* (Bogs et al. 2007). Also, the MYB transcription factor VvMYB5a was shown to affect metabolism of anthocyanins, tannins, flavonols and lignin when expressed in tobacco, suggesting it may control different branches of the flavonoid biosynthetic pathway (Deluc et al. 2006). Presently, no transcriptional regulator(s) specific for flavonol biosynthesis in grapevines have been isolated.

1.8 Factors affecting the flavonoid composition of grapes

The level of phenolic compounds in grapes is influenced by numerous cultural, climatic and environmental factors, including; light, temperature, altitude, soil type, nutritional status, microbial interactions, osmotic stress, defoliation, wounding, pathogenesis and plant growth regulators. The impact of these factors on flavonoid content and composition is extensively reviewed in Jackson and Lombard (1993), and more recently in Downey et al. (2006). Many of these factors are closely intertwined and difficult to isolate experimentally and with the genetic

variation between plant species and varieties, results in enormous diversity in flavonoid content and composition (Downey et al. 2006). However, despite the variability conferred by these factors, the greatest influence on the flavonoid content and composition are site and season (Bakker 1986; de Freitas and Glories 1999; Downey et al. 2006; Gonzalez-San-Jose et al. 1990; Guidoni et al. 2002; McDonald et al. 1998; Revilla et al. 1997). For a given site, assuming some of these variables are by and large invariant (i.e. soil type, irrigation, nutrition), the primary difference between sites is climatic, predominantly sunlight and temperature. Consequently, the focus of this investigation is the relationship between sunlight exposure and temperature of grape bunches and the importance to berry composition and metabolism.

1.8.1 Light exposure

There have been several studies into the effect of bunch exposure and particularly the impact of increased or decreased light exposure on the fruit and resultant wine quality. It is generally accepted that as fruit exposure to sunlight increases, fruit development, fruit composition, wine colour (anthocyanins) and quality improve (Buttrose 1970; Buttrose and Hale 1973; Buttrose et al. 1971; Crippen and Morrison 1986a; Crippen and Morrison 1986b; Dokoozlian and Kliewer 1996; Haselgrove et al. 2000; Kliewer and Lider 1970; Kliewer and Torres 1972; Morrison and Noble 1990; Pereira et al. 2006; Price et al. 1995a; Sparvoli et al. 1994; Spayd et al. 2002).

However, it has also been shown that grape berry colour (anthocyanins) can be negatively affected by excessive light exposure, particularly in over-exposed fruit (Hunter et al. 1995; Kliewer 1977; Kliewer and Torres 1972) and recently it was observed that high light resulted in decreased wine colour (Bergqvist 2001). Possible explanations for these differences include differences in cultivar, site and season as well as differences in sampling and analytical techniques or that there may be an optimum exposure for grape bunches.

Light exposure has been shown to significantly increase flavonol accumulation in grapes and wine (Goldberg et al. 1998; Haselgrove et al. 2000; McDonald et al. 1998; Price et al. 1995a; Spayd et al. 2002). These observations report that fruit exposed to light, mainly via changes in canopy structure, have greater levels of flavonols, particularly quercetin glucosides, than shaded fruit. An increase in flavonols from sun exposed fruit may have implications with the stability and quality of the wine, particularly if flavonols act as co-pigments for anthocyanins.

Recently the affect of light on flavonoid biosynthesis in Shiraz grapes was investigated (Downey et al. 2004). Bunches were enclosed in polypropylene boxes just after flowering to exclude light and demonstrated that the design of the boxes was such that bunch temperature was not altered.

Grapes from bunches grown in boxes (shaded fruit) had similar levels of anthocyanins, seed and skin tannins to control fruit exposed to sunlight but the shaded fruit had much lower levels of flavonols. Using similar boxes to shade Pinot noir vines, Cortell and Kennedy (2006) found that total anthocyanin and skin tannin content was slightly reduced, while flavonol content was significantly reduced. These results indicate that synthesis of flavonols in grapes is dependent on light, whereas accumulation of anthocyanins and tannins can occur even in highly shaded fruit. Presently, the molecular mechanism of light induction of flavonol synthesis is unknown.

1.8.2 Temperature

Separating the effects of temperature and sunlight, in the field, on grape berry composition is difficult because of the many biochemical pathways which are both light and temperature sensitive. There are only a few studies that have been able to separate the effects of sunlight and temperature. These include studies on the effects of temperature on grape berry development and composition in growth chambers, glasshouses and phytotrons to compare day/night temperatures (Buttrose 1970; Buttrose and Hale 1973; Buttrose et al. 1971; Dokoozlian and Kliewer 1996; Kliewer and Torres 1972). These studies found that an increase in ambient air temperature increased the metabolism of total soluble solids (sugar) and grape berry development.

High and low temperatures have been shown to effect the anthocyanin content and composition of grapes and this may also be influenced by diurnal changes in temperatures. In one phytotron study, using potted Pinot noir vines and maintaining a cool night temperature of 10-15^{o}C during grape ripening, cool day temperatures (15^{o}C) improved anthocyanin levels while hot day temperatures (35^{o}C) reduced formation of anthocyanins (Kliewer and Torres 1972). In another study it was reported that anthocyanin levels during Cabernet Sauvignon grape development were greater when day temperature was a constant 20^{o}C rather than a constant 30^{o}C in glasshouse experiments with a constant night temperature of 15^{o}C (Buttrose et al. 1971). More recently it has been shown that lower night temperatures (15^{o}C) resulted in greater anthocyanin accumulation than constant temperatures of 30^{o}C (Mori et al. 2005). Also, it has been shown that grapes exposed to high temperatures, have a higher coumaroyl-glucoside profile compared to the mono and acetyl glucosides (Downey et al. 2004; Spayd et al. 2002). However, whether these changes in anthocyanin content or composition occurs through degradation or reduced biosynthesis, is not known. Presently, there are limited studies investigating the effect of temperature, independent of light or changes in canopy structure, on the synthesis of flavonoid compounds (anthocyanins, flavonols and tannins) in bunches throughout berry development.

1.9 The definition of grape & wine quality

Internationally, no clear definition exists for optimum 'quality' of grapes or wine, and the classification of quality in both remains largely subjective. This is primarily due to the vast array of compounds in grapes, such as acids, sugars, phenolics and aroma/flavour compounds, and the complex interactions between these compounds in wines. Possibly, one of the best explanations of grape and wine quality written by Coombe and Iland (2004) includes;

> "The *objective measurement* of the chemical composition of grapes at harvest, and preliminary sensory assessments, largely influence the winemakers decision about the wine style, and hence the fermentation methods that are appropriate. Within any wine style category, grades of wine quality are recognised by *subjective assessments*, of the concentrations, balance and interactions of these compounds in wines evaluated sensorily by humans against a set of criteria. Thus, grape quality is defined as the suitability of a batch of grapes to produce a wine of the highest quality in a targeted style."

Historically, measures of grape juice characteristics have been used to predict wine quality, particularly total soluble solids (TSS/°Brix), pH and titratable acidity (TA), and in more recent years grape colour has also been used as a major factor in determining grape quality (Sas and Lim 2003; Swinburn 2003). There are a variety of cultural, climatic and environmental factors that can affect grape berry ripening and impact on the composition of fruit at harvest (Downey et al. 2006; Jackson and Lombard 1993). Recently, there has been a move within the wine industry toward a more objective definition and measurement of 'grape quality' in terms of composition factors other than sugar, acidity and colour, which affect wine properties. To a large extent, this has centred around the study of secondary metabolic pathways in grape berries, which are responsible for the biosynthesis of many of the compounds responsible for the sensory properties of wine.

2.0 Summary & project aims

Flavonoid metabolism has attracted significant interest over the last decade, with the recognition that the products of the flavonoid biosynthetic pathway (anthocyanins, tannins and flavonols) significantly contribute to grape and wine quality with respect to colour and mouthfeel of red wine. While it has been demonstrated that anthocyanins, tannins and flavonols are synthesized in different parts of the berry and during different stages of berry development, coordination of synthesis of these compounds along with how they influence grape and wine quality has yet to be elucidated.

There are strong environmental and viticultural factors, which may determine flavonoid content and composition in grapes and wine. Grape and wine colour is influenced by climate, particularly temperature and light exposure. There is also evidence that bunch light exposure alone influences flavonol synthase (*VvFLS1*) expression and flavonol accumulation at harvest, however less is known about the affect of bunch light exposure on flavonol biosynthesis during grape berry development.

Furthermore, whilst the transcriptional control of the flavonoid biosynthetic pathway genes in model plants is well documented, less is known about flavonoid transcriptional control in grapevines. Although recently the grapevine transcription factors controlling anthocyanin and tannin accumulation have been identified, the transcription factors controlling flavonol biosynthesis have yet to be determined.

This project aims to investigate the relationship between the different products of the flavonoid biosynthetic pathway and to determine their role in grape and wine quality. It will also investigate how changes in bunch light exposure at different stages in grape berry development influences the levels of these flavonoids in grapes. The research involves viticultural trials in the vineyard and analysis of anthocyanins, flavonols and tannins in grapes to determine the effects of fruit composition on wine quality. Lastly, with a focus on the light induced expression of flavonol biosynthesis in grapevines, the project sought to investigate the molecular mechanism of flavonol transcriptional control. Thus in summation, the three key aims (and corresponding relevant questions) addressed in this project were:

1) To investigate the flavonoid content and composition of *Vitis vinifera* L. cv. Shiraz grapes from a warm and cool region, and determine their role in grape and wine quality

-What is the flavonoid content and composition of grapes from a warm and cool region?

-How is synthesis of anthocyanins, flavonols and tannins coordinated?

-How do the flavonoid compounds contribute to grape and wine quality?

2) To investigate the influence of bunch light exposure on the flavonoid biosynthetic pathway in *Vitis vinifera* L. cv. Shiraz and Chardonnay grapes

-Can light exposure override the developmental expression of *VvFLS1* so that flavonols are synthesized at times when they are not normally being accumulated?

3) To explore the molecular mechanisms of flavonol biosynthesis in grapevines

-What regulates flavonol biosynthesis in grapevines?

-Will regulation be similar to that in other plant species?

CHAPTER 2

General methods

2.1 Sample processing & preparation

2.1.1 Grape samples

In this investigation, grape samples (*Vitis vinifera* L.) generally consisted of 100 whole berries. These 100 berries were weighed and average berry weight calculated. Berries were dissected for skin, by gently squeezing the berry to disrupt cell wall integrity in the flesh of the berry and the contents expelled through the pedicel opening. Seeds were removed from flesh and juice with tweezers, counted, and cleaned by gently rubbing with paper towel to remove excess flesh. The remaining flesh and juice was bulked and collected for total soluble solid (TSS/^{o}Brix) determination by a hand-held refractometer (Reichert, USA). The weight of each component was determined by dividing the bulk weight of seeds or skin by berry number, and for seeds also by seed number. All material was weighed and frozen immediately in liquid nitrogen and stored at –80^{o}C, to minimise degradation and unwanted artefacts forming in samples (Dixon et al. 1995; Sambrook and Russell 2001; Vogt et al. 1994). For use in HPLC analysis and RNA extraction, frozen grape tissue samples (skin, seed, flowers, leaves) were ground to a fine powder under liquid N_2, in a pre-cooled coffee grinder (Sunbeam, Australia) (unless stated otherwise) and returned to –80°C storage.

2.1.2 Wine samples

Samples of wine (100-500 mL) were inverted (to resuspend any sediments) and three 10 mL aliquots were frozen in liquid N_2 and stored at –80^{o}C.

2.2 Analytical methods

Methods significantly modified from their published form are outlined. Buffers, solutions, reagents and media (and the suppliers there-of) used in experiments are listed in **Appendix 2A**. Such items are underlined in the text.

2.2.1 Determination of anthocyanin & flavonol content

2.2.1.1 Extraction: grape skins & wine

To extract anthocyanins and flavonols, three separate 0.1 g aliquots (replicates) of frozen sample (see **Section 2.1.1**) were extracted in 1.5 mL Eppendorf tubes with acidified methanol (1.0 mL)(Harborne and Sherratt 1958). Samples were extracted for one hour on a spin wheel (30 revolutions/min) in darkness at room temperature. Extracts were centrifuged (15 min at 13,000 x *g*) and the supernatant used for anthocyanin and flavonol determination by HPLC and UV-VIS spectrophotometric analysis. Due to the labile nature of anthocyanins in an acidic extraction solvent, 6 samples were extracted at a time and measured.

Frozen wine samples (see **Section 2.1.2**) were thawed at room temperature in the dark, and occasionally inverted to mix. 1.0mL of wine was pipetted into an Eppendorf tube and centrifuged (15 min at 13,000 x *g*). The supernatant wine was used for anthocyanin and flavonol determination by HPLC and UV-VIS spectrophotometric analysis.

2.2.1.2 High Performance Liquid Chromatography (HPLC) analysis

The method of HPLC separation for grape and wine anthocyanins and flavonols was kindly provided by Mark Downey (2002), and is also described in Bogs et al. (2005) and Takos et al. (2006). A 200 µL aliquot of sample supernatant (**Section 2.2.1.1**) was transferred to HPLC auto-sampler vials. Samples were analysed using a Hewlett Packard 1100 high-performance liquid chromatograph (HPLC) (HP-1100) with a Wakosil C18 analytical column (3 µm, 150 mm x 4.6 mm)(SGE USA) protected by an C18 guard column (SGE USA). The separation used 10% (v/v) formic acid in water (solvent A) with methanol (solvent B). The gradient of solvent B was: 0 min, 17%; 1 min, 17%; 15 min, 35%; 40 min, 37%; 42 min, 100%; 48 min, 100%; 50 min, 17%; 53 min, 17%. The gradient was run at a flow rate of 1 mL/min, at a column temerpature of 40^{o}C and a 10 µL of sample was injected. Absorbance was measured at 520 nm (anthocyanins), 353 nm (flavonols), 320 nm (hyroxycinnamaic acids) and 280 nm (total pheonolics).

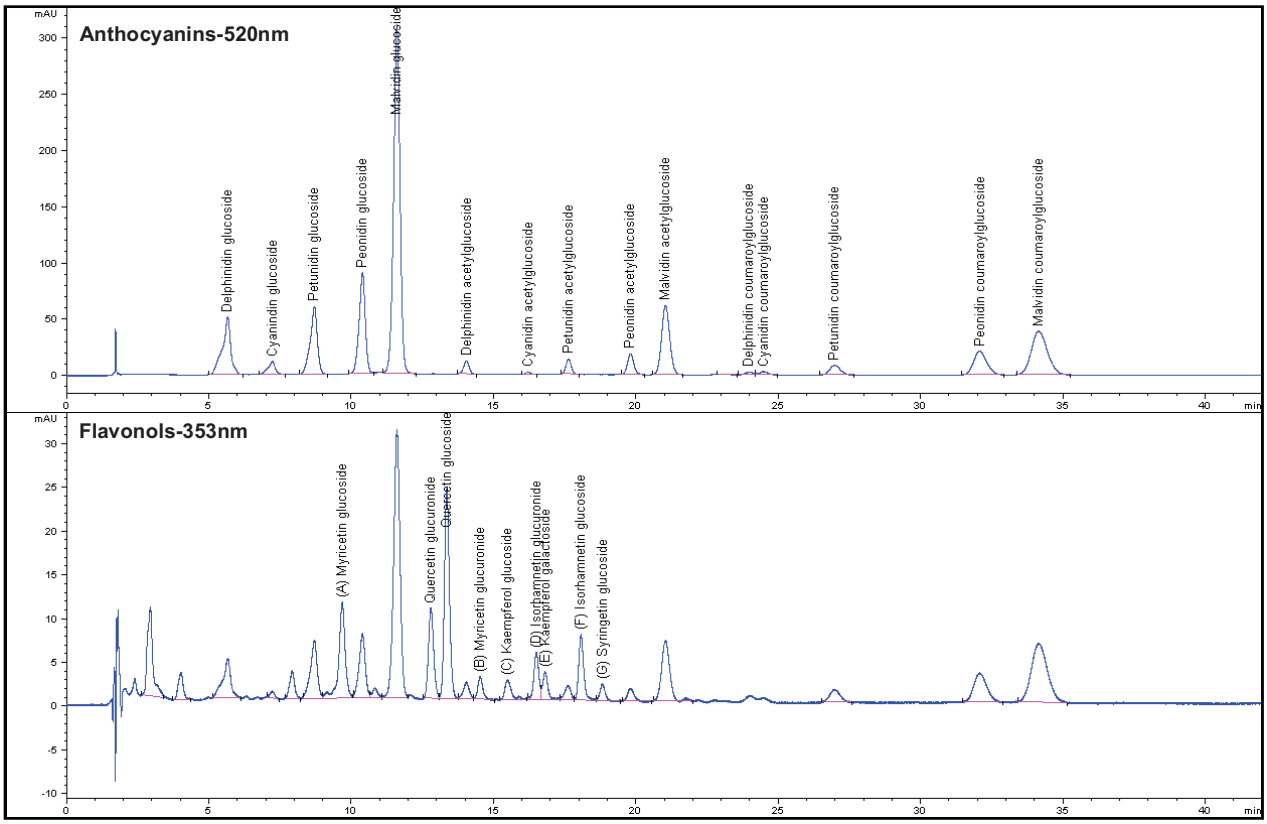
A typical HPLC separation of Shiraz skin at harvest and in wines is shown in **Appendix 2B, Figure 2B.1** and separation of anthocyanins and flavonols (alone) in Shiraz skins is shown in **Figure 2.1**. Anthocyanin and flavonol peaks were identified by three means: a) comparison of their elution order with the published separations (Cheynier and Rigaud 1986; Wulf and Nagel 1978), b) comparison of the elution time and absorbance spectra with commercial standards of malvidin-3-glucoside and quercetin-3-glucoside (Extrasynthese, France) c) LC-MS analysis (**Section 2.2.1.3**). In both grapes and wine, the anthocyanin and flavonol compounds identified were expressed as malvidin-3-glucoside and quercetin-3-glucoside equivalents respectively, based on commercial standards (Extrasynthase, France).

In Shiraz grape skin samples fifteen different anthocyanin compounds were identified. The anthocyanins comprised five anthocyanin pigments; delphinidin, cyanidin, petunidin, peonidin, and malvidin, as mono-3-glucosides, acetyl-3-glucosides and coumaryl-3-glucosides. Overall, malvidin was the predominant anthocyanin derivative, comprising ~70% of total anthocyanin content, while cyanidin was the least abundant (~2% of total). The mono-3-glucosides generally contributed ~60% of total anthocyanin content, followed by the coumaroyl-3-glucosides ~25%. Overall, the total anthocyanin content and the relative proportion of each compound reported in Shiraz skins in this investigation are similar to values reported by Downey et al. (2004).

Based on similar absorbance spectra to the flavonol standard (quercetin-3-glucoside), nine flavonol compounds were detected in Shiraz skin at commercial harvest. The peaks corresponding to quercetin-3-glucoside and quercetin-3-glucuronide were identified by comparison of the elution order with a published separation (Cheynier and Rigaud 1986; Downey et al. 2003b; Wulf and Nagel 1978). In this investigation, the quercetin glycosides were the major flavonols detected in grape tissues, and in berries skins at harvest comprised around 50% of the total flavonol content. The quercetin glycosides have been previously reported to be the predominant flavonols in numerous grape tissues in many cultivars (Castillo-Munoz et al. 2007; Cheynier and Rigaud 1986; Cortell and Kennedy 2006; Downey et al. 2003b; Price et al. 1995a). Of the remaining seven unidentified flavonol compounds, these were tentatively labelled flavonols A-G, until analysis by LC-MS analysis (see **Table 2.1**). While the majority of these unidentified peaks were only present in minor amounts, those which contributed significantly to total flavonol content in Shiraz skins at harvest included myricetin-3-glucoside (~25%) and isorhamnetin-3-glucoside (~15%).

In wines, a similar pattern of anthocyanin and flavonol separation was observed to that in grapes (see **Appendix 2B, Figure 2B.2**), however, the relative proportion of these compounds (as a % of totals) was different to that observed in grapes. For wine anthocyanins, there was a higher relative proportion of mono-3-glucosides (~70%) and a lower proportion of coumaroyl-3-glycosides (~10%). It has been previously suggested that the non-acetylated anthocyanins are more readily extracted from the fruit than the coumaroyl-3-glucosides (Leone et al. 1984; Roggero et al. 1984). It should also be noted that for anthocyanin separation of wines a large peak at the end of the chromatogram was measured and identified as 'pigmented polymers'. While pigmented polymers have previously been reported in red wines and shown to contribute to wine colour (Boulton 2001; Harbertson and Spayd 2006; Hayaska and Kennedy 2003; Peng et al. 2001) this peak was not included in the measure of total wine anthocyanins reported in the results (unless stated otherwise).

Figure 2.1 Typical HPLC separation of anthocyanins & flavonols in Shiraz skin at commercial harvest. Anthocyanins were measured at 520nm & flavonols at 353nm. Fifteen anthocyanin compounds were identified (as noted for each peak). Nine flavonol compounds were detected where the predominant flavonols, quercetin glucoside & quercetin glucuronide were identified. The remaining seven unidentified flavonol compounds were tentatively labelled flavonols A-G, until LC-MS analysis (see **Section 2.2.1.3**) identified these compound (as noted for each peak).



The proportion of the quercetin-3-glycosides (as a % of totals) in wines was reduced compared to that in the grapes suggested to be due to the presence of the flavonol aglycone quercetin, which was

detected in wines. There was also a reduction in the proportion of myricetin-3-glucoside and isohamertin-3-glucoside in wines compared to grapes. Isorhamnetin-3-glucuronide and kaemperforol-3-galactoside were not detected in wines, however the flavonol aglycone, kaempferol and three other unknown compounds tentatively labelled Flavonols A1, A2 and H were detected (see **Appendix 2B, Figure 2B.1**). These unknown wine flavonol compounds have not had their identity confirmed. The flavonol aglycones, quercetin, myricetin and kaempferol have been previously reported in commercial wines from a variety of cultivars (Castillo-Munoz et al. 2007; Goldberg et al. 1998; McDonald et al. 1998), and it has been suggested that the flavonol aglycones are produced by acid hydrolysis of the flavonol glycosides during the winemaking process and/or wine aging (Castillo-Munoz et al. 2007; Price et al. 1995a; Price et al. 1995b).

Two types of hydroxycinnamaic acids were tentatively identified in grapes and wines based on their similar absorbance spectra with commercial standards of caffeic acid and *p*-coumaric acid, which were used to quantitate these peaks (Sigma-Aldrich, USA). The hydroxycinnamates caftaric acid (referred to as *trans*-caffeoyltartaric acid (i.e. caffeic acid)) and coutaric acid (referred to as *trans*-p-coumaroyltartaric acid (i.e. coumaric acid)) are located in the skin and pulp of grape berries and have been shown to be in highest amounts in ripe fruit (Adams 2006; Romeyer et al. 1983). The concentration of hydroxycinnamates, in relation to the other flavonoid compounds (anthocyanins and tannins) is relatively low (Adams 2006; Kennedy et al. 2006a) and as a consequence these compounds are not discussed in detail, although where significant correlations are observed these are noted.

2.2.1.3 Liquid Chromatography-Mass Spectrometry (LC-MS) analysis

To confirm and identify anthocyanin and flavonol peaks in the HPLC chromatogram, LC-MS was performed with the kind assistance of Yoji Hayasaka and Gale Baldock (AWRI, Australia; (Hayasaka et al. 2005)). Shiraz skin was extracted according to **Section 2.2.1.1** and the method for LC-MS analysis was essentially the same as HPLC analysis. However, differences that were applied to the LC-MS method included: the column was Synergy Hydro C18 analytical column (3 µm, 150 mm x 250 mm)(Phenomenex, USA) and the eluent from the LC was split by the use of a T piece and delivered at 25% of the total flow to the mass spectrometer (MS) and 75% to the UV-detector (HP-1100). Both positive and negative ion mass spectra were measured for the analysis of anthocyanins and flavonols, respectively. The mass spectrum for each anthocyanin (+ mode) and flavonol (–ve mode) peak was recorded as the total mass as well as the ionisation products corresponding to the aglycone mass. This enabled the mass of the sugar fragment to be calculated

(i.e. total mass-aglycone mass). The identity of anthocyanin peaks was confirmed by comparison of their mass spectral data with published data (Krammerer et al. 2004) (data not shown).

Flavonol peaks had their identity confirmed in samples after corresponding published masses, as shown in **Table 2.1**. The identity of the most abundant flavonols, quercetin glucoside and quercetin glucuronide was confirmed by LC-MS analysis. Based on the LC-MS analysis, flavonol A was myricetin glucoside, flavonol B: myricetin glucuronide, flavonol C: kaempferol glucoside, flavonol D: isorhamnetin-3-glucuronide, flavonol E: kaempferol galactoside, flavonol F: isorhamnetin glucoside and flavonol G: syringetin-3-glucoside. The flavonols, myricetin, quercetin, kaempferol and isorhamnetin have been previously reported present in grape berries, leaves and stems as glycosides, commonly glucosides and glucuronides in variety of different cultivars (Cantos et al. 2002; Cheynier and Rigaud 1986; Cho et al. 2004; Downey et al. 2003b; Hmamouchi et al. 1996; Krammerer et al. 2004; Price et al. 1995a; Souquet et al. 2000). However only recently have the glycosides of the flavonol compound, syringetin (a derivative of myricetin) been identified in red grapes and wines (Castillo-Munoz et al. 2007; Mattivi et al. 2006).

Table 2.1 LC-MS analysis of flavonols in Shiraz grape skins. Headings include: Peak letter (tentative HPLC assignment), LC-MS retention time (RT), LC-MS total mass & LC-MS aglycone mass. The sugar mass was calculated by subtracting the aglycone mass from total mass. Flavonol compounds were identified based on corresponding masses with published data from key grapevine references.

Peak letter	LC-MS RT (min)	LC-MS Total Mass (m/z^-)	LC-MS Aglycone Mass (m/z^-)	Calculated Sugar Mass (m/z^-)	Identified Compound	References
A	10.75	479	317	162	Myricetin-3-glucoside	(Castillo-Munoz et al. 2007; Cheynier & Rigaud 1986; Cho et al. 2004)
Q-Gr	11.39	477	301	176	Quercetin-3-glucuronide	(Castillo-Munoz et al. 2007; Cheynier & Rigaud 1986; Downey et al. 2003b; Krammerer et al. 2004)
Q-Gs	11.98	463	301	162	Quercetin-3-glucoside	(Castillo-Munoz et al. 2007; Cheynier & Rigaud 1986; Cho et al. 2004; Downey et al. 2003b; Krammerer et al. 2004)
B	13.11	493	317	176	Myricetin-3-glucuronide	(Castillo-Munoz et al. 2007; Cheynier & Rigaud 1986)
C	14.02	447	285	162	Kaempferol-3-glucoside	(Castillo-Munoz et al. 2007; Krammerer et al. 2004)
D	15.05	493	331	176	Isorhamnetin-3-glucuronide	(Castillo-Munoz et al. 2007)
E	15.40	447	285	162	Kaempferol-3-galactoside	(Cantos et al. 2002; Castillo-Munoz et al. 2007; Cheynier & Rigaud 1986)
F	16.58	477	315	162	Isorhamnetin-3-glucoside	(Cantos et al. 2002; Cheynier & Rigaud 1986; Krammerer et al. 2004)
G	17.40	507	345	162	Syringetin-3-glucoside	(Castillo-Munoz et al. 2007; Mattivi et al. 2006)

2.2.1.4 UV-VIS Spectrophotometric analysis

In addition to HPLC analysis, samples were measured for anthocyanins and phenolic content on the UV-VIS spectrophotometer, based on the methods described by Iland et al. (2000). The grape sample supernatant (from HPLC analysis see **Section 2.2.1.1**) was diluted 1:20 with acidulated methanol, and transferred to a 1.0 mL quartz cuvette. Wine samples were diluted 1:40 with acidulated methanol. Using acidulated methanol as a reference, the absorbance of grape and wine samples were measured at 520 nm and 280 nm on the UV-VIS spectrophotometer (Shimadzu-160, Japan). In 2004, wine samples were also analysed for colour (and other wine parameters; inc. density, hue and phenolics) according to the methods by Bakker (1986) and Somers and Evans (1977).

2.2.2 Determination of tannin content

Two methods were employed for tannin analysis of samples. The first developed by Kennedy and Jones (2001) measures tannin composition in skin and seeds (after 70% aqueous acetone extraction) and involves the acid-catalysed cleavage of the proanthocyanidins (into individual subunits) and the subsequent attachment of a phloroglucinol moiety, allowing detection by HPLC. For the purpose of this investigation this method is referred to as phloroglucinol analysis (i.e. PGA). The second method is based on the protein precipitation assay developed by Harbertson et al. (2002), which measures total tannin content in whole berries (after model wine extraction) following precipitation by BSA and detection by a UV-VIS spectrophotometer (Shimadzu-160, Japan). For the purpose of this investigation this method is referred to as protein precipitation analysis (i.e. PPA). These methods, as described by Downey et al. (2003a) and Downey and Adams (2005) are detailed below, together with significant changes/modifications made for this investigation.

2.2.2.1 HPLC analysis of tannin composition following acid-catalysis in the presence of excess phloroglucinol (i.e. Phloroglucinol Analysis (PGA))

2.2.2.1.1 Extraction

To extract proanthocyanidins, three separate 0.1 g aliquots (replicates) of frozen sample (see **Section 2.1.1**) were extracted in darkness in 2 mL screw-top Eppendorf tubes for 24 hours with 70% aqueous acetone extraction solvent (1.0 mL)(Kallithraka et al. 1995).

2.2.2.1.2 Acid-cleavage in the presence of phloroglucinol

Samples were centrifuged (15 min at 13,000 x *g*) and two 200 µL aliquots of the supernatant of each replicate were transferred to fresh tubes and dried down under vacuum at 35°C for 60 min. One of these aliquots was redissolved in 100 µL acidulated methanol then neutralised with 100 µL sodium acetate for the analysis of free monomers. The other aliquot underwent acid-catalysed cleavage of the proanthocyanidins in the presence of excess phloroglucinol following the method of Kennedy and Jones (2001). Briefly, the dried sample was redissolved in 100 µL of phloroglucinol buffer and incubated at 50°C for 20 min, then neutralised with 100 µL of sodium acetate and centrifuged (15 min at 13,000 x *g*). 200 µL of the supernatant was then transferred to 250 µL volume HPLC autosampler vials.

2.2.2.1.3 HPLC separation

Samples were run on HPLC (HP-1100) with a LiCrospher C18 analytical column (5 µm, 250 mm × 4 mm)(Merck, Germany) protected by a C18 guard column (SGE USA). A separate reversed-phase HPLC method was developed for the cleaved and uncleaved proanthocyanidin samples. Uncleaved samples: solvent A, 0.2% (v/v) phosphoric acid, solvent B, 4:1 acetonitrile:0.2% (v/v) phosphoric acid (gradient of solvent B: 0 min, 0%; 5 min, 10%; 40 min, 10%; 55 min, 17%; 65 min, 19%; 75 min, 19%; 80 min, 100%; 85 min, 100%; 86 min, 0%). Cleaved samples: solvent A, 0.2% (v/v) acetic acid, solvent B, methanol (gradient of solvent B: 0 min, 1%; 40 min, 1%; 120 min 30%; 120.1 min, 100%; 125 min, 100%; 126 min, 1%). For both methods, 25 µL of sample was injected and run at 25°C with a flow rate of 1 mL/min. Concentrations of free monomers and hydrolysed terminal subunits were determined from standard curves prepared from commercial standards of (+) catechin, (-) epicatechin and (-) epicatechin-3-O-gallate (Extrasynthese, France). The concentration of extension subunit-phloroglucinol adducts was calculated from published molar extinction coefficients (Kennedy and Jones 2001). In this investigation the analysis of free monomers was not performed. This is due to the fact that previous reports show free monomers comprise of approximately 5% of the total tannins detected in Shiraz berries at harvest (Downey et al. 2003a).

2.2.2.2 Total tannin determination following protein precipitation & spectrophotometric detection (i.e. Protein Precipitation Analysis (PPA))

2.2.2.2.1 Extraction

For extraction of total tannins, three separate 1.0 g aliquots (replicates) of frozen berry homogenate was weighed out into a 15 mL Falcon tube and 10 mL of wine-like solution (Buffer A) added. This was incubated at room temperature for 20 min with occasional mixing and centrifuged at maximum speed for 10 min. Transfer 500 µL of the supernatant to an fresh Eppendorf tube. It is worth noting that initial modifications made to this extraction protocol (for which experimental data is not shown) include:

a) To reduce the large volumes and material required in extraction, 100 mg was extracted in 1 mL of model wine in a 1.5 mL Eppendorf tube.
b) To improve extraction efficiency, samples were sonicated (in extraction solvent) for 20 min.
c) To accommodate for finely ground seed and skin samples (see **Section 2.1.1**), the method was developed to measure these tissue types. Skin tannins were assayed according to the protocol however, seed tannin extracts were diluted 4-fold with model wine, prior to addition into the PPA.

2.2.2.2.2 Protein precipitation and spectrophotometric detection

Add 1.0 mL of protein solution to the Eppendorf tube and incubate at room temperature for 15 min with occasional inversion. Centrifuge (5 min at 13,000 x *g*) to form a pellet and carefully remove the supernatant (discard). Add 500 µL of washing buffer (Buffer B) and gently invert several times to rinse pellet. Centrifuge (5 min at 13,000 x *g*) and remove supernatant (discard). Add 875 µL of resuspension buffer (Buffer C) to the tube with the pellet and incubate at room temperature for 15 min without mixing or inverting. After 15 min resuspend pellet using a vortex mixer and allow to stand for 10 min at room temperature. Transfer the resuspended pellet to a disposable cuvette (10 mm pathlength). Zero spectrophotometer with 875 mL of Buffer C plus 125 mL ferric chloride reagent. Read absorbance at 510 nm (Background Absorbance). Add 125 mL ferric chloride reagent to the resuspended pellet and incubate for 10 min at room temperature. Read absorbance on spectrophotometer at 510 nm (Final Absorbance). Total tannins calculated by:

Absorbance due to Tannin = Final Absorbance - Background Absorbance

Total tannin (catechin eg. mg/g berries) = Absorbance due to tannin × 3.656

2.3 Detailed molecular methods

Methods significantly modified from their published form are outlined. Molecular methods used in this chapter are essentially as described by Sambrook and Russell (2001) or according to the manufacturer's instructions. Buffers, solutions, reagents and media (and the suppliers there-of) used in experiments are listed in **Appendix 2A**. Such items are underlined in the text.

2.3.1 Primer design

All primers generated were designed using the Primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) and synthesized by Invitrogen (USA). Standard primer design parameters for RT-PCR experiments included, primer size 18-22 bp and Tm 57-60^{o}C and regions amplified ranging from 150-250 bp. All primers used are listed in **Appendix 2C**.

2.3.2 Polymerase Chain Reaction (PCR)

Typical components of a PCR reaction (50 µL) were as follows: 1X PCR Buffer (1.5 mM $MgCl_2$ as supplied), 0.2 mM of each dNTP (Invitrogen, USA), 10 mM of forward and reverse primers (**Appendix 2C, Table 2C.4**), 1U of Taq DNA polymerase and DNA (2 µL of cDNA, 10 ng of DNA). A variety of Taq DNA Polymerases were used including: *Platinum, High Fidelity, Pfu* and *Pfx* (Invitrogen, USA) and *PicoMaxx* (Integrated Sciences, Australia). Reactions were performed in a PCR thermocycler (Perkin Elmer-2400, USA) and standard cycling parameters consisted of: 1 min at 95°C (one cycle); 30 s at 95°C, 30 s at 55-58°C, 1 min at 72°C (25-35 cycles); 10 min at 72°C (1 cycle).

2.3.3 Agarose gel electrophoresis of DNA

Biorad EasyCast horizontal minigel tanks (OWL Scientific, UK) were used for electrophoresis of DNA. Agarose gels, 0.8-1.2% (w/v) were prepared with TBE buffer and contained 0.5 µg mL (w/v) ethidium bromide. Before application to wells, DNA loading dye was added to each sample to a final concentration of 2X. 1 Kb molecular weight markers (Promega, USA) were also loaded and gels were electrophoresed at approximately 100 V in TBE running buffer before being visualized and photographed using a short wavelength UV transilluminator. The size of the DNA isolated was estimated from the mobility of the bands compared to a molecular weight marker.

2.3.4 Purification & quantifying DNA

DNA was purified by agarose gel electrophoresis (run with TAE buffer), and bands of interest were cut from the agarose gel, using a sterile scalpel blade under UV light. Following this, the QIAquick gel Extraction DNA purification Kit (Qiagen, USA) was used to further purify DNA. After integrity of DNA samples was checked by agarose gel electrophoresis, products were quantified on a UV-VIS spectrophotometer (Nanodrop Technologies, USA).

2.3.5 DNA ligation

For sequencing reactions, purified DNA was ligated into the T-tailed vectors pDRIVE (Qiagen, USA) using the PCR Cloning Kit (Qiagen, USA) according to the manufacturer's instructions. Other standard ligation reactions (into vectors, pLUC/pART) consisted of 1X Ligation Buffer (Promega, USA), 1U of T4 DNA ligase and DNA (at a molar ratio of $_{vector}$ 1:3 $_{insert}$) in a 10 μL reaction volume. Reactions were incubated overnight at 16°C and stored at -20ºC.

2.3.6 Transformation of bacteria & overnight cultures

Electro-competent *E. coli* XL1-Blue cells (laboratory stock) were transformed by electroporation using a Gene-Pulser apparatus (Bio-Rad, USA). 1 μL ligation reaction (~10 ng of plasmid) was mixed with a 50 μL aliquot of cells and transferred to an ice-cold electroporation cuvette (path length 1 mm). This was then given a single pulse (1.8 kV, 125 μFD, 200 Ohms), and immediately resuspended in 100 μL of SOC media. Transformed cells were spread on LB agar plates with appropriate antibiotic selection (Ampicillin/XGal) and incubated at 37°C overnight.

Single bacterial colonies were inoculated into 5 mL of LB media (selective) in 5 mL Falcon tubes and incubated overnight at 37ºC overnight, shaking at 225 rpm. Plasmid DNA was purified from cells using QIAprep Spin Miniprep Kit (Qiagen, USA).

2.3.7 Restriction enzyme (RE) digestion of DNA

To check the success of transformation and to isolate fragments from vectors, DNA was digested with various restriction endonucleases (Quantum Scientific, Australia; Roche Diagnostics, USA). A standard restriction enzyme digestion reaction contained 500-1000 ng of DNA, 1X Reaction Buffer and 1U restriction enzyme(s). Digestions were performed at 37ºC for 3 hr, and stopped by heat deactivation at 50^{o}C for 5 min. All restriction enzyme reactions were separated on 1% (w/v) agarose gels.

2.3.8 Sequencing & sequence analysis

DNA samples (as single PCR products or contained in vectors) were sent for sequencing at AGRF (Australian Genome Research Facility, Australia). Samples were prepared in separate Eppendorf tubes consisting of 1 µg of DNA, 6.4 pmoles of primers, in a total reaction volume 8 µL. Sequencing accuracy was verified by sequencing separate clones.

Resulting sequences were opened in Chromas program (http://www.technelysium.com.au) and imported to Clone Manager 5 (Sci Ed Software, USA), for sequence storage and simple sequence manipulations (determination of ORF, translation into protein sequence, inversion of sequence, RE site searching). DNA sequences were analysed using Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST/) served at the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/). Multiple sequence alignments (DNA and protein) were displayed by ClustalW Alignment program (http://www.ebi.ac.uk/clustralW/) (Thompson et al. 1994) and further manipulated using GeneDoc Display program (http://www.psc.edu/biomed/genedoc).

2.3.9 Preparation of total grape RNA

Total RNA was isolated from grape tissues using a sodium perchlorate method by Rezaian and Krake (1987) with modifications. Finely ground plant material (100-500 mg) was added to 5 mL of RNA extraction buffer 1 and mixed by shaking at 200 rpm on an orbital shaker at room temperature for 30 min. The homogenate was then passed through a separation apparatus consisting of a 20 mL disposable syringe plugged with approximately 2 cm^3 of silicon-treated glass wool (Alltech, Australia) wrapped in Miracloth (Calbiochem, Australia) and collected in a 50 mL Falcon tube. 5 mL of RNA extraction buffer 2 was added to the separation apparatus and passed thought the syringe, as before. The combined eluate was mixed with 2.5 vol of cold 100% (v/v) ethanol and stored at -20°C for 3-15 hr. The precipitate was pelleted via centrifugation (20 min at 10,000 x *g*, 4°C) and the resulting pellet was washed twice in 70% (v/v) cold aqueous ethanol, between centrifuging (5 min at 10,000 x *g*). The pellet was dried at room temperature for 20 min and re-suspended in 100 µL Rnase free water. Crude RNA samples were further purified and DNase treated using an RNeasy Mini Kit (Qiagen, USA) and an RNase-Free DNase Set (Qiagen, USA) according to the manufacturer's instructions. RNA was eluted in 60 µL of RNase free water and stored at –80°C.

Electrophoresis of RNA was essentially the same as that described for DNA except RNA loading dye was added and each sample was heated at 65°C for 5 min and then chilled on ice for 2 min before loading into wells. After the integrity of RNA was checked by agarose gel electrophoresis (see **Section 2.3.3**), RNA products were quantified using UV-VIS spectrophotometer (Nanodrop Technologies, USA).

2.3.10 First-strand cDNA synthesis

First strand cDNA synthesis was carried out from RNA (2μg) in a PCR thermocycler (Perkin Elmer-2400, USA), using SUPERSCRIPT II reverse transcriptase and Oligo(dT)$_{10\text{-}18}$ primer (Invitrogen, USA), following the manufactures protocol. Reagents required for the reverse transcription reactions were made as a master mix and aliquoted into each reaction in a single step to avoid pipetting errors that may have affected the efficiency of the reactions in different samples. The 20 μl cDNA reactions were diluted 1:1 with water and stored at –20^{o}C. Before use in RT-PCR experiments, cDNA reactions were further diluted by 20-fold.

2.3.11 Real Time PCR (RT-PCR) analysis of gene expression

Gene expression was measured by Real Time PCR (RT-PCR) on a Rotor-Gene^{TM} 2000 Real-Time PCR thermal cycler, using a SYBR green dye to quantitate PCR products (Corbett Research, Australia). Reactions were run in triplicate and each 15 μL reaction contained: 333 nM of each primer, 5 μL of diluted cDNA, 1 x Absolute^{TM} QPCR SYBR$^{®}$ Green ROX Mix (Integrated Sciences, Australia) and water. The thermal cycling conditions used were 95°C for 15 min followed by 40 cycles of: 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s, followed by a melt cycle of 1°C increments from 55 to 96°C.

RT-PCR primer sequences are listed in **Appendix 2C (**see **Table 2C.1, Table 2C.2** & **Table 2C.3)**. All primer pairs used in RT-PCR analyses amplified a single product of the expected size and sequence, which was confirmed by melt-curve analysis, agarose gel electrophoresis and DNA sequencing. The efficiency of primers was tested with dilutions of the purified PCR products, maintaining a R^2 value of $\geq$0.98. All samples were measured in triplicate and the expression of grapevine genes were normalised to the expression of *VvUbiquitin1* ((TC32075), abbreviated *VvUBIQ1*), and therefore has no units. In each experiment expression of *VvUBIQ1* remained constant during the sampling period (data not shown).

CHAPTER 3

Flavonoid composition of Shiraz grapes from a warm & cool climate region & their role in grape & wine quality

3.1 Introduction

The grape flavonoids include anthocyanins, tannins and flavonols and most contribute to grape and wine quality by influencing the colour and mouthfeel of red wine. Anthocyanins are the pigmented compounds responsible for the colour of red wine and grapes (Ribereau-Gayon and Glories 1986). Tannins contribute mouthfeel and astringency to red wines, as well as colour stability by forming complexes with anthocyanins (Gawel et al. 1998; Malien-Aubert et al. 2001; Mateus et al. 2002b; Vidal et al. 2002). The exact role of flavonols in grapes and wine is uncertain. In grapes, they may act as UV protectants (Jordan 2004; Smith and Markham 1998) and although colourless are thought to contribute to wine colour as copigments for the anthocyanins (Asen et al. 1972; Scheffeldt and Hrazdina 1978). Of the three classes of flavonoids, tannins are present in the greatest proportion in grapes and wine, followed by anthocyanins, with flavonols being present at relatively low levels (Souquet et al. 1996).

These compounds are synthesised via the flavonoid biosynthetic pathway (see **Chapter 1, Figure 1.3**). This pathway consists of several steps that are commonly regarded as the main route to the synthesis of anthocyanins. Also produced as branch point products from the anthocyanin biosynthetic pathway, are tannins and flavonols. It has been demonstrated that these three classes of flavonoids are synthesized in different parts of the berry and during different stages of berry development (Robinson and Davies 2000). For example in Shiraz, anthocyanins are synthesised in berry skins after veraison while flavonols, also synthesised in berry skins, accumulate early in development (around flowering) and later in development (during ripening) (Boss et al. 1996b; Downey et al. 2003b). Tannins are synthesised in both the skin and seed and synthesis primarily occurs between flowering and veraison (Downey et al. 2003a).

There are also strong environmental and viticultural influences on the flavonoid pathway, including light, temperature, soil type, water, nutritional status and canopy management (see reviews Downey et al. (2006) and Jackson and Lombard (1993), which may influence the flavonoid content and composition of grapes. Considering that the majority of these characteristics (i.e. soil type, irrigation, nutrition) are by and large invariant for a given site, the greatest influence is season, predominantly climate (sunlight and temperature).

Research into the effect of climate on grapevines indicates a strong relationship between grape colour, wine colour and wine quality from fruit grown in warm vineyards, where as anthocyanin levels increase, so too does wine quality (Arozarena et al. 2002; Boulton 2001; Price et al. 1995a; Spayd et al. 2002). However, this trend may not be upheld for fruit grown in cool region vineyards, where higher anthocyanin levels do not always reflect an increase in wine quality (Mazza and Miniati 1993; Sas and Lim 2003). A schematic representation of the relationship between anthocyanins and wine quality of fruit from warm and cool vineyards is shown in **Figure 3.1**.

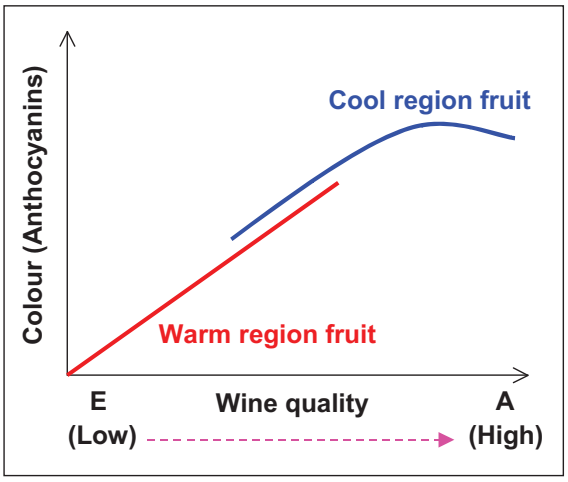


Figure 3.1 Schematic representation showing the relationship between wine quality & anthocyanins. The X-axis= wine quality, where E=low grade & A=high grade. The Y-axis= grape or wine colour (anthocyanins). In warm regions, colour fits to a linear relationship with wine grade, whereas in cool regions a colour limit is reached & an increase in colour does not increase wine grade.

Historically, grape juice characteristics have been used to predict wine quality (i.e. °Brix, pH, TA) and more recently grape colour has been used as one of the major factors in assessing grape quality and predicting wine quality (Sas and Lim 2003; Swinburn 2003) **(Figure 3.1)**. However, it is important to point out that there is no clear definition for 'quality' of grapes or wine, rather the classification of 'quality' remains largely subjective. This is primarily due to the vast array of compounds in grapes, such as acids, sugars, phenolics and aroma/flavour compounds, the complex interactions between these compounds in wines and the somewhat subjective nature of sensory evaluation. The wine industry continually seek new and improved of measurements of 'grape quality' in terms of composition factors which affect wine properties.

Therefore, with knowledge of the synthesis of the flavonoid compounds during development combined with an understanding that climatic factors can influence these compounds, areas of unknowns include:

-What is the flavonoid content and composition of grapes from a warm and cool region?

-How is synthesis of anthocyanins, flavonols and tannins coordinated?

-How do these compounds contribute to grape and wine quality?

Therefore this study aims to investigate the products of the flavonoid biosynthesis pathway (anthocyanins, flavonols and tannins) in *Vitis vinifera* L. cv. Shiraz from two climatic regions (warm and cool) and determine their role in red wine quality.

3.1.1 Background information

In collaboration with the Hardy Wine Company (HWC), Shiraz grape samples were provided from two climatic regions, the Riverland (warm) and McLaren Vale (cool) in two seasons, 2003 and 2004. These two regions were selected by HWC for investigation as they represented two viticultural regions which were historically distinct from each other in relation to different climate and site characteristics. Background information regarding key differences in climate, culture and production for these regions are described below and are summarised in **Table 3A.1** (in **Appendix 3A**). A South Australian map showing the location of these two regions is also shown in **Appendix 3A**.

3.1.1.1 Regional descriptions

3.1.1.1.1 Riverland

The Riverland is one of Australia's most productive wine grape growing regions. It consists of a number of horticultural regions, including Waikerie, Barmera, Berri, Loxton and Renmark. The central location (Berri) is located 200 Km from Adelaide and is situated inland, on the edge of the River Murray. As the region is inland, the climate predominantly consists of high summer temperatures with low summer rainfall (Dry et al. 2004b). The Mean January Temperature (MJT) is 23.2^{o}C (Bureau of Meteorology 2005) and for the purpose of this investigation is referred to as a 'warm' climate. Grapevines were first established in the region in the 1890s, primarily for the dried fruit market. However in the 1970s in response to the demand for more table wines, there was rapid planting of grapevines.

The Riverland produces approximately 25% of the total grapes crushed nationally and 60% of the total grapes crushed in South Australia (Grape and Wine Research Development Corporation 2003). The region comprises of ~20,000 Ha of vineyards, with close to 370, 000 tonnes of grapes crushed, worth an estimated $213 million (Phylloxera and Grape Industry Board of South Australia 2003). The main grapevine varieties grown in the Riverland include Shiraz, Cabernet Sauvignon and Chardonnay with Shiraz making up around 25% of the total tonnes of grapes grown and crushed. Grapevines are predominately planted on own roots, either close to the rivers edge ('river flats') or on the mallee highlands. The grape vines are predominantly drip-irrigated from the River Murray, resulting in some vineyards with elevated levels of salt in the soil. Vines are generally grown on two-wire vertical trellis with sprawling canopies that require little shoot positioning. More than 90% of the region is mechanically pruned and harvested. Typical Shiraz yields range from 10-25 T/Ha (Dry et al. 2004b).

Wines made at HWC Berri Estates Winery are predominantly fermented in large batches, in closed stainless steel fermenting vessels. Oak chips are generally added to wines (contained in large vessels), and in most cases, wines are available to consumers within 12 months of harvest. The Riverland has a reputation for producing good quality wine grapes used for premium bottled (~$20/bottle) and bulk wine (<$5/litre) sales, the majority of which is marketed overseas.

3.1.1.1.2 McLaren Vale

McLaren Vale is a small wine grape producing region located 40 Km south of Adelaide and approximately 6 Km inland of the Southern ocean. The climate is characterised by warm summers with cool afternoon sea breezes, moderate winters and winter dominant rainfall (**Table 3A.1)**(Dry et al. 2004b). The estimated MJT for this region is 21.4^{o}C (Sas and Lim 2003), and for the purpose of this investigation is referred to as a 'cool' climate. The first grapevines were planted in this region in 1836 primarily for fortified wines, however it was not until the 1960s that the region expanded with major grapevine planting for table wine production.

The region comprises of ~6,500 Ha of vineyards, with close to 45,000 tonnes of grapes crushed worth an estimated $70 million. As a proportion of South Australia, McLaren Vale generally contributes around 10% of the total vineyard area, tonnes crushed and crush value (Phylloxera and Grape Industry Board of South Australia 2003). The main grapevine varieties grown in McLaren Vale include Shiraz, Cabernet Sauvignon and Chardonnay. Shiraz makes up ~45% of the grapes

grown and crushed in the region, worth an estimated 50% of the total crush value in this region. Most grapevines are planted on own roots, on flat, undulating land with soil-types varying from restrictive clays to free draining sands. Generally vines are drip irrigated and grown on two-wire vertical trellis with either sprawling or vertical shoot positioning (VSP) canopies. Both mechanical and hand pruning and harvesting are used and Shiraz yields range from 5-15 T/Ha (Dry et al. 2004b).

Wines made at McLaren Vale's HWC Tintara Winery are predominantly fermented in small-scale batches in both open and closed fermentation vessels, made from either stainless steel or cement. The majority of wines are aged in oak barrels and for the higher quality wines can take up to 36 months before the product reaches consumers. Typically, McLaren Vale has a reputation for producing premium and super premium quality wines, ranging from $15/bottle to $100/bottle (retail price).

3.1.1.2 Differences in grape sampling

Due to the differences in production and processing between the two regions, an understanding of HWC berry sampling procedures is essential for the interpretation of the results. A schematic representation of the grape sampling process in the both regions is shown in **Figure 3.2**.

In the warm region (Riverland) berry samples are taken at three different times during berry ripening and are measured for berry weight, ^{o}Brix, and colour (by near-infared spectroscopy (NIRS)). The vineyard grower provides 3 berry samples to the winery for analysis, when the fruit is at 18, 20 and 22 ^{o}Brix, respectively. In addition a winery representative also performs a vineyard assessment where attributes such as vine vigour, bunch exposure and leaf health are recorded. The berry measurements (berry weight, ^{o}Brix, colour) taken during berry ripening are then used to forecast the potential harvest date, in combination with the vineyard assessment (vine vigour, bunch exposure, leaf health) to determine the 'quality' stream of the fruit. Fruit is divided into different quality streams; A (High quality) → E (Low quality) which influences wine style.

At harvest, when the bins of fruit arrive at the weighbridge, two fruit sampling devices are used. The first involves a Maselli sampler. A stainless steel pipe is placed into the core of the bin, removing a proportion of the fruit while simultaneously extracting the juice to measure ^{o}Brix, pH

and TA concurrently (see **Figure 3.3A**). The second sampling device is a YUBA sampler, a set of mechanical jaws designed to remove a 5 Kg core sample of whole fruit (see **Figure 3.3B**). This 5 Kg core sample is then deposited onto a sorting tray and a sub-sample of ~500 whole berries are hand selected. The 500 whole berry sample is then frozen (-20^{o}C), and sent to the winery's laboratory for subsequent NIRS analysis (colour, moisture, sugar). In the warm region, the harvested fruit is 'streamed' to tanks based on a pre-determined grape stream.

In the cool region (McLaren Vale), vineyards are monitored and the grower takes berry samples at 18, 20 and 22 ^{o}Brix. Berry weight, ^{o}Brix, pH, TA and colour are measured on these samples by the winery. In addition, vineyard assessment (i.e. benchmarking) data is collected such as bunches/m and shoots/m. A winemaker will also visit the vineyard to assess the quality of fruit taking into consideration the vineyard history and wine style. Tannin structure and fruit flavour ripeness are the basis for determining the harvest date and this is predominantly assessed by sensory evaluation in the vineyard. At harvest, as occurs in the warm region, random fruit samples are taken at the winery weighbridge to determine ^{o}Brix, pH and TA (Maselli analysis) and a sub-sample (YUBA sample) of whole berries is sent for NIR analysis. Fruit parcels are generally kept separate in the winery and if combining parcels is necessary, this is determined by the winemaker.

In summary, while both regions have similar methods of sampling fruit (Maselli and YUBA) different methods of streaming fruit are employed to manage the different intakes in each region. In the warm region, the quality is pre-determined and fruit is combined for fermentation. In the cool region, the winemaker has much more control over the direction of the fruit, where fruit is kept in separate batches. It is important to point out that the factors that HWC used to determine the grape stream allocations included a number of different berry and vineyard factors as well as the winemaker assessment (as discussed above).

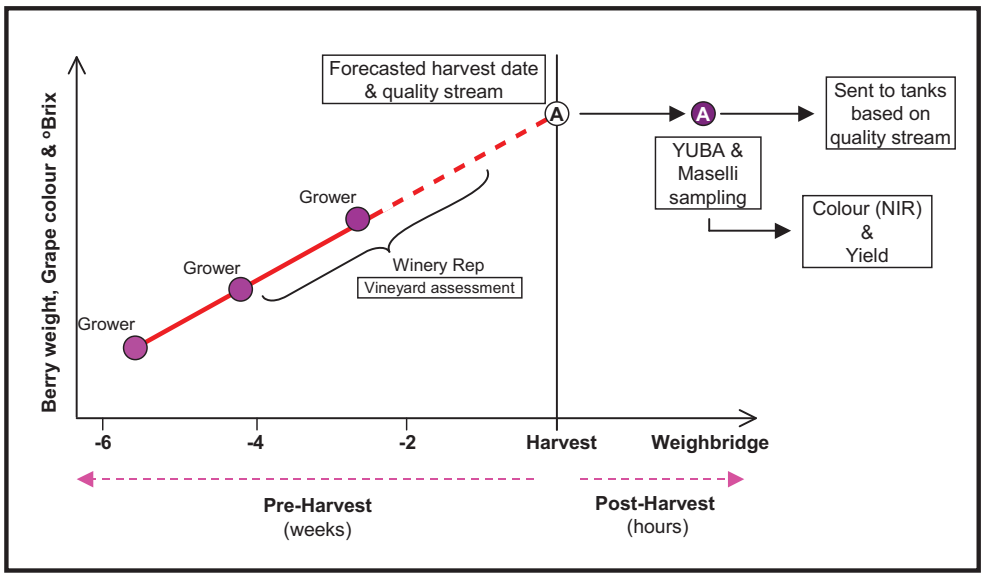


Figure 3.2 Schematic representation of the fruit streaming process in warm & cool regions. During berry ripening (pre-harvest) 3 berry samples are taken. The samples are measured for berry weight, grape colour, pH, TA & °Brix. A vineyard assessment is conducted by a winery representative within 4 weeks of harvest. In the warm region, fruit is streamed into the winery based on chemical analyses and vineyard assessment; in the cool region fruit is harvested based on vineyard sensory assessment for tannin and fruit ripeness. After fruit has been harvested & delivered to the winery weighbridge fruit is sampled by the YUBA & Maselli samplers. Fruit is then sent to fermentation tanks based on the quality stream allocation.

A Maselli Sampling

B YUBA Sampling

Figure 3.3 Maselli & YUBA sampling at the weighbridge in warm & cool regions.

A Maselli sampler. The Maselli sampler, removes fruit, extracts the juice & concurrently analyses °Brix, pH & TA.

B YUBA sampler. The YUBA is a set of mechanical jaws designed to take a fruit (~5 Kg) core sample from trucks. The fruit sample is deposited onto a sorting tray & a 500 whole berry sample is taken for NIR analysis.

3.2 Methods

3.2.1 Sample selection, collection & processing

In collaboration with the HWC, Shiraz grape samples from streams of differing fruit quality were provided from two climatic regions, warm (Riverland) and cool (McLaren Vale) in two seasons 2003 and 2004. Approximately 80 grape samples were provided each season by HWC. There were 5 differing quality streams (with ~10 samples in each stream) from the warm region and 3 differing quality streams (~10 samples in each stream) from the cool region. The vineyards selected to sample covered a broad range of small and large sites and were a mixture of both winery owned and independent grapegrower operations. Where possible, samples from the same vineyards were collected in both seasons and the selection process also ensured there was a large spread of samples in each quality stream. This provided a good representation of samples for each region. In addition, wine samples from the cool region were also provided for analysis. These samples were understood to be representative of the fermented grape samples and were taken at pressing, prior to blending, malolactic fermentation, and oak/tannin treatment.

At the weighbridge, at the time of whole berry sampling for HWC colour determination (by NIR), an extra sub-sample (~200 whole berries) was taken and kept refrigerated until collected/couriered for analysis at the CSIRO laboratory. Samples from the cool region were collected and processed within 6 hours of sampling, while samples from the warm region were packaged on ice and couriered overnight to Adelaide, processed within 12-24 hrs of sampling. On arrival of the refrigerated grape samples at the laboratory, a sub-sample of 100 whole berries was randomly taken and processed according to **Chapter 2, Section 2.1.1**.

3.2.2 Sample measurements

In both regions and seasons, grape skin samples were analysed for anthocyanin and flavonol content by HPLC as described in **Chapter 2 (Section 2.2.1)**. In 2003, skin and seed samples from McLaren Vale (not Riverland) were analysed for tannins, via phloroglucinol analysis (PGA) **(Chapter 2, Section 2.2.2.1))**. In 2004, skin and seed samples from both regions were analysed for tannins according to the protein precipitation assay (PPA)(**Chapter 2, Section 2.2.2.2**). Unfortunately in 2003, after the initial analytical measurements (anthocyanins, flavonols, tannins), samples from McLaren Vale and Riverland thawed and re-froze, over an unknown time-period due to a freezer

breakdown. Cooley et al. (in press) showed anthocyanin loss occurred when the temperature increased above 4°C during storage or defrosting of whole berries. Subsequently, no further analysis was performed on these samples.

All samples were measured in triplicate and represented in graphs are the mean values for each sample. While the standard error of the mean (±SEM) indicating the technical variation in repeated sample measurements is not shown for each sample, the average % coefficient of variation (%CV) for each chemical analysis was determined and is discussed in **Section 3.3.1**. Grape flavonoid measurements (anthocyanins, flavonols, tannins) are expressed on a per berry weight basis (mg/g berry) and wine flavonoid measurements (anthocyanins, flavonols) on a g/L basis (unless stated otherwise). Where individual flavonoid compounds were measured for a particular class of flavonoids (i.e. the HPLC measurements of anthocyanins, flavonols, tannins composition) the sum of each compound belonging to that class was determined, expressed as the total for each flavonoid class (i.e. total anthocyanins, total flavonols or total tannins). As the composition in each flavonoid class did not greatly vary, represented in graphs (below) is total of each flavonoid class (unless stated otherwise).

3.2.3 Data details

In this investigation, a 'data set' refers to all the separate measurements recorded for a particular region in one season (i.e. McLaren Vale 2003 (MV03), McLaren Vale 2004 (MV04), Riverland 2003 (RL03), Riverland 2004 (RL04)). Measurements made in this investigation, referred to as 'CSIRO data', include grape berry factors (berry weight, skin weight, seed weight, number of seeds, °Brix) and grape and wine flavonoid content and composition (anthocyanins, flavonols, tannins). Measurements provided by HWC (referred to as 'HWC data') include those made on grapes, pre-harvest (grape quality stream, vineyard assessment factors), at the weighbridge (Maselli analysis, berry weight, colour (NIR)) and on wines (wine grade, wine colour density, hue). It should also be noted that in some data sets, several measurements are missing for samples as not all measurements were provided for all samples by HWC. Thus, data sets may be incomplete for the full range of samples. In McLaren Vale, some fruit samples were fermented together (i.e. blended), therefore the wine data is identical for these grapes samples. As such, this data should be treated with caution. In this investigation, all measured variables are referred to as 'factors'. A detailed list of the factors recorded by CSIRO and HWC for each sample is provided in **Appendix 3B.** Noted in this list are those samples, which the data is incomplete and/or blended.

3.2.4 Statistical analysis

To compare the mean values and determine statistically significant differences between region and season (and the interaction between region and season), an unbalanced ANOVA was performed using the statistical package GenStat (9th Edition). Statistically significant differences were represented as p values and were used to describe the probability that an observation was not due to chance, but was a true relationship between the factors. Within this chapter, statistically significant results are denoted by *, **, ***, where $p<0.05$, $p<0.01$ and $p<0.001$, respectively. Non-significant results are denoted ns.

Data presented in this investigation was compared using standard statistical correlations. Statisticians commonly use R and R^2 values to describe the certainty of a relationship between two variables. The R-value is a relationship coefficient (Pearson's relationship) and represents the linear relationships between two variables. When R-values are squared, the resulting value (R^2) represents the proportion of the common variation in the two variables (i.e. indicates the 'strength' or 'magnitude' of the relationship). R and R^2 values close to 1 indicate there is a strong positive relationship and that two variables are positively correlated, whereas values close to -1 indicate there is a strong negative relationship and two variables are negatively correlated. R and R^2 values close to zero, indicate that there is very little relationship between two variables. In this investigation, tables of R^2 values are used to compare different variables (i.e. 'factors'). Additionally, the nature of each relationship is given as either positive or negative and R^2 values greater than 0.2 are shaded (light green) and are used as an indicator of a possible 'relationship'.

It should also be noted that the relationship between grape quality stream and wine grade was further analysed via two statistical analyses, the Spearman's P relationship coefficient and Kendall's T relationship coefficients, in the statistical package Genstat (9th Edition). While these statistical tests are considered better suited to ordinal data (i.e. ordered categorical; 1-5)(Zwart 2006), both tests in general agreed with the strength of the relationship indicated by the R^2 value.

3.3 Results

3.3.1 Grape & wine sampling

In both regions, HWC representatives performed a series of pre-harvest assessments. The pre-harvest assessments combined measurements on berry samples (i.e. berry weight, ^{o}Brix, pH, colour) and vineyard attributes (i.e. bunches/m, shoots/m, bunch exposure and leaf health), which in the warm region was used to stream fruit. It is important to highlight that the berry sample data (i.e. berry weight, ^{o}Brix, pH, colour) from the series of the pre-harvest assessments, including the final assessment was not provided by HWC. However the vineyard assessment, which is also referred to as a 'benchmarking' assessment was provided by HWC and a complete list of these factors is provided in **Appendix 3B (Table 3B.1)**. Interestingly, more quantitative measurements (i.e. bunches/m, shoots/m) were recorded in McLaren Vale vineyards, whilst subjective measurements (i.e. fruit exposure, leaf health) were recorded in Riverland vineyards.

It is important to point out that in both regions HWC decided on the grape quality stream allocation. In McLaren Vale the quality streams range from 1=high →3=low, whereas in Riverland 1=high →5=low. The actual method that HWC used to determine the grape quality stream allocation was not disclosed, however, it was indicated that different berry and vineyard factors (as noted in this investigation) as well as the winemaker's assessment were considered in allocation of grape stream. Also, the quality streams assigned to the grapes samples are independently rated in each region such that there is no link or overlapping of streams between the two regions.

At the winery weighbridge, the process of sampling whole berries was the same in both regions; the Maselli sampler measures baume, pH and TA (real time), while the YUBA sampler removes whole berries, which are deposited onto a bench and whole berries hand selected for colour analysis (delayed measurement)(see **Figure 3.3**). Both the Maselli and YUBA sampler have been shown to sample randomly and give an accurate representation of the grapes in a particular bin (Anderson 2003). The CSIRO sample was taken at this stage as a sub-sample of the YUBA sample, so essentially is a replicate of the sample normally taken by HWC for analysis.

While it was desirable to have ~10 samples in each quality stream and receive the samples from the same vineyards in both seasons, this was not always possible due to commercial constraints. Accordingly, there are an uneven number of samples in each quality stream from different vineyards

(see **Appendix 3B**). In McLaren Vale in 2004, there was one outlier sample that had significantly lower flavonoid content compared to the remaining samples. While this sample is included in all graphs, when relationships are significantly skewed by this outlier sample, this is noted.

Wine samples provided from McLaren Vale were understood to be representative of the fermented grape samples and were to be taken at pressing, prior to blending, malolactic fermentation, and oak/tannin treatment. However, wine samples received in 2003 were provided at different stages post-fermentation and some wines had been blended (see **Appendix 3B**). Therefore any relationships between grape and wine in this data set should be treated with caution.

In 2004, wine samples were collected at pressing (pre malolactic fermentation, prior to blending) and therefore this data set may be better for evaluating flavonoid content and composition from grape to wine. In both regions the wine grade allocated to each wine sample was provided by HWC. In McLaren Vale the wine grade ranged from 1=high →7=low, whereas in Riverland 1=high →5=low. Again, the wine grade assigned to the wine samples are independently rated in each region, such that there is no link or overlap of grades between the two regions.

Grapes and wine received from McLaren Vale were collected and stored at 4^{o}C. Because of the close proximity to the laboratory these samples were generally processed within 12 hr of sampling. Riverland grapes were collected and stored at 4^{o}C and couriered to the laboratory overnight, so these samples were generally stored at 4^{o}C for more than 24 hr before processing. Samples from both regions were received at a similar time in both seasons, from late February through to mid April. Due to the large volume of samples arriving in the laboratory during this time, berries were processed by a variety of people, however strict guidelines for processing the berries were set to minimise sample variation.

Of the CSIRO measurements, the berry factors (berry, skin, seed weight, ^{o}Brix) are expressed as a pooled measurement (i.e. average of 100 berries). For all chemical analyses, measurements were performed in triplicate and the coefficient of variation (%CV) was determined. The %CV is calculated by expressing the standard deviation as a % of the mean and is commonly used to indicate the variation between samples and sampling methods (Krstic et al. 2002). The %CV between replicates for anthocyanin and flavonol was less than 10%. In tannin analyses, the %CV between replicates analysed by PGA was around 10-20%, whereas for PPA it was around 10%. It should be noted that in 2003, grinding seeds with the coffee grinder, resulted in a high variation in repeated seed measurements (by PGA), associated with uneven seed particle size (i.e. seed

%CV=20% vs. skin %CV=10%). Therefore in 2004 seed samples were ground in a pre-cooled (liquid N_2) Wesch Grinder (USA), which, through centrifugal force passes ground material through a 0.5 mm filter. This reduced the %CV to around 10% (similar to skin) in repeated PGA measurements. Accordingly, with understanding of the variation between repeated sample measurements, only the mean data for each sample is plotted in graphs.

3.3.2 Analysis of tannins

In this investigation two methods were employed to measure grape seed and skins tannins, phloroglucinol analysis (PGA) as described by Kennedy and Jones (2001) and Downey et al. (2003a) and a protein precipitation assay (PPA) as described by Harbertson et al. (2002) and Downey and Adams (2005). In 2003, samples from McLaren Vale were analysed for tannins by PGA, however this method is time consuming. Therefore, in 2004 samples from McLaren Vale and Riverland were analysed for tannins by PPA. Tannins were not measured in Riverland samples in 2003, due to the fact that samples may have been compromised by a freezer breakdown. Thus, as two different methods of tannin determination were used to measure skin and seed samples, it was important to ensure they were correlative.

3.3.3.1 Comparisons of extraction solvents in the PPA

It was important (in correlative experiments) to first, adjust for the different solvents used to extract tannins. The PPA uses model wine in extractions whereas the PGA uses 70% aqueous acetone in extractions (Downey and Adams 2005; Downey et al. 2003a). Acetone (aqueous 50-70%) has been shown to be an efficacious solvent system for the extraction of total tannins and has been extensively used in the analysis of grape procyanthocyandins (Downey et al. 2003a; Kallithraka et al. 1995; Kennedy and Jones 2001; Kennedy et al. 2000a; Souquet et al. 2000). Likewise, model wine (~12% ethanol, tartaric acid) has been extensively used to extract phenolics from grape samples where it is suggested that extraction technique should mimic what potentially would be extracted under standard fermentation conditions (Iland 2001).

As 70% aqueous acetone was expected to extract higher amounts of tannins compared to model wine, it was necessary for the extraction component of the PPA to be slightly modified in order for it to operate efficiently. This was achieved by diluting samples prior to addition into the assay. Skin samples were diluted 5-fold (100 µL of sample extract: 400 µL H_20) while seed samples were diluted 10-fold (50 µL of sample extract: 50 µL 70% acetone: 400 µL H_20). For both skin and seed

samples, dilutions were performed with water rather than 70% aqueous acetone as it was decided to keep the volume of solvent to a minimum to reduce possible influences on the assay. Samples extracted by the different extraction solvents and analysed by the PPA, included skin and seed samples from McLaren Vale in 2004 and skin samples from Riverland in 2004.

The relationship between model wine extraction and 70% aqueous acetone extraction in McLaren Vale skin and seed samples in 2004 are shown in **Figure 3.4**. As expected, the concentration of tannins in seeds was higher than in skins (Souquet et al. 1996). There was a relationship between tannins extracted in model wine and in 70% aqueous acetone with R^2 values for skins R^2=0.65 and for seeds R^2=0.67 for McLaren Vale samples. The mean tannin content and standard error of the mean (±SEM) for McLaren Vale and Riverland samples extracted with 70% aqueous acetone and model wine is shown in **Table 3.1**. Clearly, 70% aqueous acetone extracted more tannins than model wine in both skin and seed samples. Skin samples from both regions, model wine extracted ~30% of that extracted in acetone, while seed samples model wine extracted ~50% of acetone.

These results confirm the merits of 70% aqueous acetone as an exhaustive extraction solvent (compared to model wine) (Downey et al. 2003a; Kallithraka et al. 1995), and also demonstrate that 70% aqueous acetone can be successfully used in the PPA, without altering the assay conditions. Subsequently, all samples were extracted in 70% aqueous acetone for use in the PPA.

Table 3.1 Mean tannin content of McLaren Vale (seed & skin) & Riverland (skin) samples in 2004 extracted with 70% aqueous acetone & model wine. Mean values are expressed mg/g berry & in brackets is the Error of the Mean (±SEM) where the number of samples in McLaren Vale was n=24 & in Riverland n=55. Calculated is the percent model wine/acetone.

	Model wine	70% Aqueous Acetone	Model wine/ Acetone %
Riverland (Skin)	0.427 (±0.016)	1.484 (±0.026)	29
McLaren Vale (Skin)	0.589 (±0.032)	1.682 (±0.077)	35
McLaren Vale (Seed)	1.190 (±0.051)	2.453 (±0.116)	49

3.3.3.2 Comparison of PPA & PGA

Once it was established that 70% aqueous acetone could be used in the PPA, it was necessary to compare the PPA to PGA. Skin and seed samples (analysed for tannins by PPA after extraction in 70% aqueous acetone (above)) were ranked in order of tannin content. Ten samples of each (skin and seed from McLaren Vale) were selected based on obtaining a large range of tannin concentrations. Samples were extracted (70% aqueous acetone) and analysed according to the PGA

(Downey et al. 2003a)**(Chapter 2, Section 2.2.2**). For both tissue types, there was a good relationship between PGA and PPA with R^2 values of 0.76 (skin) and 0.79 (seed) (**Figure 3.4B**), thus similar tannin concentrations were obtained by both methods. Slightly higher tannin values were measured by the PPA in skins, while higher tannin values were measured by the PGA in seeds. These results indicate either PGA and PPA could be used to measure skin and seed samples, and that these methods are highly correlated for measuring total tannin content in these tissue types. Recently, a correlation between PGA and PPA in red wines (R^2=0.91), and in grape skin extracts (R^2=0.62) has been reported (Downey 2007; Kennedy et al. 2006b).

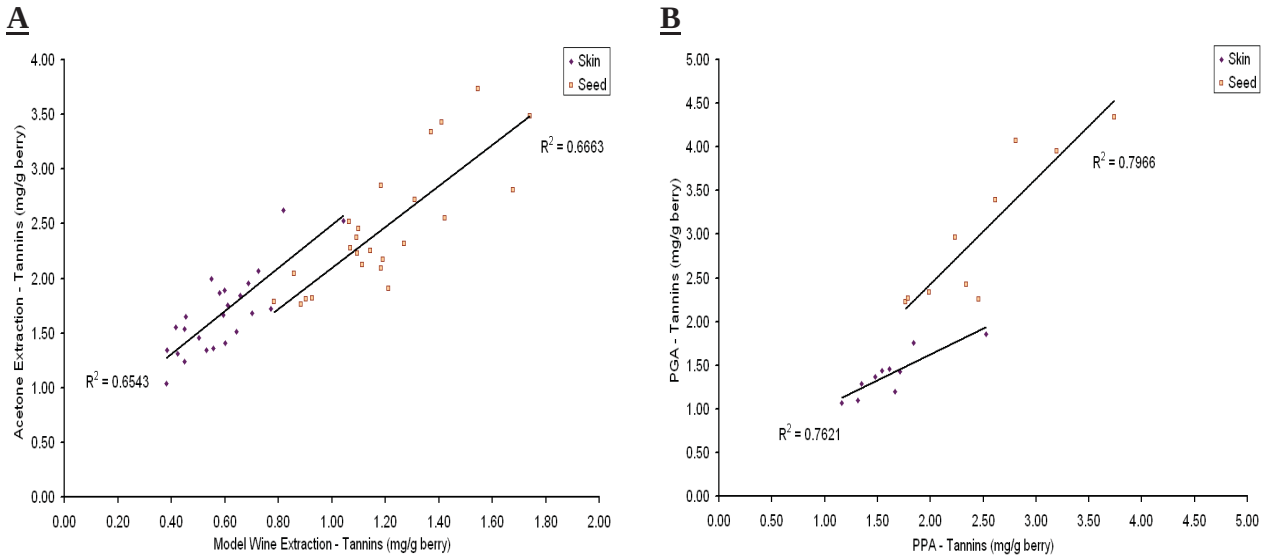


Figure 3.4 Comparison of tannin assays & extraction solvents.

A Comparison of 70% aqueous acetone v model wine extraction solvents in the PPA. Samples assayed included Shiraz skin & seed from McLaren Vale in 2004. Tannin concentration is expressed on an mg/g berry basis.

B Comparison of tannin methods; PPA v PGA (both extracted in 70% aqueous acetone). Samples assayed included a range of samples of Shiraz skin & seed from McLaren Vale in 2004. Tannin concentration is expressed on an mg/g berry basis.

3.3.3 Grape & wine phenolic measurements

The anthocyanin and flavonol content and composition of Shiraz grapes and wines was analysed by HPLC (**Chapter 2, Section 2.2.1**). In addition to HPLC analysis, total anthocyanin content was also measured by alternative methods including:

a) Absorbance of light at 520 nm on the UV-VIS spectrophotometer. The absorbance of light at 280 nm was additionally recorded and used as a measurement of total phenolics. This method is largely based on the method described by Iland et al. (2000)(see **Chapter 2, Section 2.2.2.1**)

and was performed on both grape and wine samples. For the purpose of this investigation this method is referred to as the 'Iland method'.

b) Similar to the 'Iland method' the absorbance of light at 520 nm and 280 nm on the UV-VIS spectrophotometer was measured as described by Somers (1971). This method was performed on wines only by CSIRO and HWC in 2004. For the purpose of this investigation this method is referred to as the 'Somers method'.

c) Near infrared spectroscopy (NIR). NIRS is a relatively new technology used in major wineries, (such as HWC) to measure grape and wine components including sugar/alcohol, moisture, acids and colour pigments (anthocyanins) (Gishen and Dambergs 1998).

The relationship between these different analytical measurements of anthocyanins and phenolics, performed by CSIRO and HWC, are represented by R^2 values as shown in **Table 3.2.** Also shown in **Table 3.2** is the relationship between the same measurements made by CSIRO and HWC.

In general, the relationships observed in both regions and seasons were similar. However, the relationships observed in the MV03 data set were noticeably different, the reason for which is unclear. In both regions and seasons, there was a relationship between anthocyanins measured in the grapes by HPLC and by the Iland method, with R^2 values around 0.85. This was expected as the same sample extract was used in both analyses (see **Chapter 2, Section 2.2.1.1**). There was also a relationship between grape anthocyanins measured by HPLC and by NIR with R^2 values around 0.45. This result indicates that the grape samples CSIRO received from HWC were to some extent representative of that which HWC analysed for anthocyanin content.

In McLaren Vale wines in both seasons there was a relationship between anthocyanins measured by HPLC and by Iland method with R^2 values greater than 0.88. In 2004, there was a relationship between wine anthocyanins as determined by the Somers method, the Iland method and HPLC with R^2 values greater than 0.65. These results indicate low method/technical variation and imply that any of these three methods could be used for total anthocyanin determination in red wines in future experiments. However, there was no relationship between the Somers method as measured by HWC and CSIRO, with an R^2 value of 0.035. The reason for this result is unclear, but may indicate that the wine samples analysed by CSIRO were different to that analysed by HWC, particularly in relation to anthocyanin content.

There was a strong relationship between total phenolics measured by the Iland and Somers methods in McLaren Vale wine samples (R^2 value 0.83), again indicating low method/technical variation. However, a weak relationship (R^2 value 0.38) between the Somers method of total phenolic measured by CSIRO and HWC was observed in wines, which may also indicate wine samples analysed by CSIRO were different to that analysed by HWC.

There was a good relationship between anthocyanins and phenolics in grapes and wines, as measured by Iland and Somers methods with R^2 values greater than 0.58. This relationship was also observed in wine samples measured at HWC by the Somers method, with an R^2 value of 0.57. Given that absorption of light at 280 nm is a non-specific assay, combined with the fact that anthocyanin content contributes to a large proportion of total phenolics content in grapes, these correlations were somewhat predictable (Gawel et al. 1998; Harbertson and Spayd 2006).

Table 3.2 Relationships between different measurements of anthocyanins & phenolics. All measurements were performed by CSIRO except where indicated as HWC, which were performed by Hardy Wine Company. The same measurements made by CSIRO & HWC are also compared. R^2 values highlighted in green are >0.2, & the +/- indicates whether the relationship is positive or negative. Blank cells indicate data was not available.

	R^2 Values			
	MV03	**MV04**	**RL03**	**RL04**
Anthocyanins (520nm)				
Grape				
HPLC v ILAND	+0.460	+0.978	+0.881	+0.885
HPLC v NIR (HWC)	+0.194	+0.471	+0.481	+0.329
Wine				
HPLC v ILAND	+0.928	+0.884		
HPLC v SOMERS		+0.667		
ILAND v SOMERS		+0.759		
SOMERS v SOMERS (HWC)		+0.035		
Phenolics (280nm)				
Wine				
ILAND v SOMERS		+0.830		
SOMERS v SOMERS (HWC)		+0.380		
Anthocyanins v Phenolics				
Grape				
ILAND	+0.003	+0.970	+0.871	+0.891
Wine				
ILAND	+0.586	+0.927		
SOMERS		+0.938		
SOMERS (HWC)		+0.572		
CSIRO v HWC measurements				
°Brix	+0.063	+0.604	+0.300	+0.369
Berry Weight (g)	+0.214		+0.690	+0.400

NOTES
HPLC -High Performance Liquid Chromatography
ILAND -UV-VIS spectrophotometric for grapes & wine (modified (Iland et al. 2000))
SOMERS -UV-VIS spectrophotometric for wine (only in 2004) (Somers and Evans 1977)
HWC -Measurements made by HWC

The same measurements made by HWC and CSIRO were also compared (**Table 3.2**). Overall, there was some relationship between CSIRO and HWC measurements of ^{o}Brix and berry weight, however this relationship was not consistent between regions and seasons, as reflected by the range of R^2 values from 0.2-0.6. While this result reiterates the similarity in the samples received from HWC, it also highlights the natural/biological variation in grape sampling, as both samples were taken from the same (5 Kg) batch of grapes.

Overall, these results show that CSIRO measurements generally correlated with measurements made by HWC and that the different methods used to analyse total anthocyanins and phenolics in grapes and wines were highly correlated. This provided an indication of the biological and technical variation that can occur in samples and the methods used to analyse them. Subsequently, only the CSIRO HPLC measurements are reported in graphs and tables (unless stated otherwise).

3.3.4 Grape berry composition in both regions & seasons

The mean values for the berry components and flavonoid composition in McLaren Vale and Riverland fruit in 2003 and 2004 are shown in **Table 3.3,** along with the statistical significance between region and season (and the interaction between region and season).

McLaren Vale berry weight was approximately 0.1 g less than Riverland fruit. There was no significant difference in skin weight per berry between the regions, rather seasonal variation had a greater affect on skin weight. While (on average) there were similar numbers of seeds in berries from both regions, significant differences were observed between seed weight per berry, with smaller seeds observed in McLaren Vale berries. Berry ripeness (^{o}Brix) was largely variable in both regions with significant differences between region and season. Overall, the total flavonoid content (anthocyanins, flavonols, and tannins) of fruit from McLaren Vale was higher than Riverland fruit. This is largely attributable to the fact that there were considerable differences in the total anthocyanin and flavonol content between the regions, whereas total tannin content was similar in both regions.

The concentration of anthocyanin pigments (delphinidin, cyanidin, petunidin, peonidin, malvidin) and anthocyanin groups (mono-glucoside, acetyl-glucosides) was significantly higher in McLaren Vale fruit than Riverland fruit. However, the concentration of the coumaroyl-glucoside group of anthocyanins was similar in both regions. Accordingly, there were a significantly higher proportion

of coumaroyl-glucosides (as a % of total anthocyanins) in Riverland fruit than in McLaren Vale fruit and this shift in composition appeared to be at the expense of the mono-glucosides, which were notably reduced in Riverland fruit. While McLaren Vale fruit (compared to Riverland fruit) had higher proportion of delphinidin, cyanidin and petunidin pigments, the proportion of malvidin pigments was significantly lower. There was also no statistically significant difference in the proportion of peonidin pigments between the regions.

Flavonol concentration was also higher in McLaren Vale fruit compared to Riverland fruit, particularly the glycosides of quercetin, myricetin and kaempferol. In contrast, the concentration of syringetin-glucoside was slightly higher in Riverland fruit than in McLaren Vale fruit. While the concentration of isorhamnetin-glycosides was the same in both regions, there was a large seasonal influence on these compounds, with higher levels in 2003 than in 2004. Although the proportion (as a % of total flavonols) of most of the flavonol glycosides was found be statistically significant between different regions, the difference in the proportion between the regions was relatively low when compared to anthocyanins.

Unfortunately tannin content and composition was not measured in all data sets, however, in 2004 the same method was used to determine total tannin content in skin and seeds. There was a slightly higher concentration of skin tannins in McLaren Vale fruit than in Riverland fruit, yet seed tannin concentration was relatively similar in both regions. There was no statistically significant difference in total tannin content between the regions, likely due to fact that seed tannins contribute a greater proportion to total tannin content than skin tannins.

The general influence of seasonal variation on anthocyanins and flavonols was also observed in **Table 3.3**. Majority of the anthocyanin compounds were higher in 2004 than in 2003, yet in contrast most of the flavonol compounds were higher in 2003 compared to 2004. The climatic data for these growing seasons was unavailable; however the Phylloxera and Grape Industry Board of South Australia (2005) reported overall the climatic conditions during these growing seasons, in each region, were not notably different from long-term averages. The influence of seasonal variation on the flavonoid content and composition is not further expanded in this investigation, rather the influence of climate as a whole,at each region is discussed.

Table 3.3 Mean values for grape berry factors & flavonoid composition in McLaren Vale & Riverland in 2003 & 2004. Grape flavonoid composition is represented as the concentration (mg/g berry) & as the proportion (as a % of total). Statistically significant differences between region & season (& interaction between region & season) were determined by an unbalanced ANOVA performed in GenStat (9th Ed). Blank cells indicate data was not available.

	MV03	MV04	RL03	RL04	Significance		
					Region	Season	Interaction
Berry factors							
Berry weight (g)	1.088	1.028	1.184	1.126	***	*	ns
Skin weight per berry (g)	0.381	0.321	0.391	0.317	ns	***	ns
Seed weight per berry (g)	0.055	0.055	0.060	0.061	***	ns	ns
Number of seeds	2.293	2.063	2.209	2.208	ns	*	**
°Brix	27.788	26.917	26.625	24.984	***	***	ns
Grape flavonoid composition (Concentration - mg/g berry)							
Total anthocyanins	**1.857**	**2.119**	**1.114**	**1.162**	***	**	*
Total mono glucosides	1.238	1.245	0.494	0.542	***	ns	ns
Total acetyl glucosides	0.283	0.383	0.215	0.233	***	***	***
Total coumaroyl glucosides	0.336	0.491	0.404	0.386	ns	***	***
Total delphinidin	0.194	0.142	0.055	0.050	***	***	***
Total cyanidin	0.045	0.040	0.013	0.021	***	**	***
Total petunidin	0.219	0.219	0.077	0.093	***	*	ns
Total peonidin	0.297	0.248	0.153	0.151	***	*	***
Total malvidin	1.101	1.469	0.816	0.846	***	***	***
Total flavonols	**0.199**	**0.180**	**0.168**	**0.124**	***	***	ns
Total quercetin glycosides	0.095	0.071	0.079	0.056	***	***	ns
Total myricetin glycosides	0.048	0.062	0.036	0.031	***	ns	***
Total kaempferol glycosides	0.016	0.014	0.009	0.006	***	***	ns
Total isorhamnetin glycosides	0.033	0.022	0.033	0.022	ns	***	ns
Syringetin glucoside	0.007	0.011	0.012	0.008	*	*	***
Total tannins		**4.135**		**3.958**	ns	Incomplete data sets for full statistical analysis	
Total skin tannins		1.682		1.484	**		
Total seed tannins		2.453		2.474	ns		
Total flavonoid content		**6.434**		**5.244**	***		
Grape flavonoid composition (Proportion - % of totals)							
Total anthocyanins (100%)							
Total mono glucosides	67	59	45	47	***	ns	***
Total acetyl glucosides	15	18	19	20	***	***	***
Total coumaroyl glucosides	18	23	36	33	***	ns	***
Total delphinidin	10	7	5	4	***	***	***
Total cyanidin	3	2	1	2	***	***	***
Total petunidin	12	10	7	8	***	ns	***
Total peonidin	16	12	14	13	ns	***	***
Total malvidin	59	69	73	73	***	***	***
Total flavonols (100%)							
Total quercetin glycosides	48	40	47	45	**	***	***
Total myricetin glycosides	24	34	21	25	***	***	***
Total kaempferol glycosides	8	8	5	5	***	ns	**
Total isorhamnetin glycosides	17	12	20	18	***	***	*
Syringetin glucoside	3	6	7	7	***	**	***

NOTES
- Tannin measurements in 2004 were by the PPA (extracted in 70% acetone)
- Number of samples (n) MV03 n=25, MV04 n=24, RL03/RL04 n= 55
- p<0.001=highly significant (***), p<0.01 (**), p<0.05 (*), p>0.05=not significant (ns)

3.3.5 Relationships with berry weight

One of the benefits of this investigation was that the relationship between berry weight and some of the berry factors (i.e. skin weight, seed weight) and flavonoid content could be observed (**Table 3.4**). For the most part, there was a positive relationship between skin and seed weight and berry weight, yet no relationship between seed number and berry weight. There was also a slight negative relationship between °Brix and berry weight, however the R^2 values were less than 0.2 and are not consistently observed in all data sets. Likewise, there was no consistent relationship between skin weight (as a proportion of berry weight) and berry weight, but there was a negative relationship between seed weight (as a proportion of berry weight) and berry weight. On the whole, there was no relationship between berry weight and anthocyanins or tannins (skin or seed), yet a negative relationship was observed between flavonols and berry weight.

Table 3.4 R^2 table of relationships between BERRY WEIGHT & berry factors & flavonoid content. R^2 values highlighted in green are >0.2, & are used as an indicator of a possible 'relationship'. +/- indicates whether the relationship is positive or negative. Blank cells indicate data was not available.

FACTOR	R^2 Values			
	MV03	MV04	RL03	RL04
Skin weight per berry	+0.192	+0.618	+0.398	+0.356
Seed weight per berry	+0.530	+0.391	+0.091	+0.218
Number of Seeds	+0.023	+0.103	+0.009	+0.110
°Brix	-0.185	-0.062	+0.093	-0.128
Skin weight as a proportion of berry weight	-0.142	-0.002	-0.050	-0.161
Seed weight as a proportion of berry weight	-0.400	-0.493	-0.568	-0.423
Anthocyanins	-0.056	-0.272	-0.057	-0.027
Flavonols	-0.325	-0.255	-0.167	-0.226
Tannins -Skin	+0.010	-0.148		-0.013
-Seed	-0.094	-0.063		-0.178

3.3.6 Relationships between the flavonoid compounds (anthocyanins, flavonols & tannins) in grapes

To gain an understanding of how the synthesis of the flavonoid compounds in grapes is coordinated, flavonoid composition was measured in a wide variety of grape samples. There was a positive relationship between total anthocyanins and flavonols in both regions and seasons, with R^2 values greater than 0.25 (**Figure 3.5**). While fruit from McLaren Vale clearly separated from those from the Riverland based on a higher anthocyanin content there was large spread of flavonols in both regions.

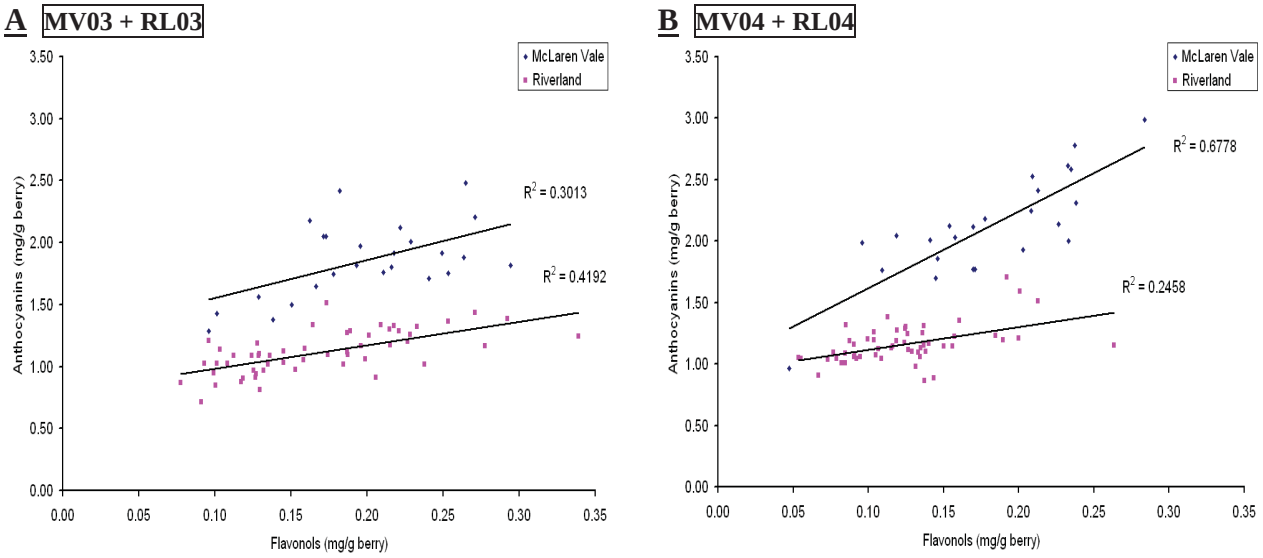


Figure 3.5 The relationship between anthocyanins & flavonols in Shiraz grape skins from McLaren Vale & Riverland. For each Shiraz grape sample, anthocyanins & flavonols in the skin were determined by HPLC. Anthocyanin & flavonol concentration is expressed on an mg/g berry basis.

A Relationship between anthocyanins & flavonols in Shiraz grape skins from McLaren Vale & Riverland in 2003
B Relationship between anthocyanins & flavonols in Shiraz grape skins from McLaren Vale & Riverland in 2004

The relationship between skin anthocyanins and tannins in both skin and seed are shown in **Figure 3.6**. All samples were extracted in 70% aqueous acetone and in 2003 grape tannins were measured by PGA (Downey et al. 2003a), while in 2004 grape tannins were measured by PPA (Downey and Adams 2005). Additionally, tannins were not measured in the 2003 Riverland fruit, as the sample integrity may have been flawed (for explanation see **Section 3.2.2**). As expected, tannin concentration in the seeds was greater than in the skin for majority of samples (Downey et al. 2003a; Souquet et al. 1996). There was a weak positive relationship between skin anthocyanins and skin tannins in both regions and seasons, but no relationship with seed tannins, which were scattered. Accordingly, there was no relationship between skin tannins and seed tannins (data not shown).

The relationship between skin flavonols and tannins (in skin and seed) is shown in **Figure 3.7**. Similar to anthocyanins, there was a positive relationship between skin flavonols and skin tannins in both regions in 2004, however this relationship was not observed in 2003. There was also no relationship between flavonols and seed tannins in most data sets, apart from in McLaren Vale (2004), when a positive relationship between flavonols and seed tannins was observed with a R^2 value of 0.23.

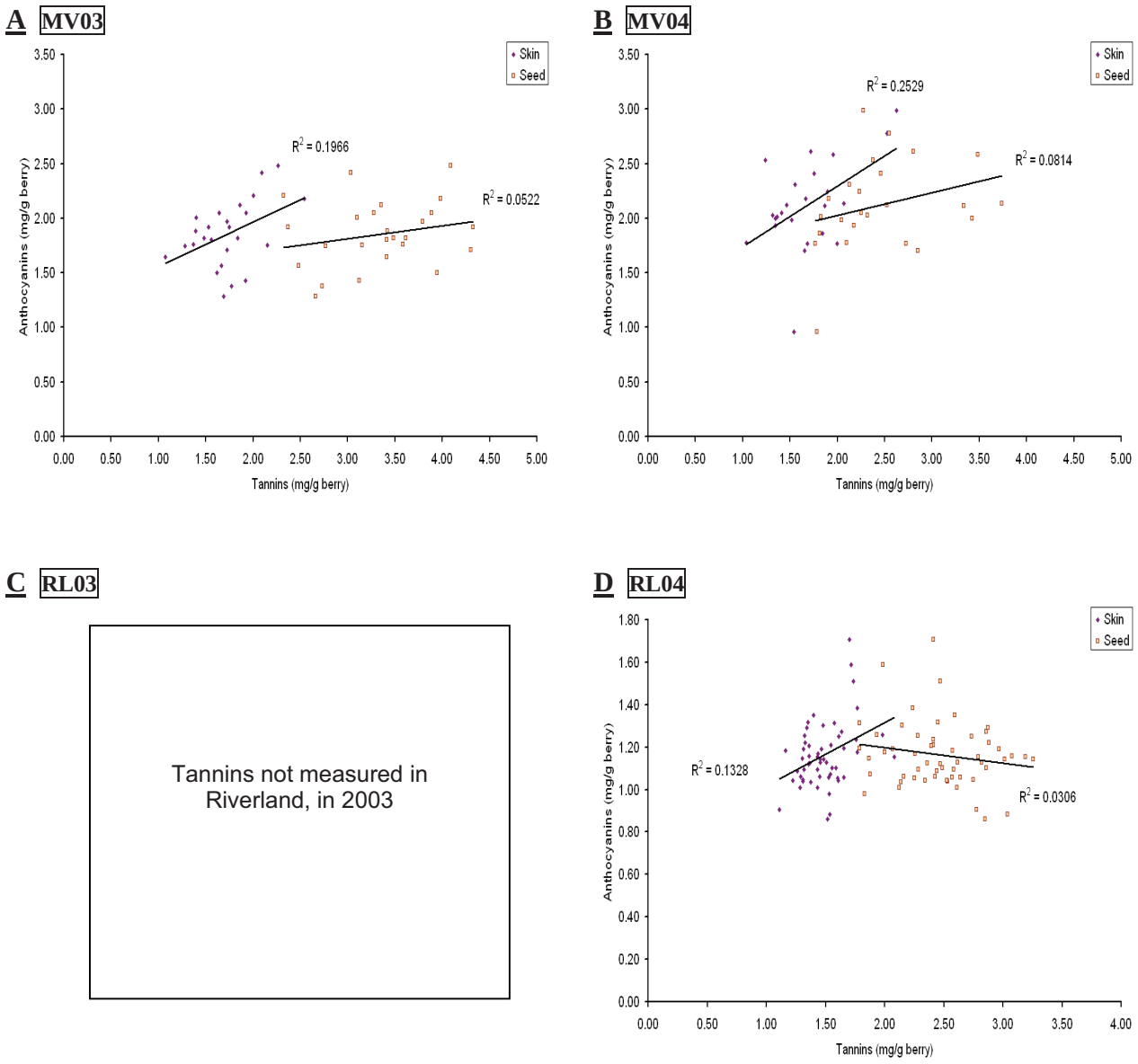


Figure 3.6 The relationship between anthocyanins & tannins in Shiraz grape skins & seeds from McLaren Vale & Riverland. For each Shiraz grape sample, anthocyanins in the skin were determined by HPLC. In 2003, in McLaren Vale, skin & seed tannins were measured by PGA, however tannins were not measured in the Riverland. In 2004 in both regions, skin & seed tannins were measured by PPA. Anthocyanin & tannin concentration is expressed on an mg/g berry basis.

A Relationship between anthocyanins & tannins in Shiraz grape skins & seeds from McLaren Vale in 2003
B Relationship between anthocyanins & tannins in Shiraz grape skins & seeds from McLaren Vale in 2004
C Tannins not measured in Riverland 2003
D Relationship between anthocyanins & tannin in Shiraz grape skins & seeds from Riverland in 2004

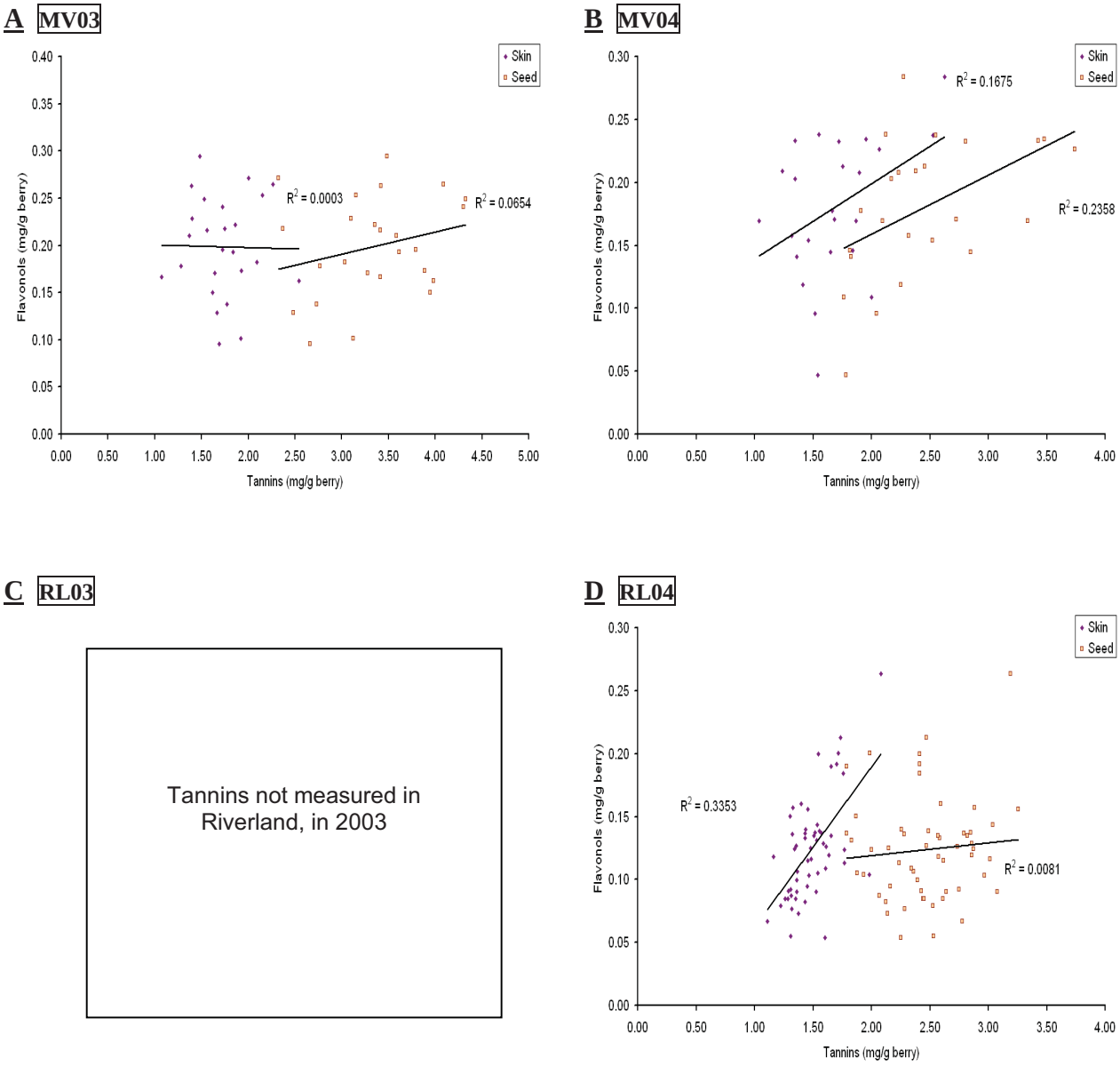


Figure 3.7 The relationship between flavonols & tannins in Shiraz grape skins & seeds from McLaren Vale & Riverland. For each Shiraz grape sample, flavonols in the skin were determined by HPLC. In 2003, in McLaren Vale, skin & seed tannins were measured by PGA, however tannins were not measured in the Riverland. In 2004 in both regions, skin & seed tannins were measured by PPA. Flavonol & tannin concentration is expressed on an mg/g berry basis.

A Relationship between flavonols & tannin in Shiraz grape skins & seeds from McLaren Vale in 2003
B Relationship between flavonols & tannin in Shiraz grape skins & seeds from McLaren Vale in 2004
C Tannins not measured in Riverland, in 2003
D Relationship between flavonols & tannin in Shiraz grape skins & seeds from Riverland in 2004

3.3.7 Relationships between the flavonoids (anthocyanins & flavonols) in wine

In addition to grape samples, wines samples from McLaren Vale were provided for analysis, enabling the relationship between grape and wine flavonoids, particularly anthocyanins and flavonols, to be investigated.

The relationship between grape and wine anthocyanins is shown in **Figure 3.8**. In both seasons there was a weak relationship between grape anthocyanins and wine anthocyanins. In general, grape and wine samples from 2004 had a higher anthocyanin concentration compared to grape and wine samples in 2003 (as expected, **Table 3.3**). The low R^2 value (0.20) in 2003 is suggested to be due to poor sampling techniques, as some wines had been blended. In 2004 a similar low R^2 value was observed (0.29), and this correlation is further reduced if the sample with the lowest anthocyanin content is removed (i.e. the outlier sample, noted in **Section 3.3.1**), resulting in a R^2 value of 0.07. It is also worth noting that while pigmented polymers were not included in the measure of total wine anthocyanins (as noted in **Section 2.2.1.2**), when summed to total wine anthocyanins the correlations did not improve (data not shown). Furthermore, there was a weak relationship between grape flavonols and wine flavonols in both 2003 and 2004 with R^2 values of 0.16 and 0.33, respectively (data not shown).

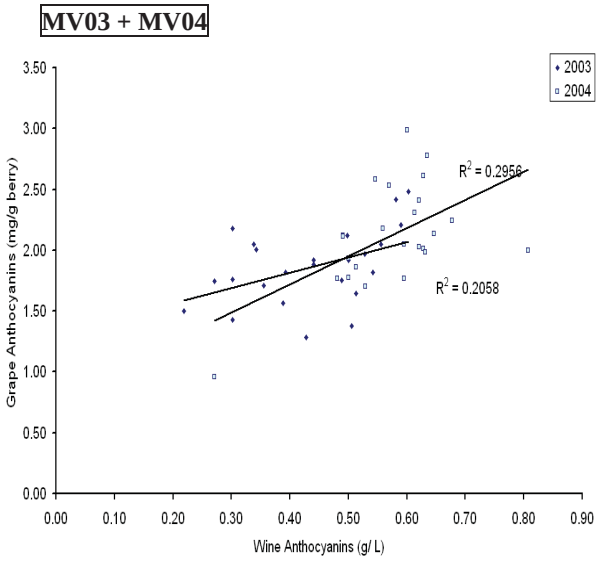


Figure 3.8 The relationship between anthocyanins in the grape & anthocyanins in the wine in Shiraz samples from McLaren Vale in 2003 & 2004. For each Shiraz sample (grape skin or wine), anthocyanins were determined by HPLC. Anthocyanin concentration in grapes is expressed on an mg/g berry basis & in wines on a g per L basis.

Following observations in grape skins, the relationship between anthocyanins and flavonols in wine was investigated (**Figure 3.9).** There was a relationship between anthocyanins and flavonols in the wine, with a higher R^2 value in 2004 compared to 2003. This result indicates that the relationship between anthocyanins and flavonols observed in grapes is also maintained during fermentation and in resultant wines.

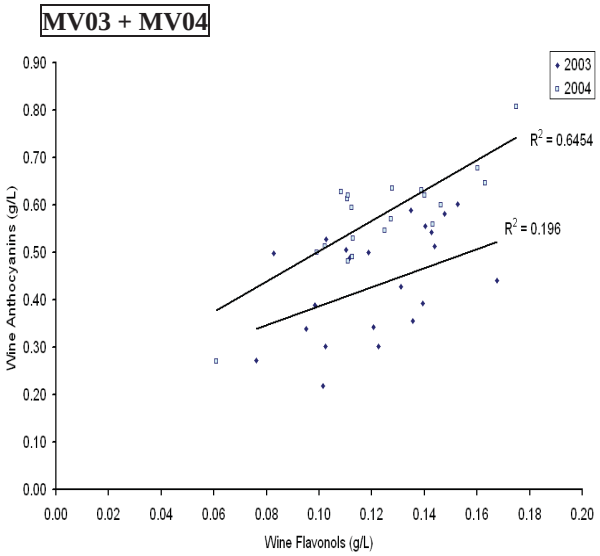


Figure 3.9 The relationship between anthocyanins & flavonols in Shiraz wine samples from McLaren Vale in 2003 & 2004. For each Shiraz sample, anthocyanins & flavonols in the wine were determined by HPLC. Anthocyanin & flavonol concentration in wines is expressed on a g per L basis.

3.3.8 Indicators of grape quality

With access to the grape quality stream allocation for each sample, it was sought to investigate potential indicators of grape quality. The relationship between grape quality stream (as allocated by HWC) and various factors (i.e. berry, flavonoid content, vineyard) as determined by HWC and CSIRO are shown in **Table 3.5**. In this table, R^2 values greater than 0.20 are shaded (light green) and are used as an indicator of a possible 'relationship'. Values with +/- in front indicate whether the relationship is positive or negative. However, it should be noted that as the grape quality stream scale is inverted (i.e. 1=high, 5=low), positive (+) values are an indication of a negative correlation (i.e. fruit quality decreases), while negative values (-) are an indication of a positive correlation (i.e. fruit quality improves).

In all data sets (except MV03, HWC berry weight) there was no relationship between grape quality stream, ^{o}Brix or berry weight as determined by HWC and CSIRO, with both positive and negative

trends observed. There was also no relationship between anthocyanins and grape quality stream, with R^2 values less than 0.13 in all data sets. This relationship (or lack thereof) is also shown in **Figure 3.10.** While the general observation could be that there is some trend towards a relationship between anthocyanins and grape quality stream, overall most of the samples are scattered for anthocyanin content in each quality stream. Similar to anthocyanins, there was no relationship between grape quality stream and any of the flavonoid compounds (flavonols, skin or seed tannins), in any data sets (except MV03, seed tannins).

There was a very weak relationship between yield and grape quality stream in 2004, where despite positive R^2 values (i.e. inverse relationship), samples from various yielding vineyards were scattered in each quality stream. In the Riverland, there was generally no relationship between the HWC vineyard assessment parameters (i.e. shoot tip growth, fruit exposure, leaf health) and grape quality stream. This could be due to the fact that these parameters are subjectively measured. In McLaren Vale, the vineyard assessment parameters were quantitative measurements of bunch and shoot numbers, and in general these measurements were positively correlated with grape quality stream with R^2 values around 0.30. Furthermore, additional vineyard factors taken by HWC in McLaren Vale such as; internode length, number of cordons, type of canopy management and type of pruning (see **Appendix 3B**), were also weakly correlated with grape quality stream, with R^2 values ranging from 0.13-0.34 (data not shown).

Of all the berry and vineyard factors measured in McLaren Vale, the highest correlating factor with grape quality stream was vine age, with R^2 values around 0.40 (note- vine age was not given for Riverland samples). This relationship is shown in **Figure 3.11.** Generally, younger vines are clustered in the lower grape quality streams, whereas the higher quality streams have an array of vines of differing ages. However, it should be noted that this data is complicated by the fact that many of the vines are of similar age and therefore some data points are overlaid.

It is generally regarded that higher yielding vineyards generally have lower anthocyanins (Coombe and Iland 2004), therefore the relationship between anthocyanins and yield was investigated (**Figure 3.12**). The two regions were clearly separated based on yield, with lower yields in McLaren Vale. The levels of total anthocyanins were inversely related to yield in both regions, although the relationships were relatively weak.

Table 3.5 R^2 table of relationships between GRAPE QUALITY STREAM & various factors as measured by HWC & CSIRO. R^2 values highlighted in green are >0.2, & are used as an indicator of a possible 'relationship'. +/- indicates whether the relationship is positive or negative, however, note that the grape quality stream allocation is inverted (i.e. 1=high-3=low). Blank cells indicate data was not available.

FACTOR	R^2 Values							
	HWC Measurements				CSIRO Measurements			
	MV03	MV04	RL03	RL04	MV03	MV04	RL03	RL04
Berry weight	-0.258		+0.001	+0.110	-0.143	+0.070	+0.001	+0.015
°Brix	-0.018	+0.007	+0.037	+0.054	+0.009	-0.020	+0.084	+0.084
Grape anthocyanins (Figure 3.10)	+0.045	-0.036	-0.094	-0.065	-0.013	-0.037	-0.011	-0.133
Grape flavonols					-0.009	-0.078	-0.021	-0.116
Grape skin tannins					-0.013	-0.029		-0.005
Grape seed tannins					+0.207	-0.023		-0.004
BM yield	+0.098	+0.151	+0.071	+0.255				
BM vine age (Figure 3.11)	-0.422	-0.442						
BM bunches /m	+0.291	+0.343						
BM shoots/m	+0.017	+0.257						
BM bunches/shoot	+0.309	+0.150						
BM shoot tip growth (S)			-0.000	+0.015				
BM fruit exposure (S)			-0.003	-0.250				
BM Leaf health (S)			-0.008	+0.018				

NOTES
-BM=Benchmarking data collected from the pre-harvest vineyard assessment
-(S) =Subjective measurements, made by winery representative

Furthermore, with understanding of the grape streaming process, combined with knowledge that the basis for payment for grape growers in McLaren Vale is based on the final wine product, it was sought to investigate any relationship between anthocyanin extraction into wine and grape quality stream. The ratio of anthocyanins in the grape/wine was plotted against grape quality stream (**Figure 3.13)**. In 2003, there was a relationship between the anthocyanin extraction and grape quality stream, with anthocyanins from the higher grape quality stream, having improved extraction (i.e. low ratio) compared to those in the lower quality streams (i.e. high ratio). However, in 2004, there was no relationship, with similar extraction ratios in each quality stream.

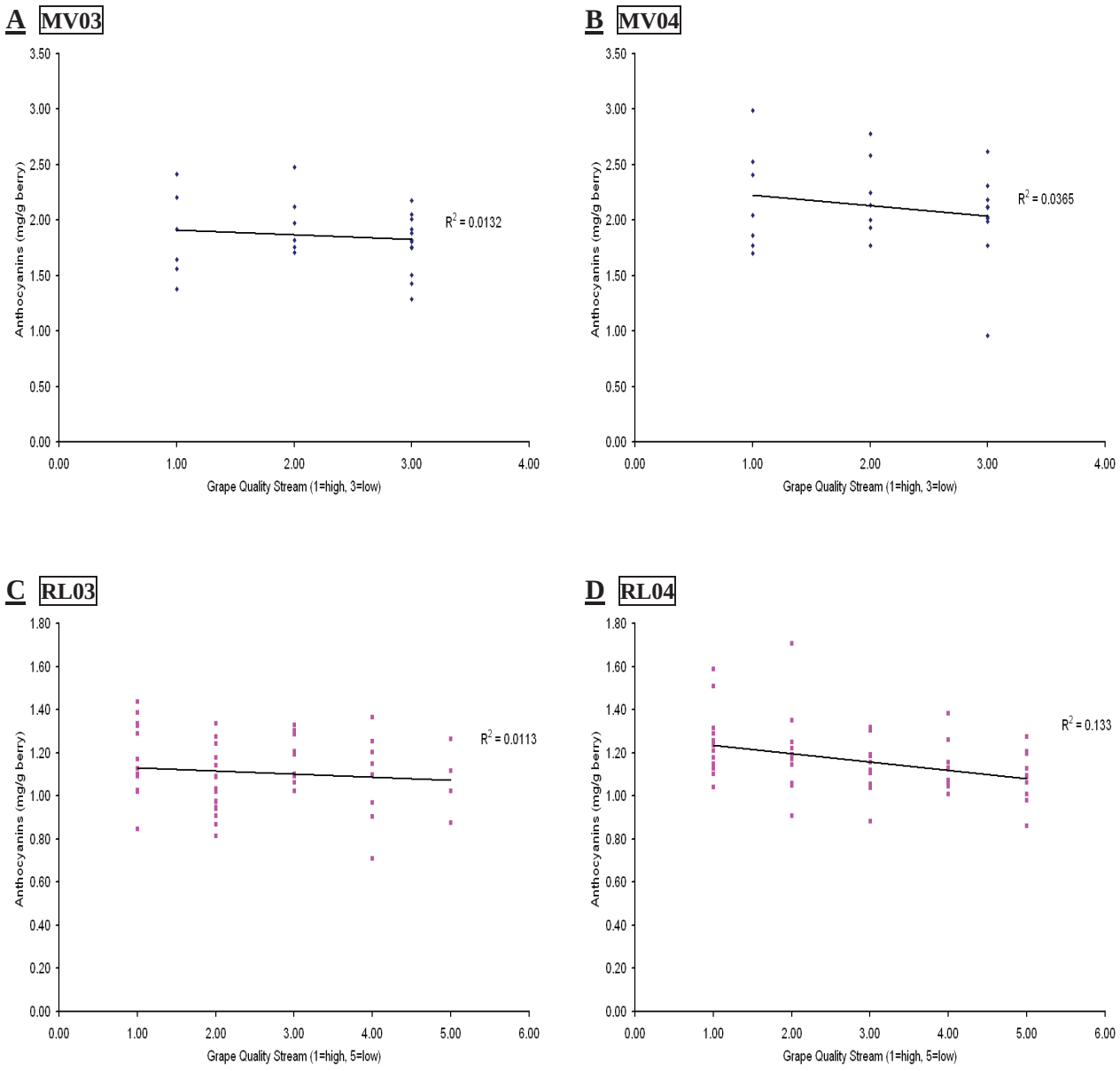


Figure 3.10 The relationship between anthocyanins & grape quality stream in Shiraz grape skins from McLaren Vale & Riverland. For each Shiraz grape sample, anthocyanins in the skin were determined by HPLC. HWC provided the grape quality stream, where 1=high→5=low. Anthocyanin concentration is expressed on an mg/g berry basis.

A Relationship between anthocyanins & grape quality stream in Shiraz grape skins from McLaren Vale in 2003
B Relationship between anthocyanins & grape quality stream in Shiraz grape skins from McLaren Vale in 2004
C Relationship between anthocyanins & grape quality stream in Shiraz grape skins from Riverland in 2003
D Relationship between anthocyanins & grape quality stream in Shiraz grape skins from Riverland in 2004

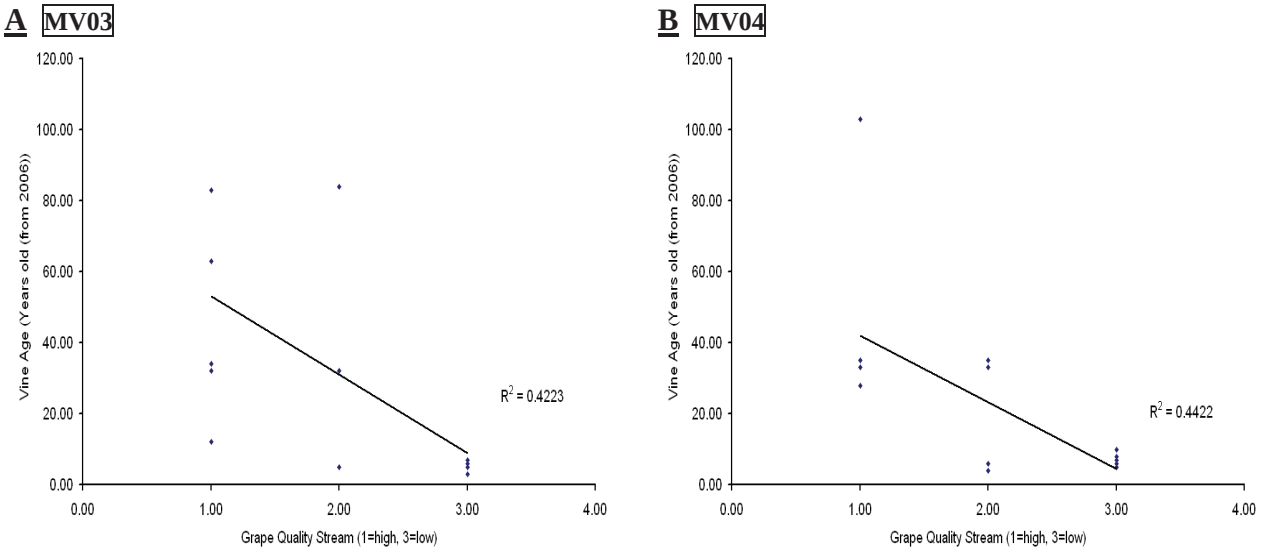


Figure 3.11 The relationship between vine age & grape quality stream in Shiraz samples from McLaren Vale. HWC provided the data for vine age (calculated from 2006) & grape quality stream, where 1=high→5=low.

A Relationship between vine age & grape quality stream in Shiraz samples from McLaren Vale in 2003
B Relationship between vine age & grape quality stream in Shiraz samples from McLaren Vale in 2004

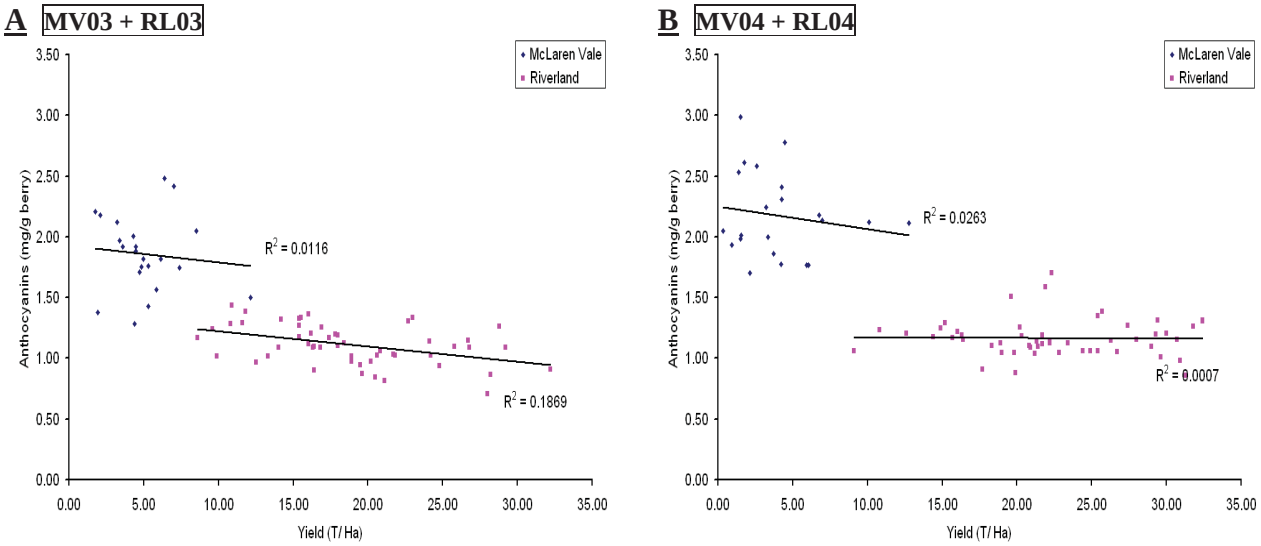


Figure 3.12 The relationship between anthocyanins & yield (T/Ha) in Shiraz grape skins from McLaren Vale & Riverland. For each Shiraz grape sample, anthocyanins in the skin were determined by HPLC. Yield data (T/Ha) was provided by HWC. Anthocyanin concentration is expressed on an mg/g berry basis.

A Relationship between anthocyanins & yield (T/Ha) in Shiraz grape skins from McLaren Vale & Riverland in 2003
B Relationship between anthocyanins & yield (T/Ha) in Shiraz grape skins from McLaren Vale & Riverland in 2004

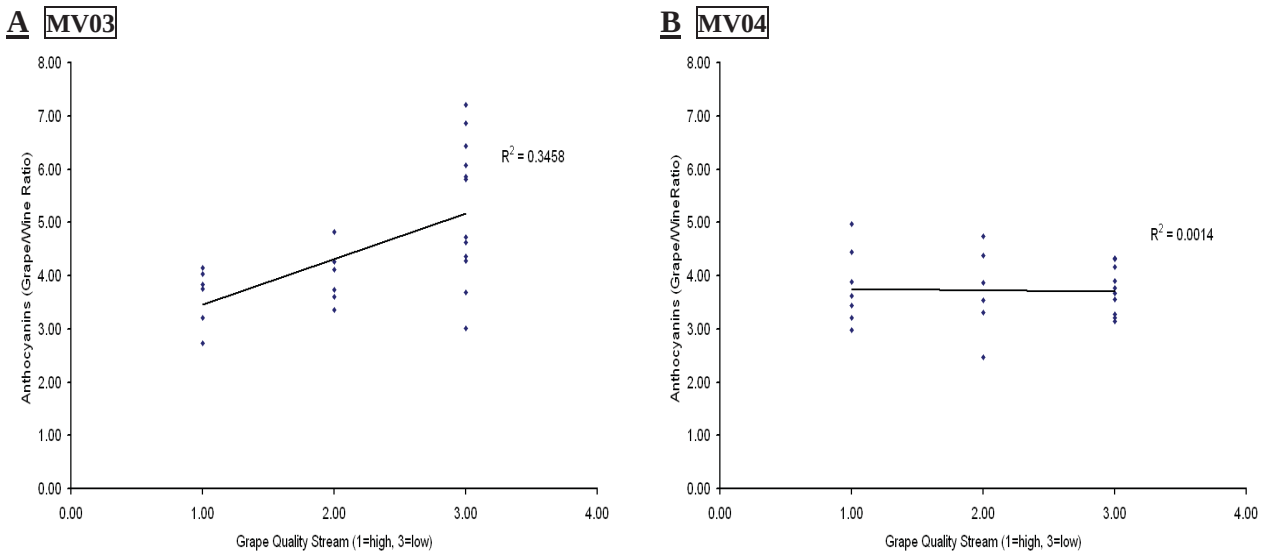


Figure 3.13 The relationship between anthocyanins (ratio of grape/wine) & grape quality stream in Shiraz samples from McLaren Vale. For each Shiraz grape sample (grape skin or wine) anthocyanins were determined by HPLC. HWC provided the grape quality stream, where 1=high→3=low.

A Relationship between anthocyanins (ratio of grape/wine) & grape quality stream in McLaren Vale in 2003
B Relationship between anthocyanins (ratio of grape/wine) & grape quality stream in McLaren Vale in 2004

3.3.9 Indicators of wine quality

After investigating relationships with grape quality stream, the next step was to explore indicators of final wine quality. The relationship between wine grade and various factors (i.e. berry, grape and wine flavonoid content, vineyard) determined by HWC and CSIRO are shown in **Table 3.6**. Again, R^2 values greater than 0.20 are shaded (light green) and are used as an indicator of a possible 'relationship'. Values with a +/- in front indicate whether a relationship is positive or negative and similar to grape quality stream, it should be pointed out that wine grade scale is inverted (i.e. 1=high, 7=low).

Overall, similar relationships to those observed with grape quality stream were observed with wine grade. In all data sets there was no relationship between wine grade and; ^{o}Brix, berry weight, grape anthocyanins, grape flavonols or skin/seed tannins, as determined by CSIRO or HWC. Yet, there were weak positive relationships between wine grade and yield, and some of the vineyard factors, particularly in McLaren Vale.

The relationship between wine grade and grape anthocyanins, and wine anthocyanins in McLaren Vale in both seasons are shown in **Figure 3.14**. In both seasons there was no relationship between wine grade and grape anthocyanins, yet, there was a relationship between wine grade and wine anthocyanins, with R^2 values greater than 0.20. Also superimposed in **Figure 3.14,** is the grape quality stream assigned to each sample. This shows the clustering of the high quality grape streams towards the high end of the wine grade scale and low quality grape streams towards the low end of the wine grade scale.

Interestingly, in 2003, it appears that as wine grade increases (i.e. closer to 1), wine anthocyanin concentrations start to level out (**Figure 3.14**). This observation is more pronounced when wine grade is converted to $/bottle (i.e. the wholesale price paid by consumers for the wine) (**Figure 3.15**). Rather than a linear relationship, a logarithmic relationship was observed with a R^2 value 0.61. It shows that generally, for wines less than $15/bottle, an increase in wine anthocyanin content will correspond with an increase in wholesale price (i.e. a linear relationship). However for wines greater than $15/bottle this relationship is not maintained; that is a increase in anthocyanins content greater than 0.50 g/L will not greatly increase the wholesale price. Once again, it was observed that there was clustering of the higher quality grape samples towards the more expensive wines. It is also worth noting, when pigmented polymers were added to total wine anthocyanins in this plot, the R^2 value significantly decreased to 0.006 (data not shown).

In the Riverland, there was no relationship between wine grade and grape anthocyanins in both seasons as shown in **Figure 3.16**. This may be due to the fact that fruit parcels were combined prior to fermentation, and accordingly the final wine grade would be based on a blended wine. There was no obvious clustering of the high grape quality streams to the high wine grades.

Finally, the relationship between grape quality stream and final wine grade was examined (**Figure 3.17**). In most data sets, there was a relationship between grape quality stream and wine grade with R^2 values greater than 0.54 (except RL03 R^2=0.02). It is important to note that in these graphs, the data is complicated by the fact that each of the data sets is ordinal (i.e. ranked 1-7), and as such the linear relationship is represented as a dashed line. Additional statistical analyses were also performed on the data, including the Spearman's P value and Kendall's T value. These relationship coefficients (P and T), along with their statistical significance are shown in **Figure 3.17.** For the most part, these additional relationship coefficients are in agreement with the R^2 values indicating

there is a highly significant relationship between wine grade and grape quality stream. However, it still must be considered that this relationship is to some extent questionable, and as such should be treated with caution.

Table 3.6 R^2 table of relationships between WINE GRADE & various factors as measured by HWC & CSIRO. R^2 values highlighted in green are >0.2, & are used as an indicator of a possible 'relationship'. +/- indicates whether the relationship is positive or negative, however, note that the wine grade allocation is inverted (i.e. 1=high-7=low). Blank cells indicate data was not available.

FACTOR	R^2 Values							
	HWC Measurements				CSIRO Measurements			
	MV03	MV04	RL03	RL04	MV03	MV04	RL03	RL04
Berry weight	No relationship				No relationship			
°Brix	No relationship				No relationship			
Grape anthocyanins (Figure 3.14 /3.16)	-0.004	-0.161	-0.028	0.037	-0.083	-0.063	-0.002	-0.071
Grape flavonols					-0.068	-0.125	-0.017	-0.043
Grape skin tannins					-0.018	-0.001		-0.001
Grape seed tannins					+0.140	-0.059		-0.003
Grape quality stream (Figure 3.17)	+0.651	+0.761	+0.022	+0.542				
BM Yield	+0.115	+0.225	-0.006	+0.165				
BM vine age	-0.255	-0.300						
BM bunches /m	+0.116	+0.531						
BM shoots /m	+0.003	+0.347						
BM bunches/shoot	+0.134	+0.500						
BM shoot tip growth (S)			-0.000	+0.003				
BM fruit exposure (S)			-0.039	-0.214				
BM leaf health (S)			-0.060	+0.000				
Wine anthocyanins (Figure 3.14)		-0.086			-0.701	-0.200		
Wine flavonols					-0.076	-0.265		

NOTES
-BM=Benchmarking data collected from the pre-harvest vineyard assessment
-(S) =Subjective measurements, made by winery representative

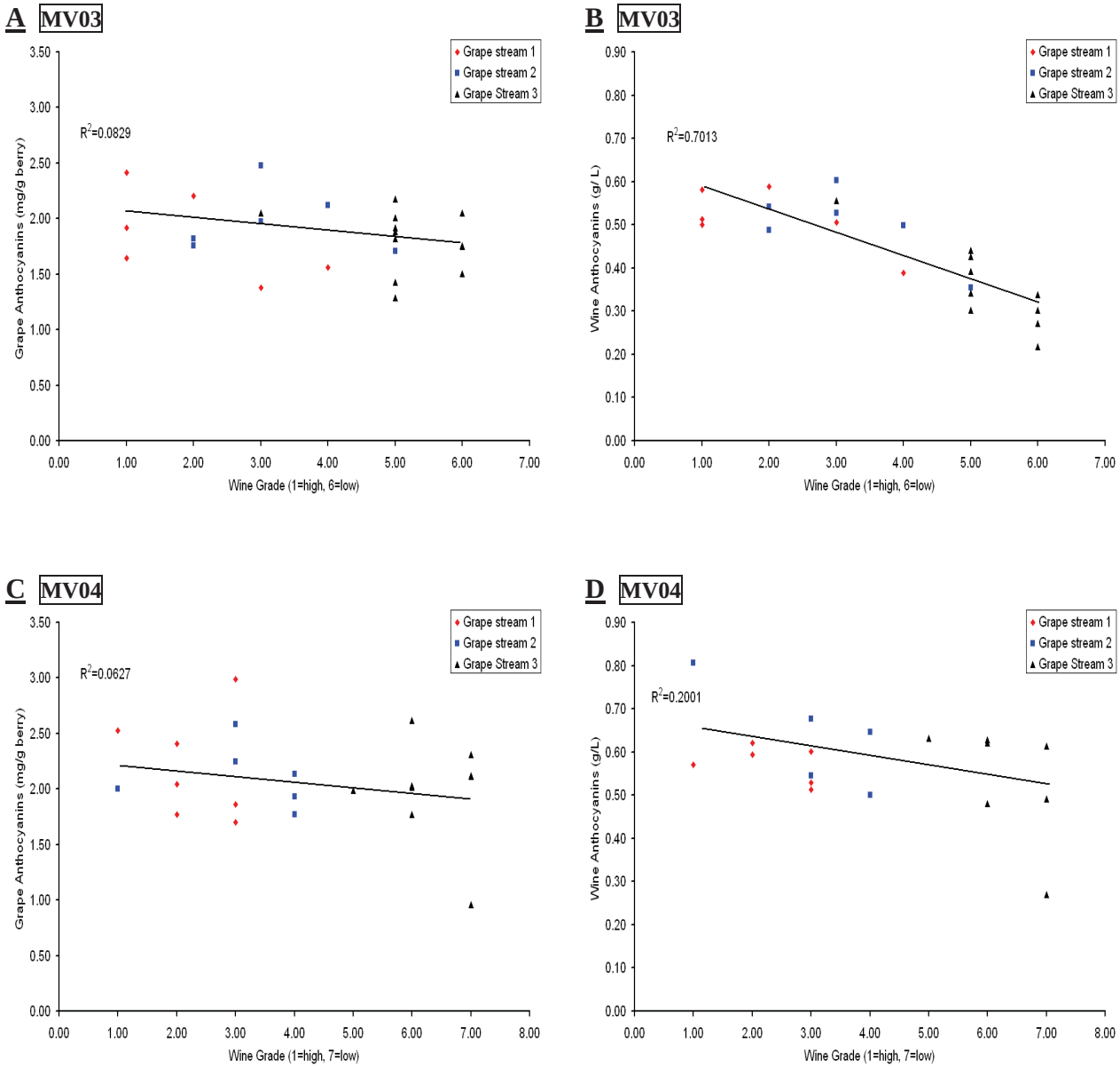


Figure 3.14 The relationship between anthocyanins (in the grape & wine) & wine grade in Shiraz samples from McLaren Vale. For each Shiraz sample (grape skin or wine) anthocyanins were determined by HPLC. HWC provided the wine grade, where 1=high→7=low. Also indicated are the grape quality streams (colored), provided by HWC, where 1=high→3=low. Anthocyanin concentration in grapes is expressed on an mg/g berry basis & in wines on a g per L basis.

A Relationship between grape anthocyanins & wine grade in Shiraz grape skins from McLaren Vale in 2003
B Relationship between wine anthocyanins & wine grade in Shiraz wines from McLaren Vale in 2003 (also, **Figure 3.15**)
C Relationship between grape anthocyanins & wine grade in Shiraz grape skins from McLaren Vale in 2004
D Relationship between wine anthocyanins & wine grade in Shiraz wines from McLaren Vale in 2004

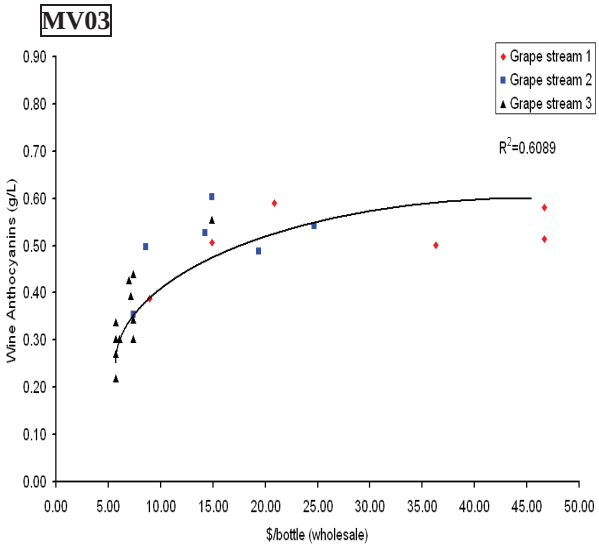


Figure 3.15 The relationship between wine anthocyanins & $/bottle (wholesale) in Shiraz samples from McLaren Vale in 2003. For each Shiraz sample, wine anthocyanins were determined by HPLC. HWC provided the $/bottle (wholesale) data. Anthocyanin concentration in wines is expressed on a g per L basis. Also indicated are the grape quality streams (colored), provided by HWC, where 1=high→3=low. The R^2 value shown is for a logarithmic relationship (not a linear relationship).

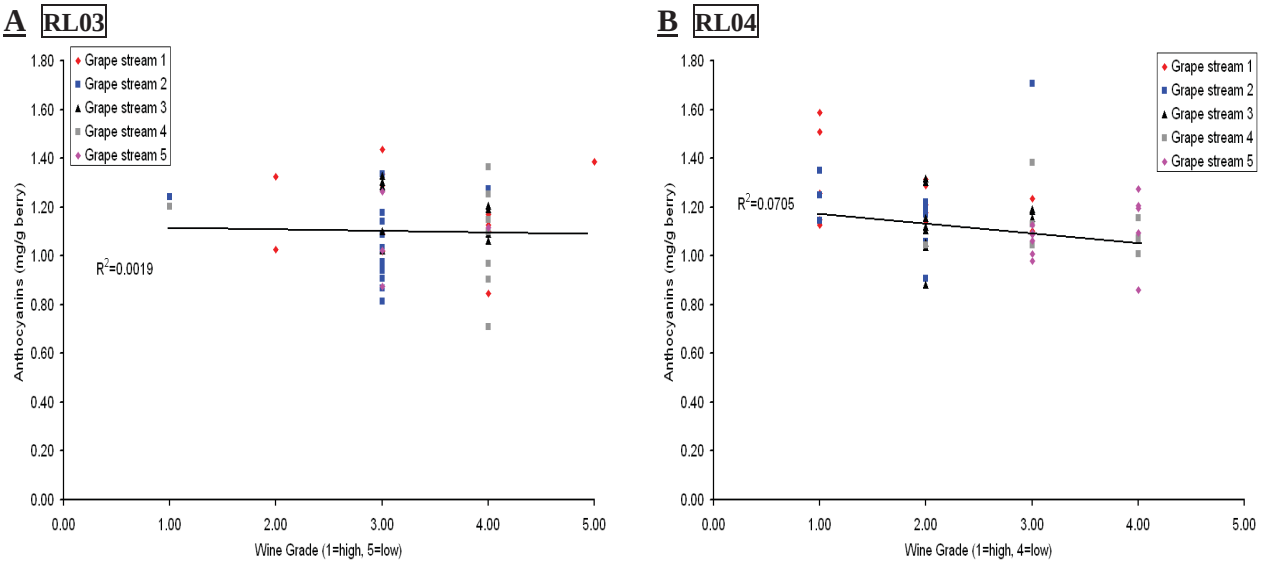


Figure 3.16 The relationship between anthocyanins & wine grade in Shiraz wine samples from the Riverland. For each Shiraz sample, skin anthocyanins were determined by HPLC. HWC provided the wine grade, where 1=high→5=low. Also indicated are the grape quality streams (colored), provided by HWC, where 1=high→5=low. Anthocyanin concentration is expressed on an mg/g berry basis.

A Relationship between anthocyanins in the grape & wine grade in Shiraz grape skins from the Riverland in 2003
B Relationship between anthocyanins in the grape & wine grade in Shiraz grape skins from the Riverland in 2004

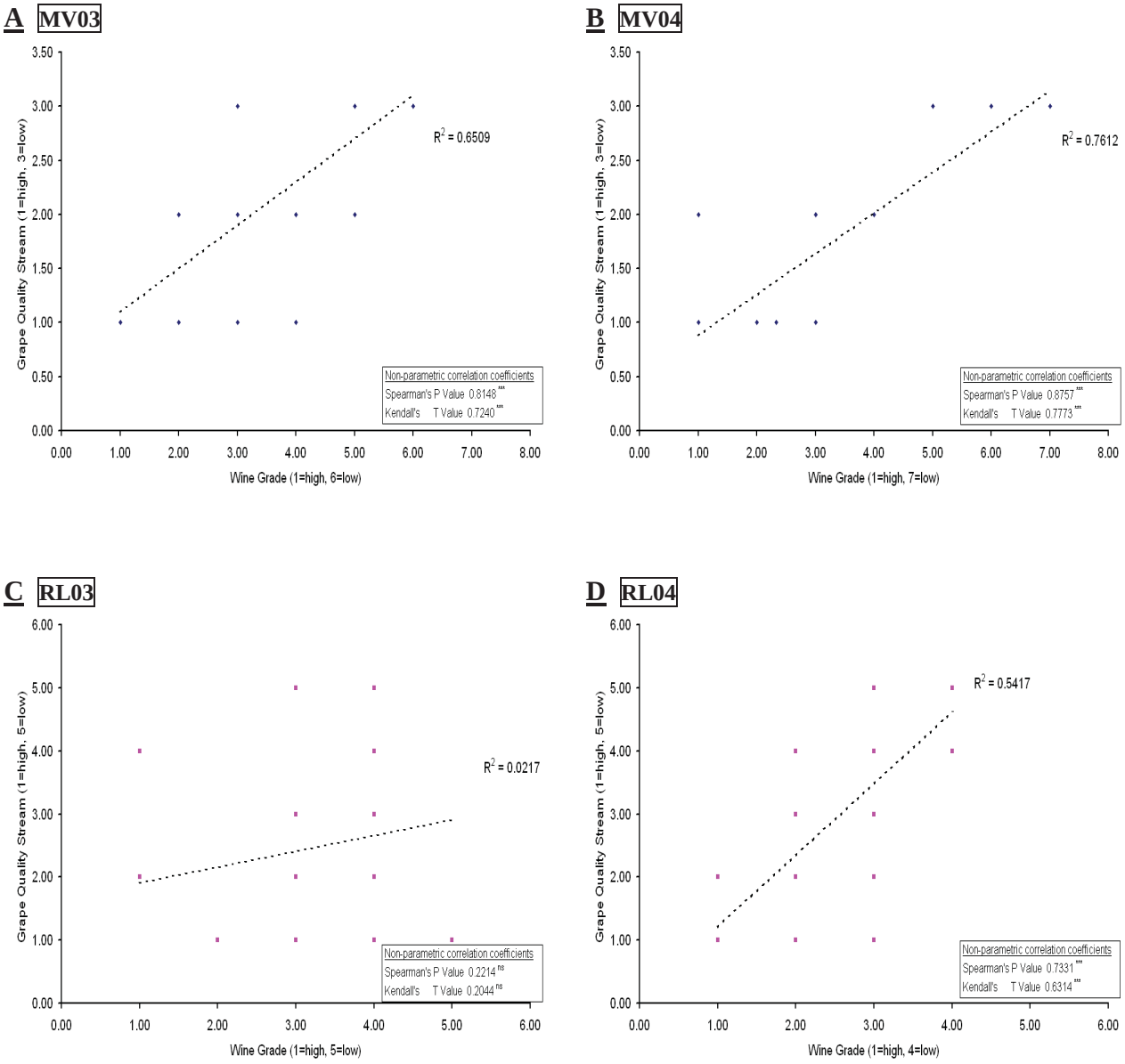


Figure 3.17 The relationship between grape quality stream & wine grade in Shiraz samples from McLaren Vale & Riverland. HWC provided grape quality stream & wine grade data, where 1=high→7=low. As data is ordinal, the strength of the linear relationship is uncertain; therefore the linear correlation is represented as a dashed line. Additional statistical tests were performed including a Spearman's & Kendall's test. The correlation coefficients for these tests & the level of significance are indicated (***) highly significant ($p<0.001$), (ns) not significant.

A Relationship between grape quality stream & wine grade in Shiraz samples from McLaren Vale in 2003
B Relationship between grape quality stream & wine grade in Shiraz samples from McLaren Vale in 2004
C Relationship between grape quality stream & wine grade in Shiraz samples from Riverland in 2003
D Relationship between grape quality stream & wine grade in Shiraz samples from Riverland in 2004

3.3.10 Miscellaneous relationships

In addition to relationships highlighted between the flavonoid compounds and grape and wine quality, additional relationships extrapolated from the data are listed in **Table 3.7**. In McLaren Vale, in both seasons, there was a weak positive relationship between ^{o}Brix and anthocyanins, however this relationship was not observed in Riverland fruit.

In this investigation, derivatives of caffeic and coumaric acid were tentatively identified (see **Chapter 2**). There was no relationship between either of these compounds and grape quality (data not shown), however caffeic acid (1) and coumaric acid (1), were strongly correlated with each other with R^2 values greater than 0.80. Furthermore, this relationship was maintained during fermentation, with R^2 values greater than 0.80 observed in McLaren Vale wines. There was also a positive relationship between caffeic acid 1 (and coumaric acid (1)) and skin weight, with R^2 values generally >0.30. The McLaren Vale vineyard factors, bunches/m and shoots/m were positively correlated with R^2 values >0.49. Interestingly, there was a negative relationship between coumaric acid (2) and these vineyard factors with R^2 values >0.30 (relationship with shoots/m shown). There was also a weak negative relationship between vine age and T/Ha. In wines, a weak positive relationship was observed between wine colour density and total tannins (R^2~0.20) and there was a correlation between wine grade and wine caffeic acid (1) with R^2 values >0.46.

Table 3.7 R^2 table of miscellaneous relationships between different factors in McLaren Vale & Riverland in 2003 & 2004. All measurements were performed by CSIRO except where indicated as HWC, which were performed by Hardy Wine Company. Missing values indicates the $R^2 < 0.2$.

FACTOR	FACTOR	R^2 Values			
		MV03	MV04	RL03	RL04
^{o}Brix	Anthocyanins	+0.279	+0.437		
Skin caffeic acid 1	Skin coumaric acid 1	+0.809	+0.854	+0.811	+0.821
Skin caffeic acid 1	Skin weight per berry	+0.344	+0.155	+0.306	+0.426
Bunches/m (HWC)	Shoots/m (HWC)	+0.490	+0.866		
Coumaric acid 2	Shoots/m (HWC)	-0.308	-0.403		
Vine age (HWC)	Yield (HWC)	-0.236	-0.128		
Wine caffeic acid 1	Wine coumaric acid 1	+0.941	+0.868		
Wine colour density (HWC)	Total tannins	+0.202	+0.210		
Wine grade (HWC)	Wine caffeic acid 1	-0.463	-0.611		

Sample details and the complete data sets for McLaren Vale and Riverland in the 2003 and 2004 seasons are available in **Appendix 3B**. Data available in **Appendix 3B** (**on CD**) includes; the mean values, a table R values (for which the nature of the relationship is noted as +/-) and a table of R^2 values (for which values greater than 0.2 are shaded (light green)).

3.4 Discussion

Grape berry development occurs primarily in two successive growth phases (Coombe and McCarthy 2000; Robinson and Davies 2000). The first phase of berry growth following flowering and fertilization involves berry expansion and seed formation, which occur in parallel. Following veraison, ripening occurs during the second growth phase and sugar accumulation occurs along with decreased acid levels, softening of the berry and accumulation of anthocyanins and flavour compounds. The flavonoid biosynthetic pathway consists of a series of common steps, whereby the common pathway is generally considered to result in anthocyanins, while flavonols and tannins are considered products of branches of the anthocyanin pathway (**Chapter 1, Figure 1.3**).

During berry development the synthesis of the flavonoid compounds occurs in different tissues and at different stages during grape berry development. Anthocyanins are located in berry skins and accumulation in Shiraz berries occurs after veraison, coinciding with increased expression of the flavonoid genes including *VvUFGT* (Boss et al. 1996a). Flavonols are also located in berry skins and in developing flowers (Downey et al. 2003b). The concentration of flavonols in grapes and the expression of the gene encoding flavonol synthase (*VvFLS1*) are found to be highest 1 week before flowering and 1-2 weeks after veraison, continuing throughout ripening (Downey et al. 2003b). Tannins are located in both seed and skin of berries and high levels have also been detected in developing flowers (Bogs et al. 2005; Coombe 1987). Tannin synthesis starts very early in berry development and continues until veraison in skins and for 1-2 weeks after veraison in seeds (Bogs et al. 2005; Downey et al. 2003a; Kennedy et al. 2001). Tannin accumulation coincides with the expression of leucoanthocyanidin reductase (*VvLAR*) and anthocyanidin reductase (*VvANR*) for formation of the tannin monomers catechin and epicatechin, respectively (Bogs et al. 2005). During the later stages of ripening, tannin maturation occurs, whereby the extractable levels of tannins start to decline, thought to be the result of complexation of tannin polymers with other cellular components (Coombe and McCarthy 2000; Downey et al. 2003a; Kennedy et al. 2000a).

However, in addition to the developmental biosynthesis of flavonols, there are also numerous cultural, climatic and environmental factors that can alter the levels of anthocyanins, flavonols and tannins, including: light, temperature, soil type, nutritional status, osmotic stress, vine vigour, defoliation, pathogenesis and plant growth regulators (see reviews, Downey et al. (2006) and Jackson and Lombard (1993)). Despite the variability conferred by these factors, one of the greatest influence on the flavonoid content and composition are site and season (Bakker 1986; de Freitas and

Glories 1999; Downey et al. 2006; Gonzalez-San-Jose et al. 1990; Guidoni et al. 2002; McDonald et al. 1998; Revilla et al. 1997).

At the start of this investigation, HWC representatives highlighted large differences in the relationship between anthocyanins and quality in red grapes and wine from different regions, where in warm regions colour can be used as an indicator of quality, whereas in the cool region colour is a less useful indicator of quality (Sas and Lim 2003)(**Figure 3.1**). The main objective of this study was to investigate the flavonoid content and composition of Shiraz grapes and determine their role in grape and wine quality in a warm and a cool region. There were three specific aims of this study, which were to investigate:

1. **The flavonoid content and composition of grapes from a range of warm and cool climate vineyards**
2. **The relationship between the flavonoid compounds in grapes**
3. **The flavonoids as indicators of grape and wine quality**

In collaboration with a major winery, more than 160 grape samples from two climatic regions (McLaren Vale and Riverland) and from two seasons (2003 and 2004) were analysed for flavonoid content and composition. With additional data provided by the winery possible relationships between different vineyard, grape and wine factors were also investigated.

3.4.1 The flavonoid content & composition of grapes from a range of warm & cool climate vineyards

There are many external variables that can influence berry development and flavonoid composition. However, one of the greatest influences on the flavonoid content and composition are site and season (Bakker 1986; de Freitas and Glories 1999; Downey et al. 2006; Gonzalez-San-Jose et al. 1990; Guidoni et al. 2002; Jackson and Lombard 1993; McDonald et al. 1998; Revilla et al. 1997). Early in this investigation it was established that the two regions; Riverland and McLaren Vale differed in site (e.g. management practices) and season (e.g. climatic differences) (**Table 3A.1**). Therefore considering the differences in site and season between these two regions, the flavonoid content and composition of grapes from the RL (warm region) and McLarn Vale (cool region) was compared. Particular focus is placed on the climatic factors, predominately temperature and light. However, the influence of management practices (e.g. pruning, irrigation) to control factors such as yield are also considered. It is important to be mindful of the fact that exploring the changes in

flavonoid content or composition between any two regions is a difficult task because of inherent complex nature of site and seasonal factors that can impact on a wide range of plant processes.

3.4.1.1 Grape berry characteristics

The flavonoids are localised in different parts of the grape berry. In skins anthocyanins, flavonols and tannins are detected, whilst in seeds only tannins are detected. Consequently berry size and the proportion of the individual parts of the berries (i.e. skin and seed) are important factors influencing the flavonoid content of grapes at harvest and of the subsequent wines produced (Coombe and Iland 2004; Downey et al. 2003a; Kennedy et al. 2000a; Souquet et al. 1996). The results presented in this investigation show that berries from McLaren Vale weighed less than berries from the Riverland (**Table 3.3**). While skin weight was relatively similar in both regions, there was a slightly higher proportion of skin weight per berry in McLaren Vale fruit, likely due to the differences in berry weight. Corresponding with smaller berries in McLaren Vale, seed weight was less in McLaren Vale compared to Riverland, while the number of seeds per berry was similar from both regions. The correlations between berry weight and some of the components of berries (i.e. skin weight and seed weight) are discussed in **Section 3.4.3** (below).

Grape berry ripening is influenced by numerous developmental and environmental factors (as reviewed in Downey et al. (2006)), and many of these factors can change the final weight of berries at harvest. Generally, changes in temperature and light exposure have shown not to greatly affect berry weight at harvest (Buttrose 1970; Buttrose et al. 1971; Cortell and Kennedy 2006; Dokoozlian and Kliewer 1996; Downey et al. 2004; Kliewer 1977; Kliewer and Torres 1972). In contrast, vineyard management techniques aimed at manipulating yield such as pruning treatments and/or deficit irrigation strategies, have shown to have both positive and negative influences on berry size (Dunn et al. 2004; McCarthy 1997; Roby et al. 2004; Walker et al. 2005). The two regions sampled in this investigation had very different viticultural management practices including different pruning techniques (i.e. warm- largely mechanised v cool- most by hand) and different irrigation requirements (i.e. warm- 5-7 ML/Ha drip irrigation v cool- 0.8-1.5 ML/Ha drip irrigation). Accordingly the average yield in each region was very different (i.e. warm ~20 T/Ha v cool ~5 T/Ha). These differences in site characteristics are likely to explain the differences observed in berry weight between the two regions. It was also observed that fruit from both regions and seasons had varying degrees of berry ripeness (^{o}Brix). This is likely due to the fact that berry flavor ripeness is a predominate factor that influences the harvest dates for a particular wine style which varies between the two regions (Coombe and McCarthy 2000).

3.4.1.2 Anthocyanin content & composition

Anthocyanins accumulate in the skins of grapes with the onset of veraison. In Shiraz, 15 different anthocyanin compounds were detected and these were separated into the mono-glucosides, acetyl-glucosides and coumaroyl-glucosides derivatives as well as the delphinidin, cyanidin, petunidin, peonidin and malvidin derivatives. There are many variables that have been shown to influence anthocyanin content and composition, including yield. In this investigation, anthocyanin content was generally higher in the low yielding vineyards (**Figure 3.12**) an observation consistent with previous reports (Clingeleffer et al. 2001; Coombe and Iland 2004; Grey et al. 1994; Holzapfel et al. 1999). The relationship between colour, yield and quality is discussed in more detail in **Section 3.4.3.4** (below).

In addition to yield the climatic factors; temperature and light have also reported to have a major effect on anthocyanin content and composition (Downey et al 2006). It has been previously shown that in hot regions, anthocyanin accumulation is inhibited in the skins of red grapes (Winkler et al. 1974). Temperature controlled experiments have also shown that exposing whole vines or bunches to high temperatures inhibits anthocyanin accumulation and biosynthesis (Buttrose et al. 1971; Haselgrove et al. 2000; Kliewer and Lider 1970; Kliewer and Torres 1972; Mori et al. 2005; Spayd et al. 2002; Yamane et al. 2006). Changes in anthocyanin composition due to elevated temperatures have also been recently studied. Downey et al. (2004) reported an increase in the proportion of coumaroyl-glucosides in Shiraz fruit in a season of high temperature, while Spayd et al. (2002) reported that cooling Merlot fruit decreased the coumaroyl-glucoside profile and heating caused a significant increase. Recently it was also shown in Cabernet Sauvignon berries that the content of individual anthocyanins, with the exception of the malvidin derivatives, decreases considerably under high temperature (35^{o}C) when compared to controls grown at (25^{o}C) (Mori et al. 2007).

It is also generally accepted that as fruit exposure to sunlight increases, fruit development, fruit composition, wine colour and quality improve, however, it has also been shown that grape berry colour and wine can be negatively affected by excessive light exposure, particularly in over-exposed fruit (Bergqvist 2001; Hunter et al. 1995; Kliewer 1977; Kliewer and Torres 1972). Recently it was shown that shading does not greatly influence total anthocyanin content, rather anthocyanin composition is altered with shaded fruit having a lower proportion of the trihydroxylated anthocyanins (the glycosides of delphinidin, petunidin and malvidin) and increased dihydroxylated anthocyanins (glycosides of peonidin and cyanidin) compared to fruit which was exposed to light (Cortell and Kennedy 2006; Downey et al. 2004; Pereira et al. 2006; Spayd et al. 2002).

Based on these previous studies on anthocyanin compositional changes under different regimes of temperature and light exposure, it was expected that fruit from the warm region might have reduced anthocyanin content and a higher proportion of malvidin, petunidin, delphinidin and coumaroyl-glucoside derivatives compared to the cool region. In part-agreement, the results in this investigation showed that the two regions could be separated based on colour (higher anthocyanin content in cool region) and that warm region fruit had a higher proportion of malvidin and coumaroyl-glucoside derivatives compared to the cool region (**Table 3.3**). However, there was no increase in the proportion of petunidin or delphinidin derivatives.

This observation suggests that the decrease in total anthocyanin content and shift in anthocyanin composition is more likely due to the temperature difference between the two regions, and not differences in light exposure. The two regions; Riverland and McLaren Vale are distinct climatic regions, with high summer temperatures and low rainfall in the Riverland compared to McLaren Vale (**Table 3A.1**). Although sunshine hours was higher in the Riverland compared to McLaren Vale, due to the array of different vineyard manipulations altering the canopy it may be that at the bunch level there is little difference in light exposure in both regions. Alternatively, significant changes in anthocyanin composition may only occur with dramatic changes to bunch light exposure (as demonstrated in the experiments by (Cortell and Kennedy 2006; Downey et al. 2004; Spayd et al. 2002)), while general climatic differences in light exposure is not sufficient to induce a large compositional change.

It was also observed that the anthocyanin composition in wines was relatively similar to that observed in grapes from McLaren Vale (data not shown). An exception however, was a reduced proportion of the anthocyanin coumaroyl-glucosides derivatives in wines compared to grapes, while the proportion of mono-glucosides derivatives appeared to increase. This could be an indication that the mono-glucoside derivatives in grapes are more readily extracted into wines, while the coumaroyl-glucoside derivatives are less extractable. Alternatively it could indicate the stability of the different types of anthocyanins in wines. This observation is in agreement with other reports that suggest the mono- and acetyl-glucosides are more readily extracted from the fruit than the coumaroyl-glucosides (Leone et al. 1984; Roggero et al. 1984). While the relative contribution of each of the grape anthocyanins components to final wine colour has yet to be established, the above observations may account for some of the reduction in grape berry colour and resultant wine colour observed in hot regions (Downey et al. 2006).

Overall, the results presented in this investigation agree with the extant literature; that temperature has a greater influence on anthocyanin accumulation and synthesis in grapes compared to changes in bunch light exposure. While the actual mechanism behind the change in anthocyanin content and composition at higher temperatures remains uncertain, the idea that it involves anthocyanin degradation and/or inhibition of anthocyanin biosynthesis is strongly supported in numerous publications (Crippen and Morrison 1986a; Downey et al. 2004; Markakis 1982; Morais et al. 2002; Mori et al. 2007; Mori et al. 2005; Pirie and Mullins 1977; Romero and Bakker 2000; Spayd et al. 2002; Yamane et al. 2006; Yamasaki et al. 1996).

3.4.1.3 Flavonol content & composition

Flavonols accumulate in the skins of Shiraz grapes during berry ripening and in this investigation the glycosides of quercetin, myricetin, kaempferol, isorhamnetin and syringetin were detected. Flavonol biosynthesis is highly light dependent and there are numerous studies showing reduced levels of flavonol glycosides in shaded grapes and higher levels in sun-exposed grapes (Cortell and Kennedy 2006; Downey et al. 2004; Haselgrove 1997; Haselgrove et al. 2000; Price et al. 1995a; Spayd et al. 2002). In this investigation total flavonol content was greater in the cool region, and in general there were no great differences in flavonol composition (**Table 3.3**). This observation may indicate that there may be higher level of bunch light exposure in the cool region, however, it is more likely that bunch light exposure would be similar in both regions (as noted earlier). Furthermore, it could be that there are other vineyard factors, such as yield that could be altering the levels of flavonols in fruit.

3.4.1.4 Tannin content

Tannins accumulate in both the skin and seeds of grape berries and synthesis primarily occurs from flowering until veraison. In 2004, total tannin content was measured in both skin and seeds in both regions. The results presented in this investigation showed there was no significant difference in total tannin content of grapes from both a warm and cool region (**Table 3.3**). While skin tannins were slightly higher in the warm region there was no difference in total seed tannin content between regions.

Tannin synthesis, accumulation and maturation during grape berry development has been shown to be complex, with considerable differences reported between the skin and seed (Amrani-Joutei et al. 1994; Bogs et al. 2005; Cheynier et al. 1997; de Freitas and Glories 1999; Downey et al. 2003a; Escribano-Bailon et al. 1995; Fujita et al. 2005; Iland 1998; Kennedy et al. 2001; Lepiniec et al.

2006). There are also many vineyard factors that have been shown to alter levels of tannins in grapes including vine vigour (Cortell et al. 2005) and vine water status (excess/deficits) (Kennedy et al. 2000a; Roby et al. 2004). However the difficulty in interpreting these factors is that they impact on a wide range of plant processes apart from flavonoid biosynthesis (i.e. alter photosynthesis and plant source-sink relationships).

There are also several studies showing the influence of general climatic conditions on the phenolic content of grapes (Arozarena et al 2002; Crippen and Morrison 1986b; Mateus et al. 2001; Wicks and Kliewer 1983), however, only a limited number have explored the effects of temperature and light exposure (independently of each other) on tannin content and composition. In Shiraz berries, tannin content and composition has been shown to be largely unaffected by bunch light exposure (Downey et al. 2004), however in Pinot noir berries a reduction in skin proanthocyanidin content and composition has been reported (Cortell and Kennedy 2006). Furthermore, while it has been suggested that temperature plays a critical role in the accumulation of some berry phenolic classes (Spayd et al. 2002), only recently has an increase in the tannin content in Pinot noir grapes and wines been linked to an increase in heat summation between fruit set and veraison (Pastor del Rio and Kennedy 2006).

3.4.1.5 Summary

This investigation clearly shows differences in flavonoid content and composition of fruit grown in warm and cool regions. In the warm region, there was a decrease in total anthocyanin content, and compositionally there were a higher proportion of malvidin and coumaroyl-glucoside compounds compared to the cooler region. This observation suggests temperature has some influence on anthocyanin accumulation, in agreement with previous studies (Buttrose et al. 1971; Downey et al. 2006; Kliewer and Torres 1972; Mori et al. 2007; Mori et al. 2005; Spayd et al. 2002; Yamane et al. 2006). Similar to anthocyanin accumulation, total flavonol content was also lower in the warm region, but there were no differences in composition. Although skin tannin content was slightly higher in the cool region, total tannin content remained similar in both regions. To date, there are limited reports on the effect of temperature (independent of light) on the synthesis of flavonoid compounds, particularly flavonols and tannins, throughout berry development and consequently this remains an area of future research (see **Chapter 6, Section 6.6.1**).

3.4.2 The relationship between the flavonoid compounds in grapes

The flavonoids are important quality components in grapes, and while the flavonoid pathway has been well studied in relation to anthocyanin synthesis (Boss et al. 1996a), only recently has the synthesis of flavonols and tannins been reported during Shiraz berry development (Bogs et al. 2005; Downey et al. 2003a; Downey et al. 2003b). However, little is known about regulation of the pathway, and whether the synthesis of the different flavonoid compounds is coordinated or independently regulated. Hence, the second aim of this investigation was to understand how the synthesis of anthocyanins, flavonols and tannins is coordinated in Shiraz grape berries.

3.4.2.1 Anthocyanins & flavonols

The results presented in this investigation showed a positive relationship between anthocyanins and flavonols in Shiraz grapes from both a warm and cool region (**Figure 3.5**). This suggests that these compounds might be co-ordinately synthesised during grape berry development. This observation, however, was somewhat surprising considering the temporal separation of anthocyanin and flavonol synthesis during grape berry development and the different response of these compounds to bunch light exposure and temperature.

Anthocyanins and flavonols are synthesised in berry skins at similar times during the ripening phase of grape berry development (Boss et al. 1996a; Downey et al. 2003b). As dihydroflavonols are common precursors for anthocyanin and flavonol synthesis acting as substrates for DFR, F3H and FLS, the potential may exist for competition between each of these pathways for common substrates. Consequently, anthocyanins would be synthesised at the expense of flavonols and as such negative coordination in synthesis may occur. This type of competition for substrates between anthocyanin and flavonol synthesis has been suggested to occur in other plant species (Davies et al. 2003a; Holton et al. 1993; Martens et al. 2001; Nielsen et al. 2002).

Anthocyanin and flavonol synthesis has been shown to respond differently to a range of climatic factors. It is widely acknowledged that flavonol synthesis and accumulation is light dependent in grapes while tannin and anthocyanin synthesis remains largely unchanged (Cortell and Kennedy 2006; Downey et al. 2004; Haselgrove et al. 2000; Price et al. 1995a; Spayd et al. 2002). It is also generally recognised that temperature has a greater influence on anthocyanin biosynthesis in grapes than light, where higher temperatures have a negative impact on anthocyanins biosynthesis (Buttrose

et al. 1971; Kliewer and Torres 1972; Mori et al. 2007; Mori et al. 2005; Spayd et al. 2002; Yamane et al. 2006). Hence, the difference in anthocyanin and flavonol biosynthesis in response light exposure and temperature was another motive to suspect separate regulation of these pathways.

There was also a relationship between anthocyanins and flavonols observed in wines from McLaren Vale, suggesting this relationship is maintained during fermentation. It also implies the total level of these flavonoid compounds are extracted proportionally to each other during fermentation. Anthocyanin extraction from grape skins occurs early in fermentation and then subsequently declines, a phenomenon that has been suggested to be due to anthocyanin instability once released and/or limited extraction from the grape skins (Boulton 2001; Boulton et al. 1998; Sacchi et al. 2005). It has also been shown that flavonols are extracted within days of the onset of fermentation, similar to anthocyanins (Price et al. 1995b). Despite numerous studies measuring flavonoid content and composition in grapes and in corresponding wines, there are limited studies that have measured extraction of anthocyanins, flavonols and tannins from different parts of the grape berry. This is likely due to the inherent amount of variation in accurately sampling these tissue types during fermentation. The interaction between the flavonoid compounds extracted from grape berry tissues during fermentation have yet to be established and remains an area of future research (see **Chapter 6, Section 6.6.4**).

3.4.2.2 Anthocyanins & tannins

The relationship between anthocyanins and tannins in berries was also observed in this investigation (**Figure 3.6**). There was no relationship between seed tannins and anthocyanins, however, there was a possible positive relationship between skin tannins and anthocyanins, although this relationship was not strong. Accordingly, the relationship between seed and skin tannins, and flavonols was for the most part, similar to that for anthocyanins (and as such is not discussed). These results indicate that there may be some positive coordination in synthesis between anthocyanin and skin tannins but no coordination with seed tannins. There are numerous issues to consider with this result including: the temporal expression of anthocyanin and tannin synthesis during grape berry development, the spatial separation between skin and seed, the influence of tannin maturation and the different response of anthocyanin and tannin biosynthesis to various external cues.

It has been previously shown that anthocyanin and tannin accumulation occurs in different grape tissues and at different times during grape berry development (Boss et al. 1996a; Downey et al.

2003a; Kennedy et al. 2001; Souquet et al. 1996). Recently, Bogs et al. (2005) showed that expression of the grapevine tannin gene, *VvANR* in tobacco plants, competed with expression of *VvUFGT* for the substrate cyanidin, diverting metabolism away from production of the anthocyanins and toward production of epicatechin. It was suggested that a relationship between anthocyanins and skin tannins might exist, whereby anthocyanins would be co-ordinately synthesized at the expense of skin tannins, via the conversion of intermediate compounds, anthocyanidins (Bogs et al. 2005). Consequently, negative coordination in synthesis may be expected between these two compounds.

It is also important to consider that the concentration of seed and skin tannins start to actually decline after veraison, as they undergo tannin maturation (Downey et al. 2003a; Kennedy et al. 2001; Kennedy et al. 2000a). Tannin maturation is thought to be the result of complexation of the tannin polymers with other cellular components, however the actual mechanism remains unknown (Amrani-Joutei et al. 1994; Coombe and McCarthy 2000; Downey et al. 2003a; Fournand et al. 2006; Gagne et al. 2006; Geny et al. 2003; Kennedy et al. 2001; Kennedy et al. 2000a; Robinson and Walker 2006; Xie and Dixon 2005). Thus, the concentration of tannin compounds detected in grapes at harvest represents a balance between accumulation of tannins through synthesis and decreased extractability.

There was no relationship between skin tannins and seed tannins or any of the skin flavonoid compounds (anthocyanins, flavonols) and seed tannins, most likely due to the spatial variation. While skins and seeds have features in common in regard to phenolics and ripening, they also have distinct functions within the berry. The skin is a fleshy tissue type and has both a protective and active metabolic role in grapes, whereas, seeds are hard structures, and play an important role in protecting the placental parts of the berry (i.e. nucellus, endosperm and embryo) (Adams 2006). It has also been well established that the composition of tannins is different in skin and seed (Downey et al. 2003a; Kennedy et al. 2001) and recently tannin synthesis was shown to occur independently in the skin and seeds (Bogs et al. 2005). Therefore, the poor relationship between tannins within these tissue types and with other flavonoid compounds was not unexpected; rather it reiterates the independent nature of tannin regulation in seeds and skins.

Lastly, there are numerous climatic conditions and viticultural management practices, which may alter the amount and type of tannins that accumulate in fruit. These include vine water status

(Kennedy et al. 2002; Roby et al. 2004), vine vigour (Cortell et al. 2005) and sun exposure (Cortell and Kennedy 2006; Downey et al. 2004; Ristic et al. 2004). While these studies, along with others (reviewed in Downey et al. (2006)) point towards a variety of factors that can influence tannin synthesis during the ripening phase, the specific nature of many of these interactions are not yet well defined. As such, the variation in tannin biosynthesis observed in response to different external cues was another reason to suspect separate regulation of the tannin and anthocyanin pathways.

3.4.2.3 Regulation of the flavonoid biosynthetic pathway

The results presented here show a positive relationship between anthocyanins and flavonols and only a slight positive trend between anthocyanins and tannins in Shiraz grape berry skins. This suggests that regulation of the flavonoid biosynthetic pathways in grape berry skins may be to some extent coordinated. There are a number of mechanisms by which the flavonoid pathway can be organised to facilitate the coordination of biosynthetic activities both on a transcriptional and post-transcriptional level, as discussed below.

3.4.2.3.1 Transcriptional regulation

Transcriptional regulation of the flavonoid pathway has been well document in a variety of plants including, *Arabidopsis*, maize, petunia and snapdragon (Winkel-Shirley 2001). Transcriptional control essentially involves the DNA-binding of different, yet specific, transcription factors to promoter regions of the flavonoid genes. One of the key classes of transcription factors involved in controlling parts of the flavonoid pathway includes the MYB proteins (Koes et al. 2005).

In grapevine, regulation of anthocyanin pathway has been shown to be controlled by two MYB proteins, VvMYBA1 and VvMYBA2, that activate *VvUFGT* in red berries (Kobayashi et al. 2002; Kobayashi et al. 2005). Only recently has regulation of the tannin pathway been reported, where the transcription factor, VvMYBPA1 activates the promoters of *VvLAR* and *VvANR* but not *VvUFGT* (Bogs et al. 2007). A MYB transcription factor VvMYB5a has been shown to induce synthesis of anthocyanins, tannins, flavonols and lignin when expressed in tobacco (Deluc et al. 2006). While the transcription factor/s responsible for flavonol biosynthesis in grapevine have not been isolated, recently in *Arabisopsis*, a MYB transcription factor, AtMYB12, was found to be a flavonol specific activator of flavonoid biosynthesis, activating *AtFLS* and *AtCHS* genes. These studies suggest that regulation of the flavonoid pathway by MYB transcription factors is likely to be specific for a

specific class of flavonoid compound. Yet, in the case of VvMYB5a, there may also be transcription factors, which have broader functions and are able to up-regulate whole parts of the pathway.

The results presented in this investigation suggest that there could be transcription factors that are able to act in coordination with each other (eg. during development) and other transcription factors that can operate independently of other factors (eg. in response to different environmental stresses). Considering there are a number of regulatory genes responsible for synthesis of anthocyanin, flavonols and tannins in plants and that the interaction of these genes and the molecular genetics of control have yet to be determined, transcriptional regulation remains an area of flavonoid biology in which much remains to be learned.

3.4.2.3.2 Post-transcriptional regulation

In addition to transcriptional changes, there are numerous post-transcriptional processes that are likely to alter the levels of flavonoids, including enzymatic regulation via protein modification(s) and control of metabolic flux by interactions with intermediate substrates. Also, flavonoid organisation in the cell may be dependent on transport and storage of these compounds on both a cellular (i.e. different cell types) and sub-cellular level (i.e. within cells).

3.4.2.3.2.1. Enzymatic regulation

a) Modification of the protein

There are many factors that can affect protein stability, including general cell environment factors such as temperature and pH, and also by particular chemicals that can specifically influence a particular enzyme (i.e. co-factors or enzyme inhibitors) (Elliot and Elliot 1997). The activity of enzymes can be modified by allosteric control and/or covalent modification (eg phosphorylation). Allosteric control works on the principle that each enzyme can potentially have more than one specific binding site, thereby increasing the flexibility of control (Elliot and Elliot 1997). Regulation by phosphorylation reactions involve enzymes called protein kinases which transfer phosphoryl groups from ATP to specific proteins and when this happens the target enzyme undergoes a conformational change and the enzyme becomes active (or may alternatively have an inhibitory effect) (Elliot and Elliot 1997). Consequently, this type of regulation requires a balance between active (phosphorylation) and non- active (non-phosphorylation) status, and is subsequently more controlled regulation. It has been previously shown that anthocyanin and tannin synthesis

requires at least one ATPase for transport of these compounds into the vacuole (Alfenito et al. 1998; Baxter et al. 2005; Muller et al.2001). Although it is widely regarded that the flavonoid biosynthetic pathway is organised as a multi-enzyme complex that could potentially have many binding sites allowing for rapid biosynthesis (Stafford 1990; Stafford 1991; Winkel-Shirley 2001), this has yet to be established.

b) Control of metabolic flux

Flavonoid biosynthesis may also be regulated at the substrate level, by feedback/forward inhibition of certain enzymes. In *Arabidopsis* it has been shown that some downstream flavonoid products function together with upstream intermediates to regulate flux through the pathway system (Li et al. 1993; Pelletier et al. 1999). This type of control is suggested to provide a means of matching the metabolic flux through the pathway to the accumulated end product (Tanner 2004; Winkel-Shirley 2002). As flavonoid intermediate levels are low and difficult to measure, most studies to date have measured the end products and related this to transcriptional control (Goes da Silca et al. 2005). The control of metabolic flux of flavonoid biosynthesis is a post-transcriptional process that is largely underestimated in plants.

3.4.2.3.2.2. Organisation in the cell

a) Sub-cellular compartmentalisation

There are many sub-cellular processes that may regulate flavonoid biosynthesis including transport and storage of flavonoid biosynthetic products. Flavonoid biosynthesis is suggested to occur near the endoplasmic reticulum where products are subsequently transported across the tonoplast into the vacuole (Abrahams et al. 2003; Schwinn and Davies 2004). It has been shown that anthocyanin intra-vacuolar transport involves glutathione-S-transferase (GTS) protein and an ATP-requiring transport protein (Alfenito et al. 1998; Mueller et al. 2001). In contrast, intra-vacuolar transport of tannins is suggested to require at least two specific transport proteins, one of which has been shown to be ATP dependent (Baxter et al. 2005; Debeaujon et al. 2001; Kitamura et al. 2004; Mueller et al. 2001; Tanner 2004). Whilst these studies indicate that different transporters are responsible for the vacuolar transfer of different flavonoids, the exact mechanism(s) of transport, the number of transporters required and precisely what type of compounds are transported (i.e. compounds modified by glycosylation/polymerisation), to date, remains largely unknown in plants (Schwinn and Davies 2004).

Coupled with transport of anthocyanins and tannins across the tonoplast is storage of these compounds once inside the vacuole. It is commonly regarded that anthocyanins are localised in pro-vacuoles, called anthocyanic vacuolar inclusions (AVIs) (Markham et al. 2000). AVIs are suggested to increase anthocyanin stability and also reduce inhibition of certain vacuolar enzymes (Zhang et al. 2006). In grapevine cell cultures, AVIs have been shown to have a different anthocyanin composition (i.e. increased coumaroyl-glucoside content) compared to the whole cell (Conn et al. 2003), suggesting selectivity of a particular anthocyanin derivative.

In this investigation (as a side experiment), the flavonoid composition of AVIs from grape cell cultures (described by Conn et al. (2003)) was determined. It was found that anthocyanins and tannins were present in the AVIs, but not flavonols (data not shown). Confirming results by Conn et al. (2003), the anthocyanin content and composition of the AVIs were higher in acylated derivatives (i.e. coumaroyl-glucosides) than non-acylated derivatives compared to the whole cell. The absence of flavonols in AVIs isolated from grape cell culture confirmed observations by Markham et al. (2000) who also did not detect flavonols in AVIs isolated from the petals of various flower species. The localisation of tannins in AVIs has not been previously reported, however it is generally recognised that tannins are localised in pro-vacuoles in plant cells (Abrahams et al. 2003; Tanner 2004). The observation that anthocyanins and tannins are localised in AVIs may be of significance, as the role of these compounds acting as co-pigments for each other in berry cells has not been firmly established.

b) Cellular compartmentalisation

While it is widely recognised that skin anthocyanins, flavonol and tannins are localised to the hypodermal cells in grape berry skins (Adams 2006; Coombe and Iland 2004; Hardie et al. 1996), there is little information surrounding the precise localisation of these compounds in different cell layers. In one study, it has been shown that anthocyanin content differs in different layers of hypodermal cells (Hrazdina and Moskowitz 1980; Hrazdina et al. 1978). It has also recently been shown that different types of tannins are localised in different cells and in different layers of the berry skin (Cadot et al. 2006; Gagne et al. 2006). These studies suggest that different cell layers in the skins of grape berries may accumulate different types and amounts of flavonoid compounds. This is an important observation when considering how accumulation of the flavonoid compounds may be controlled at the level of individual cells (Adams 2006).

It should also be noted that metabolic processes such as glycosylation and polymerisation might also play a role in the accumulation of anthocyanins, flavonols and tannins, respectively. These metabolic reactions are suggested to occur in the vacuole and are thought to provide stability for these compounds (Hagerman and Butler 1994; Hrazdina et al. 1978; Tanner 2004). However the exact mechanism, the genes/enzymes responsible and location where these reactions occur have yet to be elucidated, particularly for tannin and flavonol biosynthesis (Adams 2006; Tanner 2004; Winkel-Shirley 2002; Xie and Dixon 2005). Consequently, these processes warrant further investigation.

3.4.2.4 Summary

Based on the relationships observed between anthocyanins, flavonols and tannin in grape berry skins in this study, it was suggested that there might be some coordination in biosynthesis of these compounds. There are a number of mechanism by which metabolism can be organised so as to facilitate the coordination of biosynthetic activities, including transcriptional and post-transcriptional processes. Although significant progress has recently been made in understanding of transcriptional control of the flavonoid biosynthetic genes in grapevines, post-transcriptional processes remain poorly understood. Areas of unknowns include: enzyme structure, function and regulation and the mechanism(s) by which flavonoids are transported and stored in plant cells. Nevertheless, at both levels of regulation (pre or post transcription) it could be that the genes or enzymes, or the regulators of these, respond differently to internal and external cues, which would ultimately influence the final concentration of flavonoids that accumulate in cells. It is clear that further research is required to determine how co-ordination in flavonoid biosynthesis in grapes might be controlled within the cell and at the level of individual cells, as it is this which ultimately determines how these products are extracted into wine (see **Chapter 6, Section 6.6.2**)

3.4.3 Indicators of grape & wine quality

Although there is no clear quantifiable definition for grape or wine quality (**Introduction, Section 1.9**), some measures of grape juice characteristics that have been used to predict wine quality, include total soluble solids (TSS/°Brix), pH and titratable acidity (TA) and grape colour (Coombe and Iland 2004; Swinburn 2003). There are a variety of viticultural practices (i.e. irrigation, pruning, canopy management, use of fertilisers and pesticides) and environmental factors (i.e. temperature, rainfall, humidity, sun-exposure) that can affect grape berry ripening and the composition of fruit at harvest, resulting in changes in these juice characteristics (Downey et al. 2006; Jackson and Lombard 1993). Climate has been observed by wine industry representatives as one of the major factors for variation in relationships between colour and wine quality. In the warm regions, colour can be used as an indicator of wine quality, whereas in the cool region, colour is a less reliable indicator of wine quality (**Section 3.1**, **Figure 3.1**). Recently, there has been a move within the wine industry toward a more objective definition and measurement of grape quality in terms of composition factors in addition to sugar, acidity and colour, which affect wine properties. Hence, the aim of this study was to determine how the flavonoid compounds contribute to grape and wine quality in both warm and cool climates. Identifying potential indicators of grape and wine quality ultimately may enable viticulturists and winemakers to change management practices that may lead to better quality grapes and wines.

No obvious relationships were found between grape quality stream or wine grade and any of the berry components (berry weight, seed/skin weight), juice composition (^{o}Brix) or grape flavonoid composition (anthocyanins, flavonols, tannins) in the warm or cool region (**Table 3.5** & **Table 3.6**). This result was somewhat surprising considering the historical information regarding some of these factors as indicators of 'quality'. Although there was some relationship between 'quality' and the McLaren Vale vineyard factors (vine age, canopy assessments), these relationships were not strong. Using berry factors (i.e. berry weight, seed/skin weight, ^{o}Brix and flavonoid composition) and vineyard factors (i.e. yield, vine age and canopy assessments) as indicators of grape and wine quality, are discussed below.

3.4.3.1 Berry weight

Berry weight/size has been widely recognized as a factor determining wine grape quality (Dry et al. 2004a; Gladstones 1992; McCarthy 1997). A presumed role for berry size as a factor in wine grape

quality arises from a recognition that important constituents localized in the skin of harvested berries, become diluted by the juice released from the berry flesh upon crushing (Coombe 1987a). The implicit mechanism of this concept involves the surface area: volume ratio, whereby smaller berries, compared to large berries, have greater skin/flesh ratio, said to improve red wine colour and quality (Gladstones 1992; May 2004; Singleton 1972).

In this investigation, there was no consistent relationship between berry weight and grape and wine quality (**Table 3.5** & **Table 3.6**), with a large variation in berry size in each quality stream/grade. While there is experimental evidence supporting the idea of a relationship between berry weight and wine grape quality (Botting et al. 1996; Johnstone et al. 1996; Rolley 2004; Winter 2005), recently it was argued that the amount of skin (and seed) solutes might not be a simple function of berry volume (Roby et al. 2004). The results from an irrigation deficit experiment indicated that the source(s) of variation in berry size are more important in determining must composition and wine sensory properties than berry size *per se* (Roby and Matthews 2004). This idea was supported by Walker et al. (2005) and Walker et al. (2002), who made wine from different sized Shiraz berries, and found that wine characteristics, including colour were similar from small and large berries. They concluded that the increase in wine quality from smaller berries was primarily due to the treatments used to induce small berries rather than the smaller berries, as such.

The relationship between berry weight, the components of berries (skin, seeds) and the flavonoid content were also established in this investigation (**Table 3.4**). Skin weight per berry was linearly related to berry weight yet skin weight, as a proportion of berry weight, was almost constant among different berry sizes. This result indicates that growth of the skin and flesh appears to be coordinated, leaving the relative skin mass essentially unchanged, as also reported by Roby et al. (2004).

The amount of seed development can have a profound effect on the development on the pulp of the berry (i.e. berry weight) (Coombe and Iland 2004). A positive relationship between seed weight and berry weight was observed in all data sets (except Riverland, 2003), yet there was no relationship between seed number and berry weight (**Table 3.4**). These results suggest that individual seed size has a greater influence on berry weight rather than seed number, with a few large seeds having more impact on berry growth than a greater number of small seeds. Accordingly, there was a strong negative correlation between seed weight (as a proportion of berry weight) and berry weight

whereby smaller berries had a larger proportion of seed weight per berry. Previous studies have shown a strong positive correlation between berry weight and seed number (Boselli et al. 1995; Boulton et al. 1998; Cawthorn and Morris 1982) however, recent studies have indicated that berry weight is better correlated with seed weight per berry (Downey 2002; Roby et al. 2004). Nevertheless, in regard to any of these berry components (i.e. skin weight, skin weight (% of berry weight), seed weight, seed weight (% of berry weight)) relating to grape and wine quality there were no strong correlations observed in this study (data not shown).

The concentration of skin anthocyanins and tannins per berry were relatively insensitive to berry size for most data sets, yet there was an inverse relationship between skin flavonols per berry and berry weight (**Table 3.4**). The poor relationship between berry weight and anthocyanin content is in contrast to previous studies that found anthocyanin concentration decreased with berry size (Hardie et al. 1997; Roby and Matthews 2004; Ummarino and Di Stefano 1997). Also, no relationship between skin tannins and berry weight has been reported (Roby and Matthews 2004) where the concentration of skin tannins was essentially unchanged with berry size. The relationship between skin flavonols and berry weight has not been previously reported, and the reason behind such a relationship remains unclear.

The concentration of seed tannin did not change with berry size (**Table 3.4**). This was due to the fact that despite a strong positive correlation between seed tannins and seed weight (as a proportion of berry weight) there was a strong negative correlation between berry weight and seed weight (as a proportion of berry weight), which resulted in no relationship between seed tannins and berry weight. This is in contrast to work by Roby et al. (2004) who found that in general seed tannin content increased with berry size. However, this observation was primarily attributable to the fact that they observed seed weight (as a proportion of berry weight) increasing rather than decreasing with berry weight, unlike the results observed in this investigation.

3.4.3.2 Juice composition (^{o}Brix)

The measurement of grape total soluble solids (TSS/^{o}Brix) provides an assessment of grape ripeness in terms of sugar content, where during winemaking sugar is converted to alcohol. General observations have been that higher levels of TSS in grape juice have been associated with increases in wine quality (Caroll et al. 1978; Maujen et al. 1983). However, these improvements in wine

quality are most likely to be indirectly due to other changes in fruit composition associated with advanced ripeness, rather than due to increases in sugar content alone. It is now commonly regarded that knowledge of TSS concentrations is an essential indicator for wine style, but by itself, fails as a guide to 'quality'. This is illustrated by the fact that the same percent alcohol is seen on the labels of both high and low quality wines within each wine style (Coombe and Iland 2004). These observations are in agreement with the results presented in this investigation, where there was no relationship between TSS and grape and wine quality (**Table 3.5** & **Table 3.6**).

The relationship between TSS and berry weight was also investigated. While a slight inverse trend was observed in some data sets, overall it appeared that there was no relationship between TSS and berry weight, with varying sugar levels for different sized berries (**Table 3.4**). There was also no relationship between TSS and grape anthocyanin, flavonol or tannin content in the Riverland, however a weak relationship between TSS and anthocyanins was observed in McLaren Vale fruit (**Table 3.7**). Although an inverse relationship between ^{o}Brix and berry weight has been previously described (Cawthorn and Morris 1982; Roby and Matthews 2004), there are numerous reports suggesting sugar accumulation is largely independent of berry size (Boselli et al. 1995; Boulton et al. 1998; Coombe and Iland 2004; Coombe 1989; Glynn and Boulton 2001; Gray 2002; Trought 1996). The data presented in this investigation lends support to the notion that sugar accumulation in the fruit is independent of other processes occurring in the berry during ripening.

3.4.3.3 Grape flavonoid content & composition (anthocyanins, flavonols & tannins)

Anthocyanins are the compounds responsible for red colour observed in grapes and wines. It has long been recognized in the wine industry, that wine colour is related to red wine quality (Somers and Evans 1977). Yet, it is also understood that highly colored grapes do not necessarily produce highly colored wines, which depend on the fermentation practices and/or co-pigmentation reactions. Nevertheless, the anthocyanin content of grapes at harvest is of vital importance in achieving quality wines. Presently, some Australian wineries including HWC have adopted the use of grape berry colour as one of the factors used in determining grape quality (Barnett 2004; Bevin 2005; Swinburn 2003).

In this investigation there was no relationship between total anthocyanin content (or any of the individual anthocyanin compounds) and grape quality stream in both regions (**Table 3.5, Figure**

3.10). This was also observed by HWC, when colour was measured by NIR (**Table 3.5**). A reason for this poor relationship may be that the colour measurements made in this investigation were on fruit obtained from the weighbridge and not from fruit taken at the final stage of berry sampling, which was when the fruit was streamed into quality streams. HWC did not provide the colour data measured as part of the pre-harvest berry assessments, so the relationship between the grape colour measurements in the vineyard and quality stream allocation cannot be drawn (as schematically represented in **Figure 3.2**).

The pre-harvest vineyard assessment is performed approximately 1-4 weeks prior to harvest (see **Section 3.1.1.2**). Therefore it is plausible that there may have been climatic, cultural or viticultural factors that may have significantly altered the colour of the grape berries in the last week/s prior to harvest. For example, water deficits during the ripening phase of grape berries have been shown to alter the concentration of anthocyanins at harvest (Matthews et al. 1990). Also in Shiraz, a phenomenon has been observed whereby berries start to systematically shrivel 90 days after flowering (Coombe and McCarthy 2000; McCarthy 1999; McCarthy and Coombe 1999), an effect that could also alter the anthocyanin concentration. Additionally, it has also been shown that colour levels can significantly reduce between the vineyard and weighbridge (i.e. storage time between harvest and crushing) resulting from polyphenol oxidase activity, a problem commonly associated with in mechanically harvested fruit (Krstic et al. 2002).

There are only a small number of studies investigating the relationship between grape colour and final wine quality, and coincidentally most of these studies have been performed in Shiraz berries from warm-hot regions. Work by Francis et al. (1999) and Rolley (2004) have shown anthocyanin levels in grapes strongly correlate with final wine quality, over a wide array of growing conditions. However, the linear correlation determined by Francis et al. (1999) was confined to grapes with no more than 1.3 mg/g anthocyanins, after which the authors suggest that the linear relationship may not hold. Also, Rolley (2004) emphasised there was a strong correlation only when the fermentation process was standardized, as differences in fermentation practices significantly skewed relationships. Overall, it appears that the reasons for the good correlations between grape anthocyanins and red wine quality have yet to be elucidated, however could stem from the correlation with other compounds, such as flavonols or tannins or perhaps aroma and flavour compounds (as discussed below).

Flavonols are the least abundant class of flavonoid compounds in grapes, however in wines, they are thought to play a role in co-pigmentation and stabilisation of anthocyanins during fermentation and ageing of red wines (Baranac et al. 1997; Boulton 2001; Downey et al. 2003b; Lambert 2002; Mayen et al. 1995; Price et al. 1995b; Scheffeldt and Hrazdina 1978). It is also widely accepted that flavonol synthesis is strongly light dependent, and so the concentration of flavonols in fruit appears to be a good indicator of bunch exposure during development (Cortell and Kennedy 2006; Downey et al. 2004; Haselgrove et al. 2000; Price et al. 1995a; Spayd et al. 2002). As synthesis of some of the flavour compounds derived from other pathways, such as the norisoprenoids, are also dependent on light (Baumes et al. 2002; Bureau et al. 2000; Gerdes et al. 2000; Grotewold 2006; Hashizume and Samuta 1999; Marias et al. 1992; Razungles et al. 1998), it was thought that flavonols may be a good indicator for wine flavour compounds and wine quality. However, similar to anthocyanins, there was no relationship between flavonol content and grape and wine quality (**Table 3.5**).

Tannins are the largest contributor to grape and wine total phenolic content (Souquet et al. 1996), comprising of a range of different sized compounds from small oligomers to the large polymeric pigments extracted from both the skin and seed of berries. In wines, tannins are important as they contribute to the body and mouthfeel on wines, while also providing some stability to anthocyanins compounds (Bakker 1998; Cheynier et al. 2000; Dallas et al. 1996; Eglinton et al. 2004; Gawel et al. 2000; Glories 1988; Malien-Aubert et al. 2001; Mateus et al. 2002a; Romero and Bakker 2000; Thorngate 1997; Timberlake and Bridle 1976; Vidal et al. 2002). Thus, the tannin content and composition in grapes is likely to have some role in determining the final wine quality. Presently, tannins *per se* are not routinely measured in the wine industry; however, the total phenolic content of grapes is commonly measured (i.e. A280), and generally this measurement has been linked to the tannin content of grapes and wine, despite it being a non-specific assay. The development of robust tannin assay for use in the wine industry has been the recent focus of many research organisations (Harbertson et al. 2002; Sarneckis et al. 2006) and is discussed in **Chapter 6, Section 6.6.3**. Nevertheless, in this investigation, there was no relationship between either skin or seed tannins and grape and wine quality (**Table 3.5**).

3.4.3.4 Vineyard Characteristics (vine age, yield & canopy measurements)

Historically, it is regarded that older vines produce better quality wines; a concept based on the fact that as the vines get older the yield reduces whilst the carbohydrate supply remains high (in the form of the trunk and cordons)(Dry et al. 2004a). In this investigation, vine age was correlated with grape quality in McLaren Vale vineyards, whereby grapes from older vines were in the higher grape quality streams compared to those from younger vines (**Figure 3.11**). Coincidently, a trend was also observed between vine age and yield, whereby the older vines were typically lower yielding (**Table 3.7**). It should be noted that the correlation between vine age and grape and wine quality is somewhat limited, as there were only a few vines over 20 years of age (which skewed the data), compared to the majority of vines, which were less than 10 years old. Nevertheless, the correlation between vine age and grape and wine quality was not totally unexpected as winemakers commonly consider the history of the vineyard in determining potential grape quality, particularly in some of the smaller regions such as McLaren Vale.

It is generally accepted that the lower the yield the higher the quality of the wine (i.e. an inverse relationship) and while there are many examples supporting these generalisations, there are equally as many that do not (Coombe and Iland 2004). The basis for this historically driven generalisation has been linked to a misunderstanding of vine balance (Dry et al. 2004a), and involves the fact that having extremely high yields can result in overcropping, which is when the grape crop cannot be ripened to an appropriate composition (including anthocyanin content) for a targeted wine style, therefore decreasing overall grape and wine quality. However, the reality is that yield alone is not a good measure of vine balance, nor is it a reliable predictor of wine quality (Dry et al. 2004a).

In this investigation there was a weak inverse relationship between yield and grape and wine quality (**Table 3.5** & **Table 3.6**). However, this relationship was not consistent between region or season, with a high amount of variation observed in all data sets. Additionally a weak inverse relationship was observed between anthocyanin content and yield, with the two regions clearly separating based on colour and yield (**Figure 3.12**). Together, these results imply that the lower yielding vineyards generally have a higher anthocyanin content and may be consequently allocated into a higher grape quality stream to produce a higher value wine product.

These results are similar to previous studies, which show weak relationships between yield, colour and quality. As demonstrated in Coombe and Iland (2004)(Table 11.6), data collected from 25

irrigated Shiraz vineyards from the Riverland, indicated that vines represented by the 'lowest wine score', had grapes with less colour, longer shoots, larger berries and poorer bunch exposure and had a slightly higher yield. However it was noted that the yield/quality correlation was not significant indicating that vineyard factors other than yield were also important in the search for good quality potential. Work by Grey et al. (1994 and 1997) showed no relationship between yield and wine quality across a large sample of Shiraz growers in several regions. Similarly, Holzapfel et al. (1999) and Clingeleffer et al. (2001) reported a weak relationship between yield and berry colour in a study of Shiraz and Cabernet Sauvignon vineyards within single hot regions, with vineyard yields ranging from 8-50 T/Ha. While these authors acknowledge that low yielding vineyards generally produce better quality wines than high yielding, they suggest that it does not necessarily mean that low yields *per se* determine high grape quality, rather, where the vines are grown (i.e. climatic) and the way that they are managed (i.e. irrigation, pruning), has a greater impact on grape quality.

Assessment of the grapevine canopy has recently been adopted by many Australian wineries, to gain a better understanding of the link between vineyard attributes and grape and wine quality (Dry et al. 2004a). The importance of vineyard canopy management practices is described in detail by Smart and Robinson (1991). Within this publication, a scorecard was created to assess the components of a canopy. The principal of the scorecard is based on the excessive shading of leaves and bunches that lowered fruit quality and that such conditions were associated with shoot vigour and crowded canopies. This grapevine scorecard included some vineyard factors measured by HWC in both regions.

In McLaren Vale vineyards, there was a weak inverse relationship between grape and wine quality and the quantitative vineyards assessment factors (bunches/m, shoots/m, bunches/shoot), whereby the greater the number of shoots and bunches per meter the lower the quality stream allocated to those vineyards (**Table 3.5** & **Table 3.6**). Bunches per meter gives an approximate indication of yield, while shoot spacing per meter gives an indication of vine vigour (i.e. shading). The calculation establishing the number of bunches per shoot provides an indication as to the potentially accessible photosynthate available to bunches on a particular shoot, which to the grower/winemaker may give warning of overcropping. The fact that these canopy indicators correlate with grape and wine quality tends to indicate that moderately open canopies rather than dense shaded canopies will result in better quality fruit, confirming suggestions made by Smart and Robinson (1991).

In contrast to the correlations with the vineyard assessment factors in McLaren Vale, there was no relationship with the subjective vineyard assessment factors (shoot tip growth, fruit exposure and leaf health) and grape and wine quality in the Riverland (**Table 3.5** & **Table 3.6**). As outlined in Smart and Robinson (1991), these factors are used as indictors of vine growth, density and overall vine health, respectively. Yet, the fact that these factors are subjective and were likely to have been estimated by different winery representatives makes the non-relationship to some extent expected.

In the early 1990's some of the larger Australian wine company, tested the Smart and Robinson (1991) canopy scorecards with the aim of improving the assessment of potential grape quality from individual grape suppliers. While linear regression analysis showed small trends for lower 'quality' fruit from vineyards with leafy dense canopies and poor fruit exposure, the main outcome was that huge variability and inadequate data precluded predictions of the potential for wine quality of any vineyard lots (Grey et al. 1994; Grey et al. 1997). The authors pointed out that while they did not validate the concept of canopy assessments, it indicated that better methods are needed, particularly problems associated with within-vineyard variability (Grey et al. 1997).

It is clear that fruit composition and wine quality are improved by moderating shoot vigour and ensuring adequate but not excessive fruit exposure (Smart et al. 1990; Smart and Robinson 1991). Coupled with these characteristics is the central importance of vine balance - the ratio of vine growth to fruit production, for long-term sustainable production (Dry et al. 2004a; Howell 2001). Therefore, use of vineyard assessments by the wine industry appears to be valuable, however consideration must be given towards vineyard variability, quantitative measurements and assessments performed by experienced representatives.

3.4.3.5 Wine anthocyanins & wine grade

Wine colour has been correlated with overall wine quality in several research studies (Jackson et al. 1978; Johnstone et al. 1996; Somers and Evans 1974; Somers and Evans 1977; Somers et al. 1983; Tyrrell 1981). However, the limitations of most of these studies were that wine colour was not masked, and since visual colour is a wine quality attribute, it is inherently the case that deeper colored wines score higher quality. It is now widely acknowledged in the industry there are other factors that can influence overall wine quality particularly some of the other flavonoid compounds, as well as some of the flavour and aroma compounds.

In this investigation there was a relationship between wine anthocyanins and wine grade from samples from McLaren Vale, in both seasons (**Figure 3.14**). However there was no relationship between grape anthocyanins and wine grade in both regions (**Figure 3.14** & **Figure 3.16**). It should be noted, that in McLaren Vale, fruit samples were kept separate for fermentation and thus the resulting wine grade allocations correspond to a particular grape sample. Unfortunately, this was not the case in the Riverland, as grape samples from individual vineyards were streamed into one fermentation. Therefore wines were not provided and the specified wine grade should be treated with some caution. For this reason, the poor correlation between grape anthocyanins and wine grade in the Riverland was not unexpected as wine grade represented a mix of grape samples.

In McLaren Vale, there was a poor correlation between grape anthocyanins and wine grade, yet there was a correlation between wine anthocyanins and wine grade, indicating a weak correlation between grape and wine anthocyanins (**Figure 3.8**). While this may have been due to some sampling issues (i.e. wine blended/exposed to oak, as noted for 2003 wines), it may also be related to differences in fermentation practices (i.e. not standardized practices) involving the ease with which anthocyanins are extracted from grape skins and/or co-pigmentation associations with other compounds located in the berry (Boulton 2001).

Clearly, changes in fermentation practices are based on the decisions of the winemaker. The results presented in this investigation indicated that anthocyanins were more readily extracted into wine from the higher quality grapes than from the lower quality grapes (**Figure 3.13A**). This suggests the winemaker may preferentially apply different fermentation practices according to the different grape quality streams to produce a corresponding wine of equal quality. Furthermore, despite the poor correlations with grape colour, it was shown there was some relationship between grape quality stream and wine grade (**Figure 3.17**). While this result suggests that the grape streaming processes in the vineyard is to some degree effective in forecasting the final wine quality, this correlation itself is inherently obvious as the winemaker is largely driven by the grape quality stream to produce a corresponding wine of equal quality.

Interestingly, when wine grade was converted to the wholesale price ($/bottle)) for McLaren Vale wines (**Figure 3.15**), it was shown that for wines less than $15/bottle an increase in wine anthocyanin content corresponded with an increase in wholesale price, however for wines greater

than $15/bottle this relationship was not maintained and anthocyanin content remained unchanged. This observation confirmed earlier suggestions by HWC that indicated that there was a maximum in anthocyanin content at some intermediate level of wine quality (**Figure 3.1**).

To understand this relationship, it is necessary to appreciate the plethora of compounds in wines, their associations with each other, and how they may relate to final wine quality. Wine colour is the result of a complex series of reactions and is influenced by the amount and type of flavonoids in the fruit, the extent of extraction of these compounds during winemaking, the stability of the pigments during fermentation and subsequent aging of the wine (Boulton 2001; Canals et al. 2005; Cheynier et al. 2006; Cheynier et al. 2001; Fulcrand et al. 2006; Kennedy et al. 2006a; Ribéreau-Gayon 1982; Ristic et al. 2007; Sacchi et al. 2005; Singleton and Trousdale 1992). Thus while grape anthocyanins are initially the prominent contributor to wine colour (and not taste or astringency) (Vidal et al. 2004), the levels and composition of other flavonoids such as tannins and flavonols in the fruit are also important as they influence anthocyanin stability by acting as co-pigments (Bakker 1998; Bakker et al. 1993; Boulton 2001; Cheynier et al. 2006; Cheynier et al. 2000; Dallas et al. 1996; Eglinton et al. 2004; Gawel et al. 2000; Glories 1988; Lambert 2002; Price et al. 1995b; Romero and Bakker 2000; Schwartz et al. 2005; Somers 1971; Thorngate 1997).

It should also be noted that, in addition to the flavonoid compounds, there are also several minor wine constituents such as the aroma and flavour compounds, which considerably add to wine quality. These compounds largely contribute to the sensory characters in wines and are subsequently of great interest to wine producers as they relate to key quality attributes. Methoxypyrazine compounds are responsible for herbaceous vegetative aromas in wines (Allen and Lacey 1998). It was suggested that as the precursors to these compounds are present in the grape, there is the opportunity to assess the potential level of these aromas in grapes and possibly predict resultant levels in wines (Allen and Lacey 1998). However, it should be noted that in general, flavour and aroma compounds are not amenable to easy determination as it is an exceedingly difficult task to define and measure individual volatile aroma compounds in grapes or wines to fully predict sensory properties of wine (Francis et al. 2004; Smyth et al. 2005).

3.4.3.6 Determining grape & wine quality: a complex subject

One of the major aims of this investigation was to identify alternative indicators of grape and wine quality in both regions. However it quickly became apparent that this task was going to be difficult, based on two key reasons, the first, due to vineyard variability and the second; relating to the subjective nature of wine 'quality'.

Vineyard variation is widely recognised by the wine industry as a potential serious negative factor for grape quality (Bramley and Hamilton 2004; Coombe and Iland 2004; Trought 1996). Vineyard variability may be either temporal (season to season) or spatial (vine to vine within a vineyard, bunch to bunch within a vine, or berry to berry within a bunch) (Gray 2002; Krstic et al. 2002). Temporal variability largely depends on the climatic conditions whereas spatial variability may enter a vineyard system through differences in soil type, crop load, vine size, cluster position, temperature and sunlight exposure. As such temporal variability influences the vineyard as a whole, whereas spatial variability can generally be managed by viticultural practices. Nevertheless, factors contributing to both temporal and spatial vineyard variability can greatly influence the flavonoid content and composition of grapes at harvest. In this investigation the poor relationships observed is likely to be due to the fact that fruit samples came from a diverse range of vineyards exposed to different external variables, thereby making it difficult to determine which factors exactly are responsible.

The problems associated with vineyard variability are further compounded by the variation in vineyard sampling (i.e. how do you accurately sample a variable vineyard?). It is generally accepted in the wine industry that an 'average' sample represents a whole vineyard, however this largely ignores the effects of spatial variation. Consequently, fruit samples received by the winery may in fact not be representative of the whole vineyard, therefore limiting the opportunities to maximise the production of quality wines. In this investigation, HWC indicated that the sampling procedures at the weighbridge (i.e. Maselli and YUBA) gave an accurate representation of the grapes in a particular bin (Anderson 2003). However, the reproducibility of sampling procedures in the vineyard and how berry samples taken in the vineyard match up to those received at the weighbridge was not discussed. Therefore, acknowledging that accurately sampling a vineyard is extremely difficult (Krstic 2004; Krstic et al. 2002), the poor correlations observed in this investigation could also be due to inaccuracies in fruit sampling, whereby grape samples did not represent the vineyard as a whole.

Despite the array of vineyard, berry and wine factors measured in this investigation, no strong indicator(s) of grape and wine quality were found (**Table 3.5** & **Table 3.6**). Fundamentally, this would be due to the fact that wine 'quality' is highly subjective and thus hard to quantitatively measure and define. Wines, and the grapes they are made from are highly differentiated products (Boulton et al. 1998). Different consumer types often have different opinions on wine quality, making it impossible to remove subjectivity from their assessments of quality. While attributes most appreciated by the winemaker may involve the diversity and persistence of flavours of a wine and its ability to age, for certain consumers buying a wine for a specific occasion, the wine's appearance in the glass and types of flavours might be important (Francis et al. 2004).

It is also important to mention that another potential limitation of this investigation may be the use of the linear regression analyses to compare different factors. Generally, R^2 values of 0.2 or greater were noted as important, although care was taken not to consider these relationships as statistically significant. While this type of statistical analysis does have its benefits (easy to perform and interpret), the use a linear regression analysis may in fact skew some correlations and/or actually overlook some of the permutations of correlating factors.

Other forms of statistical analyses that may be better suited to this sort of data may include the use of; principal component analysis (PCA), which is commonly used to show the relationships between different attributes in large data sets or alternatively perform partial least squares (PLS) analysis which extracts predictive models for different attributes. These statistical tests have been previously used to investigate the relationship between volatile aroma compounds and perceived aromas in Riesling and Chardonnay wines (Smyth et al. 2005). While it was found that of the 46 compounds measured two related well to perceived aromas, it was noted that the data from the PCA and PLS statistics had high degree of variability and uncertainty associated with it (Smyth et al. 2005). Furthermore, the use of PLS models may lead to some discrepancies when applied to different regions and used in different seasons where the influence of seasonal/climatic variation may render them ineffective. Therefore, due to the fact that; a) there was high variability between samples b) that the quality (both grape and wine) scale was highly subjective and c) there were hardly any linear correlations and that those observed were mostly weak (R^2 values less that 0.5), it was probable that that the use of these types of statistical tests in this investigation, would not provide any extra value.

Therefore keeping in mind; the limitations of the statistics, the variation in vineyards and that wine 'quality' is highly subjective, the elusive question still remains:

What factors can be used to predict grape and wine quality?

Whilst winemakers would prefer to have a single measurement of composition that universally correlated with perceived quality, the reality is this is never going to be possible. Considering the above discussion regarding the diversity of grape quality indicators and requirements for winemakers, it is certain that not one analytical measurement will allow an assessment of the multiplicity of sensory attributes, rather it will require multiple quality indicators. It is worth considering a situation where several quality factors might be used by a winery in determining quality of a grape lot. Francis et al. (2004) has suggested a model using sequential consideration of grape berry factors to define quality grades. The order of the grape berry factors listed includes: TSS, colour, berry weight (considered important for extraction during fermentation) and sensory/GC-MS (which would only be performed on those samples in the highest quality streams) where if particular target requirements are not met for each factor then the grape sample is downgraded to a lower quality stream. This multiple factor approach to streaming fruit would be a good approach to allocating 'quality' to different grape lots, however added to list (after colour measurements) would be the inclusion of a grape tannin measurement.

Recently, the tannin content of grapes and wine and the different analytical methods to determine concentration has been the focus of many Australian wineries, due to their high contribution to the total phenolic content in grapes/wine, ability to influence the stability of anthocyanins in wines and influence the mouthfeel properties of wines (Bakker 1998; Bakker et al. 1993; Baranowski and Nagel 1983; Cheynier et al. 2000; Dallas et al. 1996; Eglinton et al. 2004; Gawel et al. 2000; Glories 1988; Romero and Bakker 2000; Souquet et al. 1996; Thorngate 1997). The influence of tannin content on perceived astringency has also recently been reported (Kennedy et al. 2006b; Mercurio et al. 2007b; Smith et al. 2007). In this investigation tannin content in skin and seeds was measured by two methods; the phloroglucinol HPLC method (i.e. PGA) (Downey et al. 2003a; Kennedy and Jones 2001) and the protein precipitation UV-VIS spectrophotometric method (i.e. PPA) (Downey and Adams 2005; Harbertson et al. 2002). Similar to recent observations by Downey (2007), it was found PGA and PPA correlated for total tannin content in grape skins, suggesting both these methods can be used to measure the tannin content in grape berries.

In the past, measurements of tannins in grapes and wines have been difficult (costly, time consuming, non-specific)(Herderich and Smith 2005; De Beer et al. 2004). However, development of a protein precipitation assay using BSA (i.e. PPA) (Downey and Adams 2005; Harbertson et al. 2002; Harbertson et al. 2003) as well as a precipitation assay using methyl-cellulose (Mercurio et al. 2007a; Sarneckis et al. 2006) has alleviated some of these difficulties, being relatively inexpensive and rapid to perform. It has recently been shown in grape skin extracts that these methods are correlated (R^2=0.42), as well as with HPLC analysis (eg PPA (BSA) v HPLC (PG) R^2=0.62) (Downey 2007). Also, recently these precipitation methods have been further developed into a high throughput analysis format, making tannin analysis more efficient (Heredia et al. 2006; Mercurio et al. 2007b). Providing a grape and wine tannin assay that is robust, inexpensive and rapid may prove a useful tool for winemakers in making decisions at the winery, such as when to press and when/what to blend, which may ultimately lead to the production of higher quality wines (Herderich and Smith 2005; Heredia et al. 2006).

3.4.3.8 Summary

The results presented in this investigation show that in both regions many of the historical measures of grape quality (TSS, berry weight, colour, yield) are not good predictors of grape and wine quality, and those which traditionally have not been measured, including grape tannins and flavonols also did not correlate with grape and wine quality. Those measurements, which did correlate in McLaren Vale included vine age and quantitative canopy measurements, whereby older vines with relatively open canopies were allocated into a higher quality stream. This presents a stronger argument for the usefulness of vineyard assessments. In McLaren Vale, there was a correlation between wine colour and wine quality, however this relationship was non-linear, where after a certain anthocyanin level, no further increase in wine quality can be achieved, a relationship previously observed by winery representatives (Sas and Lim 2003).

Although, grape colour was not a good predictor of grape and wine quality in this investigation, the value of the grape colour measurements should not be disregarded as an important measure of red wine quality, and perhaps in combination with other factors, may prove acceptable as a measurement of 'quality'. It is very unlikely that there will ever be a single constituent that acts as a 'magic bullet' to perfectly indicate potential grape and wine quality, rather it is suggested that

measures such as TSS, pH and TA will almost certainly remain important to the wine industry as 'first stage' quality indicators, followed by measurements of colour and tannins for 'second stage' quality indicators and lastly 'third stage' indicators those relating to particular flavour and aroma compounds.

This investigation highlights the positive and negative benefits to the wine industry, whereby undertaking objective berry measurements will improve grape and wine quality by assisting the harvest and streaming processes of fruit into the winery and optimisation of tank space in the winery. Further research is required to understand the complex association of the flavonoid compounds extracted during winemaking (see **Chapter 6, Section 6.6.4**).

3.5 Conclusion

The results presented in this investigation clearly show, in both seasons, the grapes from warm and cool climates formed two distinct data sets based on anthocyanin content. While the composition of tannins and flavonols remained relatively constant, the anthocyanin composition was slightly different with a higher proportion of anthocyanin coumaroyl-glucosides in the warmer region. This result is consistent with earlier reports that temperature can influence anthocyanin content and composition.

There was a correlation between anthocyanins and flavonols for both the warm and cool climate samples in both seasons, however those from the warm region had lower anthocyanin for a given level of flavonol. As expected, the level of tannin in the seeds was greater than in skin for all samples. In both seasons, there was a weak correlation between anthocyanin levels in the skin and skin tannins, but no relationship with seed tannins. These results suggest there is some co-ordination in the synthesis of anthocyanins, flavonols and skin tannins, but not seed tannins. Further research is required to elucidate the transcription controllers for the flavonoid genes under developmental and changing environmental conditions, as well as processes involved in post-transcriptional modification.

It was shown that there were no strong relationships with any of the flavonoids and grape quality, signifying the need for improvement in objective measures of streaming fruit for quality. Additionally, the two regions clearly separated based on yield and despite the weak correlations in both regions, the levels of total anthocyanins were inversely related to yield. In the cool region, there was a non-linear relationship between wine anthocyanins and final wine grade, indicating a maximum in anthocyanin content at some intermediate level of wine quality. These results provide a better understanding of the synthesis of the flavonoid compounds in grapes and how they may contribute to grape and wine quality, however further research is required to link wine flavonoid composition to that of the grapes at harvest, and the influence this will have on resultant wine quality.

CHAPTER 4

The influence of bunch light exposure on flavonol synthesis in Shiraz & Chardonnay grapes during berry development

4.1 Introduction

Flavonols are products of the flavonoid biosynthetic pathway, along with anthocyanins and tannins (Darne 1993; Stafford 1990). Flavonols are a relatively small proportion of total flavonoids in grapes and wine and although they are thought to contribute to wine colour by acting as co-pigments for anthocyanins, their influence on grape and wine quality remains largely unknown (Baranac et al. 1997; Boulton 2001; Brouillard et al. 1997; Cheynier and Rigaud 1986; Downey et al. 2003b; Lambert 2002; Morrison and Noble 1990).

Flavonols are found in grape skins as the glycosides of quercetin, kaempferol, myricetin and isorhamnetin. Recently, the pattern of flavonol synthesis and accumulation during berry development was investigated. It was shown that flavonol synthesis is catalysed by the flavonol synthase gene (*VvFLS*) which has two distinct periods of expression, the first around flowering and the second during ripening of the developing berries (Downey et al. 2003b; Haselgrove et al. 2000). Two *VvFLS* genes have been identified in grapevine (*VvFLS1* and *VvFLS2*), with *VvFLS1* more highly expressed during berry development compared to *VvFLS2* (Downey et al. 2003b). Furthermore, as flavonols are detected in the glycosylated form it has been proposed that there must be a glycosylation enzyme responsible for flavonol glycosylation (i.e. a flavonol specific glycosyl-transferase (FGT)(Ford et al. 1998a)), however to date no such enzyme has been isolated.

Of the many environmental and viticultural factors that affect the flavonoid composition of the fruit, bunch exposure to light has been regarded as one of the major influences (Downey et al. 2003b; Jackson and Lombard 1993; Smart and Robinson 1991). It is generally accepted that as fruit

exposure to sunlight increases, fruit development and flavonoid composition and consequently wine colour (anthocyanins) and quality improve (Dokoozlian and Kliewer 1996). However, it has also been shown that grape berry colour (anthocyanins) can be negatively affected by excessive light exposure, particularly in over-exposed fruit (Hunter et al. 1995; Kliewer 1977) and recently it was observed that high light resulted in decreased wine colour (Bergqvist et al. 2001). Possible explanations for these differences include differences in cultivar, site and season as well as differences in sampling and analytical techniques or that there may be an optimum exposure for grape bunches, beyond which fruit flavonoid composition declines.

Light exposure has been shown to significantly increase flavonol accumulation in grapes and wine (Goldberg et al. 1998; Haselgrove et al. 2000; McDonald et al. 1998; Pereira et al. 2006; Price et al. 1995; Spayd et al. 2002). Fruit exposed to light, mainly via changes in canopy structure, have greater levels of flavonols, particularly quercetin glycosides, than shaded fruit. However, it is important to note that in changing the canopy structure, leaf sun exposure may also be changed leading to altered photosynthate assimilation as well as berry exposure. An increase in flavonols from sun exposed fruit may have implications for the stability and quality of the wine, particularly if flavonols act as co-pigments for anthocyanins (Downey et al. 2003b; Price et al. 1995).

Recently, the effect of light on flavonoid biosynthesis in Shiraz grapes during development was investigated (Downey et al. 2004). Bunches were enclosed in polypropylene boxes just after flowering to exclude light. The boxes were shown to substantially reduce light transmission, without altering bunch microclimate or vine physiology. Grapes from bunches grown in boxes (shaded fruit) had similar levels of anthocyanins and seed and skin tannins to control fruit exposed to sunlight but the shaded fruit had much lower levels of flavonols (Downey et al. 2004).

Therefore, with an understanding of the developmental expression of *VvFLS1* and accumulation of flavonols during berry development along with the compelling evidence that flavonol synthesis is light dependent in grapes, the question remained:

Can light exposure override the developmental control of expression of *VvFLS1* so that flavonols are synthesized at times when they are not normally being accumulated?

Here, *VvFLS1* gene expression and flavonol accumulation was examined in exposed, shaded and light induced Shiraz and Chardonnay fruit at four separate times during development: pre-flowering, pre-veraison, post veraison and pre-harvest.

4.2 Methods

4.2.1 Experimental approach

4.2.1.1 The influence of bunch light exposure on *VvFLS1* expression & flavonol accumulation

4.2.1.1.1 Plant material

Experiments were conducted in a commercial vineyard, Harvey's Slate Creek, at Willunga, South Australia (34° 46' South, 138° 32' East) (**Appendix 4A, Figure 4A.1**). The climate is maritime with a MJT around 21.4°C (Dry et al. 2004b). Varieties sampled included *Vitis vinifera* L. cv. Shiraz and Chardonnay vines, which were grown on own roots and planted in 1988 and 1992, respectively. Vines were drip irrigated and grown on modified Scott-Henry trellises with shoots from both cordons being trained upwards. The experimental plot included a single block each for 180 Shiraz vines (6 rows and ten panels) and 72 Chardonnay vines (four rows and six panels). A two-panel and two-row buffer was also employed to avoid end effects.

4.2.1.1.2 Shading treatments

The boxes, made from white polypropylene sheeting (0.6 mm), are painted black on the interior, and are designed with outer and inner compartments which significantly reduces light transmission (<0.1% between wavelengths of 220-800 nm), while maintaining temperature and relative humidity similar to the ambient temperature as designed and described by Downey (2002)(**Figure 4.1A**). To further stabilise the boxes, a single strip of black electrical tape was wrapped around the outer compartment. Developmental grapevine growth stages were defined according to the modified Eichhorn-Lorenz (E-L) system (Coombe 1995). At the start of each growing season, around budburst (E-L Stage 4), a number of these polypropylene boxes were randomly applied to buds on vines on both sides of the row (**Figure 4.1B**). Applying the boxes at this early stage in development has been shown not to affect inflorescence and bunch development (Downey 2002). When shoots began to fill the boxes (E-L Stage 15), the boxes were quickly opened and repositioned to enclose one inflorescence allowing the remaining part of the shoot to develop in the light. Boxes were examined weekly and where shoot growth resulted in bunches emerging from boxes, these were discarded. Vines were checked weekly and for monitoring purposes, 60 berries were randomly collected to determine total soluble solids (TSS/^{o}Brix) by a hand-held refractometer.

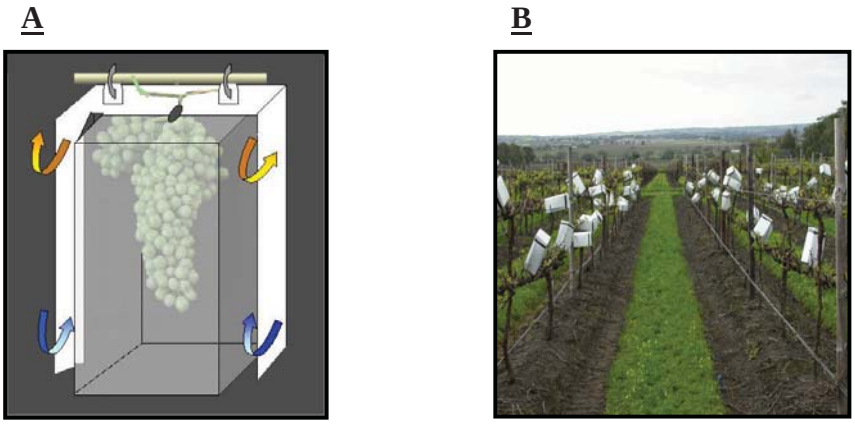


Figure 4.1 Polypropylene boxes applied to Shiraz & Chardonnay vines.

A Design of the shade box by Downey (2002). The bunch is positioned centrally & enclosed by two overlapping inner & outer compartments, which are attached to a shoot within the canopy. The design of the box creates a space between the inner & outer compartments which allowed for air flow in (blue arrow) & out (orange arrow) of the box interior. To further stabilise the boxes, a single strip of black electrical tape was wrapped around the outer compartment.

B At the start of each growing season, around budburst, boxes were randomly applied to buds on vines on both sides of the row.

4.2.1.1.3 Sampling & analysis of fruit

Three separate 'light induction' experiments, in three different seasons, were performed in this investigation including:

- **Experiment 1 (Preliminary)** Chardonnay bunch light exposure, pre-veraison (2002-2003)
- **Experiment 2** Shiraz and Chardonnay bunch light exposure during berry development (2003-2004)
- **Experiment 3 (Hourly light induction)** Shiraz bunch exposure, pre-veraison (2004-2005)

In each experiment, at a different stage in development (E-L Stages 15-38), boxes were randomly removed from bunches (1 box =1 bunch). At the start of each sampling time, when boxes were opened (day 0), shaded bunches and nearby exposed bunches were tagged for identification. Immediately following this, 100 berries were randomly sampled from tagged bunches for the shaded (now-light induced) and exposed (exposed control) bunches. The berries were weighed, the skins separated (and weighed) and ^{o}Brix determined. Skins were frozen in liquid N_2. The light induced grape berries continued to be sampled hourly/daily until the end of the experiment, when another exposed control sample was taken. It is important to note that exposed controls were sampled only on the first and last day at each sampling time, after the preliminary light induction (**Experiment 1**) demonstrated that *VvFLS1* expression and flavonol concentration in exposed controls did not significantly change over the sampling period.

The affect of light on the pathway for flavonol synthesis was investigated using RT-PCR to determine the level of *VvFLS1* gene expression and HPLC to measure flavonol concentration. These methods are described in detail in **Chapter 2**. Key details regarding these methods related to this chapter are listed below.

4.2.1.1.3.1 RT-PCR expression analysis of the flavonoid pathway genes

In RT-PCR analysis, expression of the flavonol synthase (*VvFLS1*) gene (as well as other grapevine genes (*VvUFGT, VvLDOX, VvCHI*) was normalised to the expression of *VvUbiquitin1* ((TC32075), abbreviated *VvUBIQ1*), and therefore has no units. Primer sequences are shown in Appendix 2C. *VvFLS1* gene expression was selected for analysis over *VvFLS2*, as it is more highly expressed during development. *VvUBIQ1* was chosen for normalisation of *VvFLS1* gene expression because its expression was found to be relatively constant throughout grape berry development (Bogs et al. 2005; Downey et al. 2004), and did not change in response to light (see **Chapter 5**). In each light induction experiment, expression of *VvUBIQ1* remained constant during the sampling period (data not shown). All samples were measured in triplicate, therefore the data is represented as the mean and the standard error (±SEM, n=3) for the three analytical replicates. It should be noted that RT-PCR is a semi-quantitative technique, and therefore caution should be exercised when interpreting RT-PCR results, taking into consideration the magnitude of variation in gene expression. It is generally considered that changes in gene expression of up to 2-fold are often observed as variation in gene expression between samples.

4.2.1.1.3.2 HPLC analysis of anthocyanin & flavonol composition & concentration

In HPLC analyses, samples of berry skins, flowers and leaves were extracted and analysed in triplicate for anthocyanin and flavonol content as detailed in **Chapter 2, Section 2.2.1**. In Shiraz berries after veraison, 15 anthocyanin compounds were detected, including the 5 anthocyanin pigments (malvidin, petunidin, peonidin, cyanidin, delphinidin) with the attached sugar moieties (glucosides, acetyl-glucosides and coumaroyl–glucosides). In Shiraz and Chardonnay, most of the flavonols listed in **Chapter 2, Table 2.1** were detected in inflorescences and leaves. However, as the season progressed some of the minor flavonols could not be detected. Pre-harvest all flavonol compounds were detected in Shiraz berries, however the glycosides of myricetin and kaempferol were not detected in Chardonnay berries.

Anthocyanin and flavonol compounds were expressed as malvidin-glucoside and quercetin-glucoside equivalents, respectively and in graphs are represented as the sum of all peaks in that flavonoid group (i.e. total anthocyanins and total flavonols). In most graphs, anthocyanin and flavonol concentration is expressed on a per berry weight basis (mg/g berry), however when expressed per g of skin extracted or per berry, the trends remain the same (data not shown). Inflorescence and leaf samples are expressed on a per gram of tissue basis (mg/g tissue). Data in graphs is represented as the mean and the standard error (±SEM, n=3) for the three analytical replicates.

Using the statistical package GenStat (9th Edition) a two-way ANOVA was used to test treatment effects (exposed controls and light induced) and time effects (day 0 (start) and day 6 (finish)) across three analytical replicates. A Fishers protected Least Significant Difference (LSD) test (p=0.05) was also performed on these samples. It is important to note that the statistical analysis was not performed on days where an exposed control was not taken.

4.2.1.2 Experiment 1 Chardonnay bunch light exposure, pre-veraison (2002-2003) (Preliminary experiment)

Preliminary to the major experiment conducted in the 2003-2004 season, a trial experiment was performed in 2002-2003. Twenty boxes were applied to Chardonnay inflorescences, when shoots had ~10 leaves separated (E-L Stage 16). Approximately 3.5 weeks before veraison (E-L Stage 30-31)(**Table 4.1**), 10 boxes were removed from bunches and berries skins were randomly sampled from light induced and exposed control bunches. Berries (light induced and exposed controls) continued to be sampled, every day, for 3 days. *VvFLS1* gene expression and flavonol content was determined by RT-PCR and HPLC, respectively. In HPLC analysis, only the quercetin glycosides were detected in Shiraz berry skins.

Table 4.1 Key grapevine growth stages & sampling dates for Chardonnay in the 2002-2003 season (preliminary light induction experiment).

	Date	Sampling dates [δ]	Weeks from veraison	E-L Stage	°Brix
Flowering [α, β]	22/11/02	-	-9	23	-
Pre-veraison (sample- berry skins)	-	16/12/02-19/12/02	-3.5	30-31	-
Veraison [γ]	23/1/03	-	0	35	-

NOTES
[α] Boxes were applied to vines on 1/11/02 (E-L Stage 16)
[β] Flowering date is approximate date on which 50% cap-fall occurred on the average bunch
[γ] Veraison date is the approximate date at which °Brix started to rapidly increase
[δ] 100 berries were sampled every day for 3 days

4.2.1.3 Experiment 2 Shiraz & Chardonnay bunch light exposure during berry development (2003-2004)

In the 2003-2004 growing season, at budburst (E-L Stage 4), 110 boxes were randomly applied to buds on Shiraz and Chardonnay vines. At four separate times throughout berry development; pre-flowering, pre-veraison, post-veraison (Shiraz only) and pre-harvest, boxes were removed from bunches and berries were sampled. Sampling dates are shown in **Table 4.2** and **Table 4.3**. In Shiraz, flowering occurred on 23/11/03, followed by veraison 8.5 weeks later on 22/1/04. The grapes were commercially harvested approximately 7 weeks after veraison, on 13/3/04 at 26.5 ^{o}Brix (**Table 4.2**). In Chardonnay, flowering occurred slightly earlier on 20/11/03, followed by veraison 9 weeks later, on 22/1/04 (same as in Shiraz). Chardonnay grapes were commercially harvested approximately 1 week earlier than Shiraz, on 6/3/04 at 23.5 ^{o}Brix (**Table 4.3**).

At the start of each sampling period, approximately 30 boxes were removed from bunches and 100 berries were randomly sampled from shaded and exposed control bunches. Light induced berries were sampled every 2nd day, up until day 6 after the beginning of light exposure, when another exposed control sample was taken. An additional sample was also taken on day 6, pre-veraison and post-veraison, from berries still contained in boxes (shaded control). Pre-flowering, the samples comprised of inflorescences, and by stripping the rachis, unopened flowers were collected. A separate sample of young leaves at Stage 2 (Downey et al. 2003b) was also collected at this time. *VvFLS1* gene expression, along with expression of other genes (*VvUFGT, VvLDOX, VvCHI*) was determined by RT-PCR and anthocyanin and flavonol content was determined by HPLC.

Table 4.2 Key grapevine growth stages & sampling dates for Shiraz in the 2003-2004 season (light induction experiment).

	Date	Sampling dates $^{\delta}$	Weeks from veraison	E-L Stage	^{o}Brix
Budburst $^{\alpha}$	18/9/03	-	-22.5	4	-
Pre-flowering (sample-inflorescences & leaves)	-	12/11/03-18/11/03	-9.5	15-20	-
Flowering $^{\beta}$	23/11/03	-	-8.5	23	-
Pre-veraison (sample-berry skins)	-	12/1/04-18/1/04	-1.5	33	5.7
Veraison $^{\gamma}$	22/1/04	-	0	35	8.0
Post-veraison (sample- berry skins)	-	2/2/04-8/2/04	+1.5	36	13-17
Pre-harvest (sample- berry skins)	-	23/2/04-29/2/04	+4.5	37	20-22
Harvest	13/3/04	-	+7	38	26.5

NOTES
$^{\alpha}$ Boxes were applied to vines at budburst, on 18/9/03 (E-L Stage 4) & re-orientated 12/11/03 (E-L Stage 15)
$^{\beta}$ Flowering date is approximate date at which 50% cap-fall occurred on the average bunch
$^{\gamma}$ Veraison date is the approximate date at which ^{o}Brix started to rapidly increase
$^{\delta}$ From the start of each sampling date, berries were sampled every 2nd day for 6 days

Table 4.3 Key grapevine growth stages & sampling dates for Chardonnay in the 2003-2004 season (light induction experiment).

	Date	Sampling dates [δ]	Weeks from veraison	E-L Stage	°Brix
Budburst [α]	~10/9/03	-	-24	4	-
Pre-flowering (sample-inflorescences & leaves)	-	10/11/03-16/11/03	-10	16-21	-
Flowering [β]	20/11/03	-	-9	23	-
Veraison [γ]	22/1/04	-	0	35	8.7
Post-veraison (sample- berry skins)	-	2/2/04-8/2/04	+1.5	36	15-19
Pre-harvest (sample- berry skins)	-	23/2/04-29/2/04	+5	37	20-22
Harvest	6/3/04	-	+7	38	23.5

NOTES
[α] Boxes were applied to vines post-budburst on 18/9/03 (E-L Stage 7) & re-orientated on 12/11/03 (E-L Stage 16)
[β] Flowering date is approximate date on which 50% cap-fall occurred on the average bunch
[γ] Veraison date is the approximate date at which °Brix started to rapidly increase
[δ] From the start of each sampling date, berries were sampled every 2nd day for 6 days

4.2.1.4 Experiment 3 Hourly light induction of Shiraz berries, pre-veraison (2004-2005)

Following sampling in the 2003-2004 season, another light induction experiment was performed in 2004-2005, where boxes were removed pre-veraison and samples taken over a shorter time scale. Sixty boxes were randomly applied to Shiraz inflorescences, when shoots had ~8 leaves separated (E-L Stage 15). Approximately 2 weeks pre-veraison (E-L Stage 31-32) (**Table 4.4**), the boxes were removed from bunches and 100 berries were randomly sampled from light induced and exposed control bunches at 9:00am. Light induced berries continued to be sampled 3, 6 and 11 hrs after induction and daily at 9:00am, on day 1, 2, 4 and 6. Another exposed control sample was taken on day 6. *VvFLS1* gene expression and flavonol content was determined by RT-PCR and HPLC, respectively. In HPLC analysis, only the quercetin glycosides were detected in Shiraz berry skins.

Table 4.4 Key grapevine growth stages & sampling dates for Shiraz in the 2004-2005 season (hourly light induction experiment).

	Date	Sampling dates [δ]	Weeks from veraison	E-L Stage	°Brix
Flowering [α,β]	15/11/04	-	-10	23	-
Pre-veraison (sample- berry skins)	-	9/1/05-15/1/05	-2	31-32	5.5
Veraison [γ]	27/1/05	-	0	35	-

NOTES
[α] Boxes were applied to vines on 27/10/04 (E-L Stage 15)
[β] Flowering date is approximate date on which 50% cap-fall occurred on the average bunch
[γ] Veraison date is the approximate date at which °Brix started to rapidly increase
[δ] 100 berries were randomly sampled 3, 6 & 11 hrs after induction, followed by days 1, 2, 4 & 6

4.2.1.5 The diurnal pattern of *VvFLS1* gene expression in Shiraz bunches at different stages during development

Experiments were conducted in 2003-2004 at the Coombe vineyard on the Waite Campus of Adelaide University (Adelaide, South Australia, latitude 34° 56' south, longitude 138° 36' east (**Appendix 4A, Figure 4A.2**). Tissue from *Vitis vinifera* L. cv. Shiraz (clone BVRC12) vines were

sampled. The 13 year-old own rooted vines, were trained to a single bi-lateral cordon, VSP trained and drip irrigated. The experimental plot included two rows that face N-S consisting of 180 vines, and a two-panel and five-row buffer was employed to avoid end effects. Temperature was logged over the experimental period (TinyTag Data Loggers, Australia) and light was measured as photosynthetically active radiation (PAR; 400-700 nm) at each sampling time (LI-COR Biosciences, UK). The Shiraz vines were monitored throughout the growing season along with weekly ^{o}Brix measurements for determination of growth stage (Coombe 1995).

Samples were collected at three separate times during berry development, post-flowering, post-veraison and pre-harvest. Sampling dates are shown in **Table 4.5**. Flowering occurred on 12/11/03, followed by veraison 10 weeks later on 20/1/04. The grapes were harvested approximately 6 weeks after veraison, on 7/3/04 at 24.0 ^{o}Brix. Starting at 05:00, a 100 berry sample was randomly collected from Shiraz bunches. Samples continued to be collected every 4 hrs until 17:00 the following day (total of 40 hrs). Post-flowering, 10-15 inflorescences were sampled at each time-point, and all material was processed according to **Section 4.2.1.3**. Expression of the flavonoid genes (*VvFLS1, VvUFGT, VvLDOX*) was determined by RT-PCR. Genes were normalised to the expression of *VvUBIQ1*, which, remained relatively constant throughout sampling (data not shown). All samples were measured in triplicate therefore data is represented as the average and the standard error of the mean (±SEM). Similar to the light induction experiments, caution should be exercised when interpreting RT-PCR results, taking into consideration the magnitude of variation in gene expression. It is generally considered that changes in gene expression of up to 2-fold are often observed as variation in gene expression between samples.

Table 4.5 Key grapevine growth stages & sampling dates for Shiraz in the 2003-2004 season (diurnal pattern of *VvFLS1* expression experiment).

	Date	Sampling dates [δ]	Weeks from veraison	E-L Stage	°Brix
Flowering [β]	12/11/03	-	-10	23	-
Post-flowering (sample-inflorescences)	-	19/11/03-20/11/03	-9	24	-
Veraison [γ]	20/1/03	-	0	35	11.0
Post-veraison (sample-berry skins)	-	28/1/04-29/1/04	+1	36	13-15
Pre-harvest (sample- berry skins)	-	25/2/04-26/2/04	+5	37	20-22
Harvest	7/3/04	-	+6	38	24.0

NOTES
[β] Flowering date is approximate date on which 50% cap-fall occurred on the average bunch
[γ] Veraison date is the approximate date at which °Brix started to rapidly increase
[δ] From the start of each sampling date, 100 berries were randomly selected from bunches every 4 hrs over 40 hrs

4.3 Results

4.3.1 Experiment 1- The influence of bunch light exposure on flavonol synthesis in pre-veraison Chardonnay berries (Preliminary experiment)

In the 2002-2003 season, in a trial experiment, the influence of light on the activity of the flavonol pathway in Chardonnay berries was determined. The influence of bunch light exposure on *VvFLS1* expression and flavonol accumulation in Chardonnay berries, pre-veraison is shown in **Figure 4.2**. On day 0, *VvFLS1* expression in the shaded berries was 110-fold lower than in exposed berries, and flavonol levels were 6-fold less than in exposed berries. After the removal of the boxes on day 0, *VvFLS1* expression in light induced berries increased by 430-fold on day 1. On day 2, *VvFLS1* expression was at the maximum, with levels 2290-fold greater compared to day 0 shaded berries. By day 3, *VvFLS1* expression in light induced berries had started to reduce. Corresponding with the increase in *VvFLS1* expression, flavonol concentration increased, reaching levels similar to the exposed berries by day 3. Throughout the 3-day sampling period, *VvFLS1* expression and flavonol concentration in the exposed berries remained relatively constant compared to light induced berries.

CHARDONNAY

A **B**

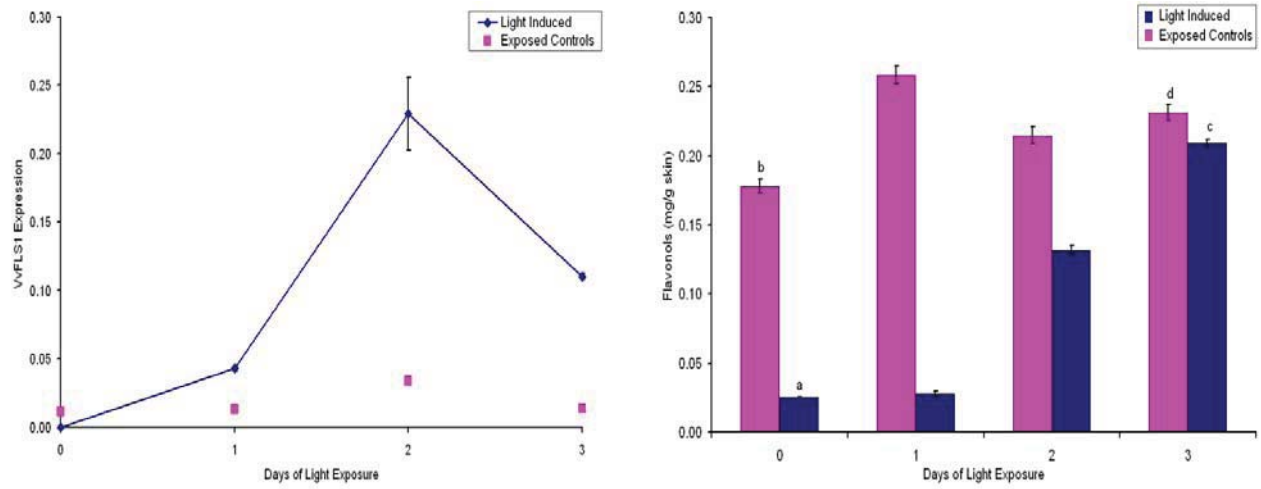


Figure 4.2 The influence of bunch light exposure on *VvFLS1* expression & flavonol accumulation in pre-veraison Chardonnay berries. Boxes were applied to vines when shoots had ~10 leaves separated in 2002-2003 season. Approximately 3.5 weeks prior to veraison, 10 boxes were removed from vines & 100 berry skins randomly sampled from shaded (now Light Induced (LI)) & nearby exposed berries (Exposed Controls (EC)). LI & EC samples were subsequently taken every day for 3 days. For each sample 100 berries were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates.

A Expression of *VvFLS1* relative to *VvUBIQ* expression in LI & EC berry skins determined by RT-PCR.

B Accumulation of total flavonols (expressed per g of skin) determined by HPLC. Quercetin glycosides detected in berry skins were summed to total flavonols. Different letters indicate significant differences between LI & EC on days 0 & 3 determined by a 2-way ANOVA followed by a Fishers protected Least Significant Difference (LSD) test (p=0.05).

4.3.2 Experiment 2- The influence of bunch light exposure on flavonol synthesis in Shiraz & Chardonnay bunches during berry development

4.3.2.1 Pre-flowering sampling

In the 2003-2004 season, the major light induction experiment was performed on Shiraz and Chardonnay vines. The first sampling time was approximately 1 week prior to flowering, when *VvFLS1* expression and flavonol accumulation was expected to be high compared to later stages in development (Downey et al. 2003b). The influence of bunch light exposure on *VvFLS1* expression and flavonol accumulation in Shiraz and Chardonnay inflorescences is shown in **Figure 4.3**.

In Shiraz, on day 0, *VvFLS1* expression in the shaded inflorescences was 11-fold lower than the exposed inflorescences, corresponding with a 4-fold reduction in flavonol concentration. Two days after the removal of the boxes, *VvFLS1* expression in light induced inflorescences increased by 13-fold relative to day 0, reaching levels similar to exposed inflorescences. Following this, *VvFLS1* expression decreased on days 4 and 6 but was still higher than the shaded inflorescences (day 0) and was similar to exposed inflorescences on day 6. The increase in *VvFLS1* expression in light induced inflorescences on day 2 coincided with the gradual accumulation of flavonols to levels similar to exposed inflorescences by day 6. Over the 6-day sampling period, *VvFLS1* expression in exposed inflorescences decreased 2-fold, while flavonol concentration remained unchanged.

In contrast to Shiraz, in Chardonnay on day 0, *VvFLS1* expression in the shaded inflorescences was only slightly less than in the exposed inflorescences; however flavonol levels were around 2-fold less than in exposed inflorescences. Following removal of the boxes on day 0, *VvFLS1* expression in light induced inflorescences increased by 2.6-fold on day 2 returning to levels similar to the exposed inflorescences by day 4. On day 6, *VvFLS1* expression in light induced inflorescences increased again, by 2-fold, to levels much greater than the exposed inflorescences. Flavonol concentration in light induced inflorescences gradually increased over the 6-day sampling period, to reach levels similar to the exposed inflorescences. Over the sampling period, *VvFLS1* expression and flavonol concentration in the exposed inflorescences remained relatively constant.

In both varieties, it was noticeable that shaded inflorescences were a lighter green colour compared to exposed controls (**Figure 4.4**). This suggests there is less chlorophyll present in shaded samples and implies a reduction in light exposure. Additionally, in Shiraz, the shaded inflorescences were

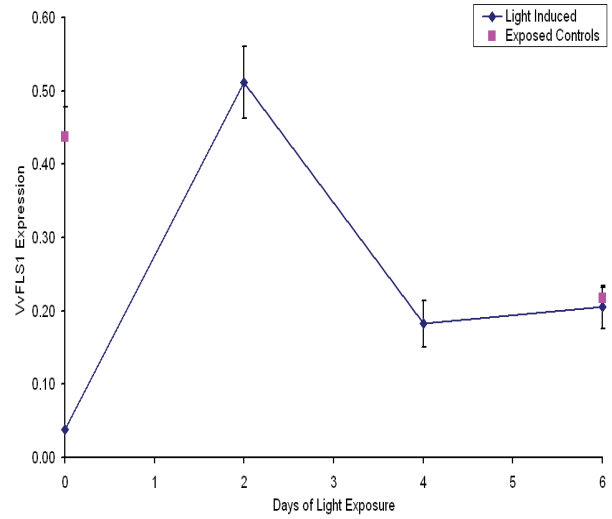
slightly more elongated and had extended rachis laterals compared to exposed inflorescences. There was no obvious effect on inflorescence architecture in Chardonnay (**Figure 4.4**).

Flavonol synthesis was also light induced in leaves as shown in **Figure 4.5**. In Shiraz, on day 0, *VvFLS1* expression in shaded leaves was 7-fold lower than in exposed leaves, corresponding with a 33-fold reduction in flavonol concentration. After the removal of the boxes on day 0, *VvFLS1* expression, increased by 5-fold on day 2 and remained high on day 4 before declining to levels similar to exposed leaves on day 6. Flavonol concentration in light induced leaves gradually increased after removal of the boxes, and on day 6 levels were 80% of that in exposed leaves. While flavonol concentration in exposed leaves remained unchanged over the 6-day sampling period, *VvFLS1* expression decreased 2-fold by day 6.

In Chardonnay, on day 0, *VvFLS1* expression in shaded leaves was around 4-fold less than in exposed leaves and there was a 36-fold reduction in flavonol concentration. Following removal of the boxes on day 0, *VvFLS1* expression increased around 10-fold on day 2, returning to levels similar to exposed leaves by day 4 and day 6. The increase in *VvFLS1* expression in light induced leaves on day 2 coincided with a rapid increase in flavonol accumulation where flavonol levels reached approximately 50% of exposed leaves. However, flavonol concentration in light induced leaves did not significantly change after day 2, with the total amount accumulated remaining less than 50% of exposed leaves on day 4 and 6. Over the 6-day sampling period, *VvFLS1* expression and flavonol concentration in exposed leaves remained unchanged.

SHIRAZ

A



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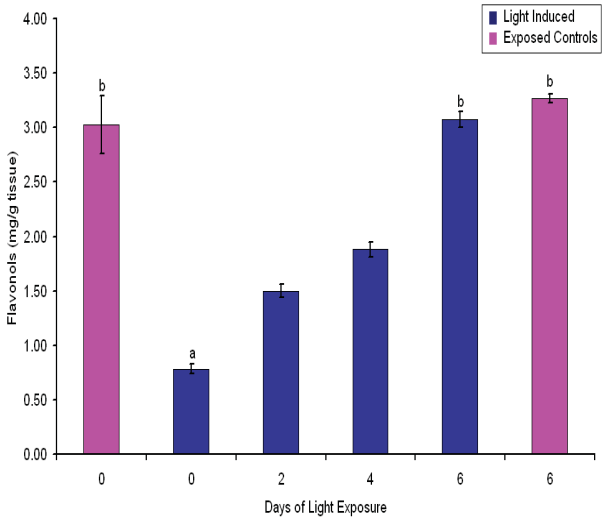
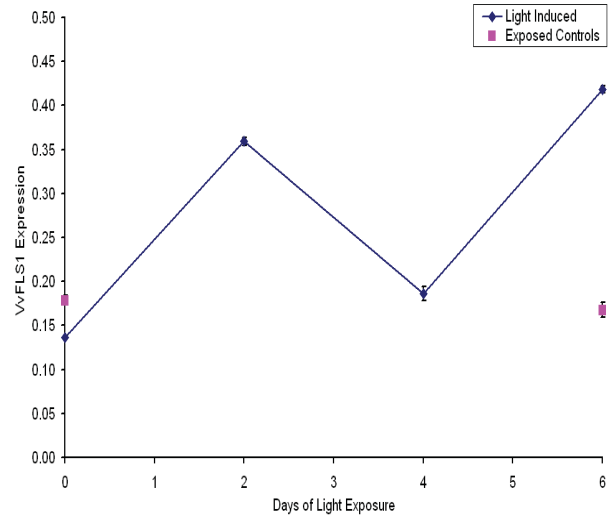


CHARDONNAY

C



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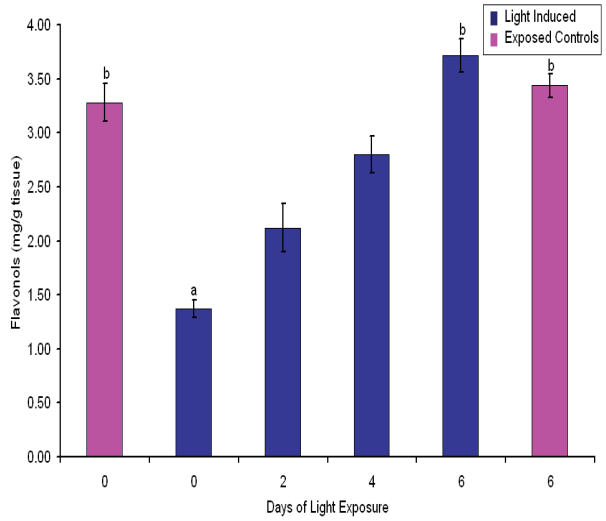


Figure 4.3 The influence of bunch light exposure on *VvFLS1* expression & flavonol accumulation in Shiraz & Chardonnay inflorescences. Boxes were applied to vines at budburst in the 2003-2004 season**.** Approximately 1 week prior to flowering, 30 boxes were removed from vines & 5-10 inflorescences were randomly sampled (Light Induced (LI)) along with nearby control inflorescences (Exposed Controls (EC)). LI samples were subsequently taken every 2nd day for 6 days, when another EC sample was taken. For each sample 5-10 inflorescences were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates.

A,C Expression of *VvFLS1* relative to *VvUBIQ1* expression in LI & EC inflorescences determined by RT-PCR.

B,D Accumulation of total flavonols (expressed per g of tissue) in LI & EC inflorescences determined by HPLC. Different letters indicate significant differences between LI & EC on days 0 & 6 determined by a 2-way ANOVA followed by a Fishers protected Least Significant Difference (LSD) test (p=0.05).

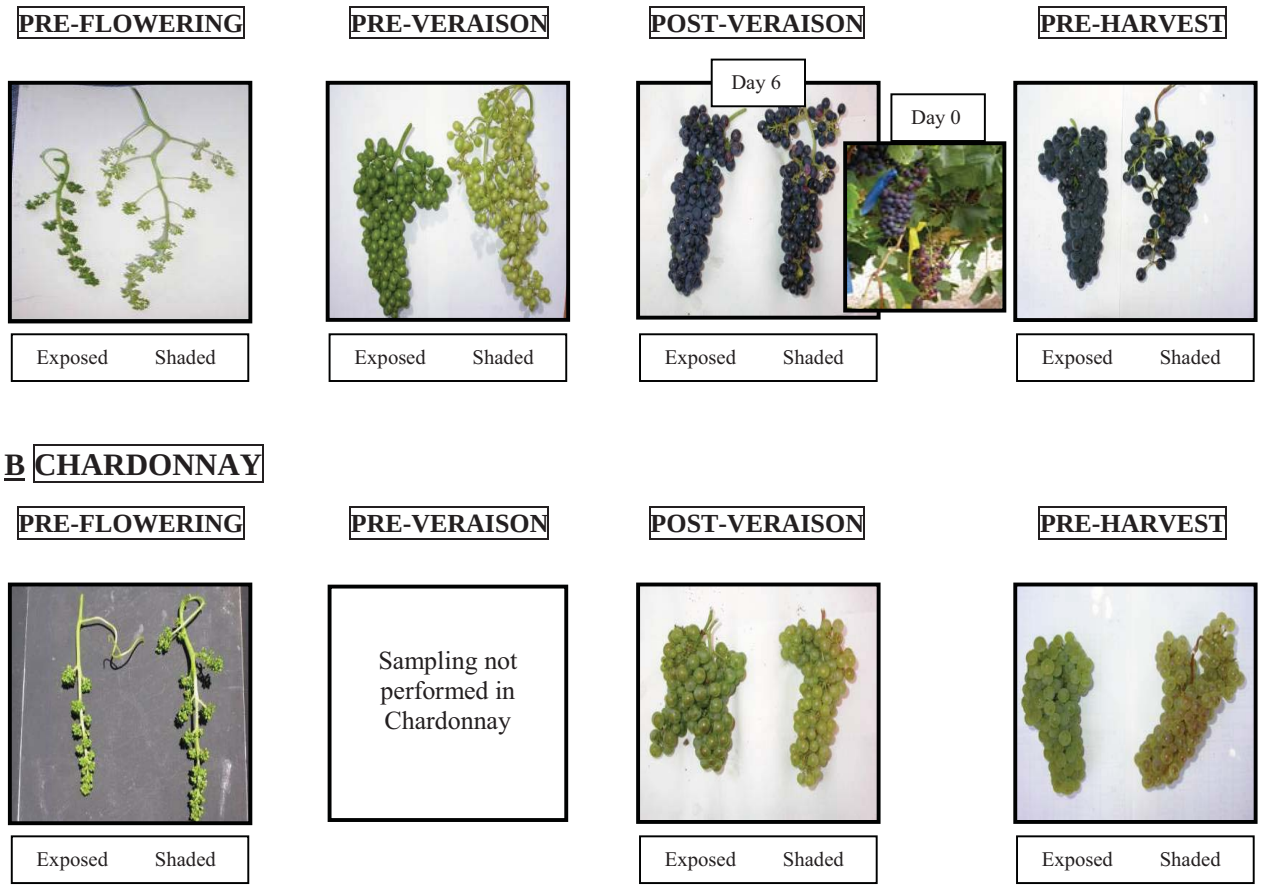


Figure 4.4 Phenotypic & developmental differences between exposed & shaded Shiraz (A) & Chardonnay (B) bunches at different stages in development. Photos show exposed control bunches compared to shaded bunches (on day 0). In Shiraz, post-veraison, the phenotypic change in anthocyanin accumulation on day 0 (insert) & day 6 is shown.

Table 4.6 Berry weight & °Brix of Shiraz & Chardonnay exposed & shaded fruit. For each sample the average berry weight & °Brix of 100 berries was determined. Represented in the table is the average berry weight (g) & the error of the mean (±SEM) for two samples taken on day 0 & 6 (i.e. n =2). °Brix is represented as a range; the average on day 0 & on day 6. Blank cells indicate data not available.

	Pre-veraison		Post-veraison		Pre-harvest	
	Exposed	**Shaded**	**Exposed**	**Shaded**	**Exposed**	**Shaded**
SHIRAZ						
Berry Weight (g)	0.610 (±0.03)	0.515 (±0.04)	0.949 (±0.001)	0.817 (±0.06)	1.002 (±0.03)	1.120*
°Brix	5.7*		13.7-16.9	16.5-17.6	20.2-22.3	21.2-22.2
CHARDONNAY						
Berry Weight (g)	Sampling not performed in Chardonnay		0.930 (±0.05)	0.747 (±0.05)	1.180 (±0.01)	1.010*
°Brix [α]			15.1-20.2	15.5-18.9	21.0-22.0	20.1-22.3

NOTES
* Indicates n =1 (average of 100 berries)
[α] Total soluble solids (°Brix) increased over the 6-day period & therefore is represented as a range (day 0 to day 6)

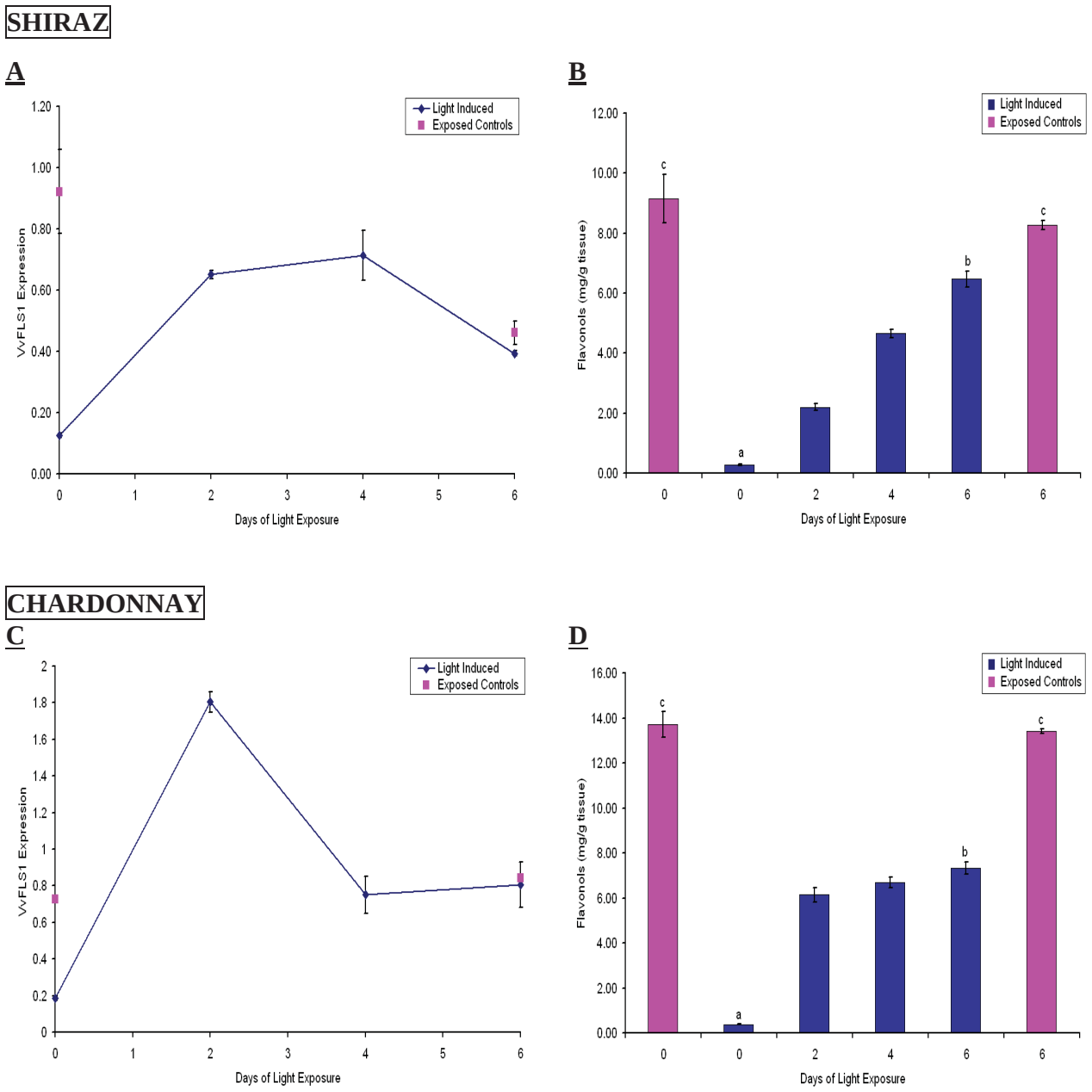


Figure 4.5 The influence of bunch light exposure on *VvFLS1* expression & flavonol accumulation in Shiraz & Chardonnay leaves. Boxes were applied to vines at budburst in the 2003-2004 season. Approximately 1 week prior to flowering, 30 boxes were removed from vines & 5-8 leaves were randomly sampled (Light Induced (LI)) along with nearby control leaves (Exposed Controls(EC)). Leaves were young, at Stage 2 (Downey et al. 2003b). LI samples were taken every 2nd day for 6 days, when another EC sample was taken. For each sample 5-8 leaves were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates.

A,C Expression of *VvFLS1* relative to the expression of *VvUBIQ1* in LI & EC leaves determined by RT-PCR.

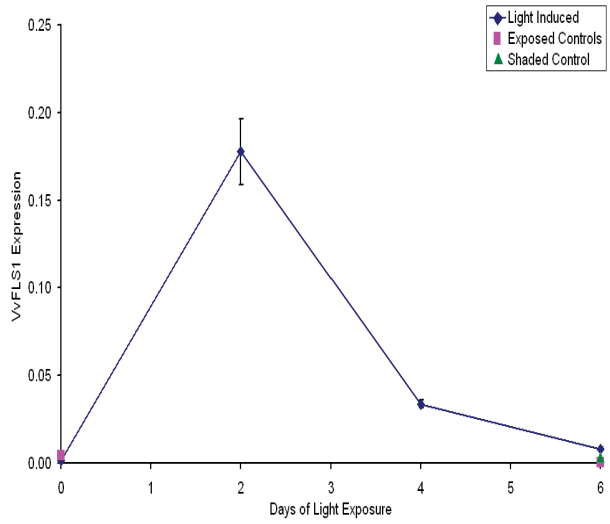
B,D Accumulation of total flavonols (expressed per g of tissue) in LI & EC leaves determined by HPLC. Different letters indicate significant differences ($p<0.001$) between LI & EC on days 0 & 6 determined by a 2-way ANOVA followed by a Fishers protected Least Significant Difference (LSD) test ($p=0.05$).

4.3.2.2 Pre-veraison sampling

The second sampling time was approximately 1.5 weeks prior to veraison, in Shiraz fruit only. As this stage in development, *VvFLS1* expression and flavonol accumulation was expected to be low (Downey et al. 2003b). The influence of bunch light exposure on *VvFLS1* expression and flavonol accumulation in Shiraz berries, pre-veraison is shown in **Figure 4.6**. On day 0, *VvFLS1* expression was around 3-fold lower in shaded berries than in exposed berries and flavonol levels were 32-fold less than in exposed controls. After the removal of the boxes on day 0, *VvFLS1* expression increased 100-fold on day 2, returning to levels similar to exposed berries on day 4 and day 6. The increase in *VvFLS1* expression in light induced berries on day 2 coincided with the rapid accumulation of flavonols to levels similar to exposed berries. Over the 6-day sampling period, *VvFLS1* expression and flavonol concentration in exposed controls and shaded controls berries remained similar.

SHIRAZ

A



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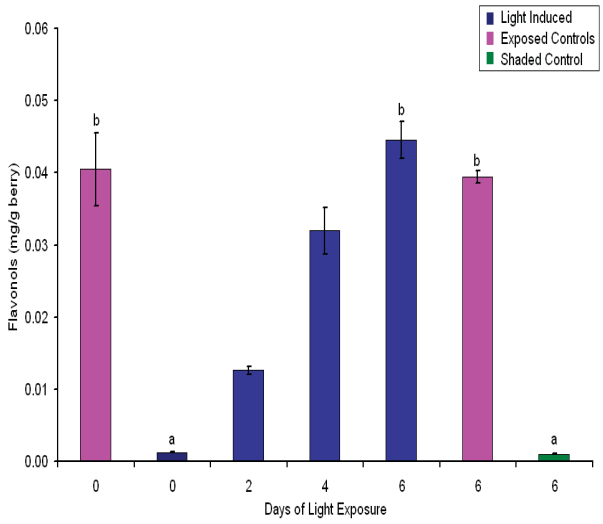


Figure 4.6 The influence of bunch light exposure, pre-veraison, on *VvFLS1* expression & flavonol accumulation in Shiraz berries. Boxes were applied to vines at budburst in the 2003-2004 season. Approximately 1.5weeks prior to veraison, 30 boxes were removed from vines & 100 berry skins randomly sampled (Light Induced (LI)) along with nearby control berries (Exposed Controls (EC)). LI samples were taken every 2nd day for 6 days, when another EC sample was taken. An additional shaded control (SC) sample was also taken on day 6. For each sample 100 berries were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates.

A Expression of *VvFLS1* relative to *VvUBIQ1* expression in LI, EC & SC berry skins determined by RT-PCR.

B Accumulation of total flavonols (expressed per g of berry) in LI, EC & SC berry skins determined by HPLC. Different letters indicate significant differences ($p<0.001$) between LI, EC & SC on days 0 & 6 determined by a 2-way ANOVA followed by a Fishers protected Least Significant Difference (LSD) test ($p=0.05$).

Pre-veraison, berry weight in shaded berries was 0.1g lower than in exposed berries (**Table 4.6**). Shaded berries were also a lighter green colour compared to exposed berries, and there were minor differences in bunch architecture, with the shaded bunches generally having slightly longer and looser bunches (**Figure 4.4**). At the start of the pre-veraison experiment ^{o}Brix was approximately 5.7 in exposed berries.

4.3.2.3 Post-veraison sampling

The third sampling time was post-veraison, by approximately 1.5 weeks, when *VvFLS1* expression and flavonol accumulation were expected to be low (Downey et al. 2003b). The influence of bunch light exposure on *VvFLS1* expression and flavonol accumulation in Shiraz and Chardonnay berries is shown in **Figure 4.7**.

In Shiraz, on day 0, *VvFLS1* expression in the shaded berries was 1400-fold lower than in exposed berries. There were also no flavonols detected in the shaded berry skins. After the removal of the boxes on day 0, *VvFLS1* expression in light induced berries increased by 40,000-fold on day 2 and then decreased on day 4 and day 6, although expression remained significantly higher than in exposed berries. The large increase in *VvFLS1* expression coincided with the rapid accumulation of flavonols to levels 40% greater than exposed berries by day 6. Over the 6-day sampling period, *VvFLS1* expression in exposed berries remained fairly constant, while flavonol concentration increased around 2-fold. It should also be noted that while on day 6 the shaded control berries had slightly higher *VvFLS1* expression and some flavonols were detected, the concentration was still less than 10% of exposed berries.

In Chardonnay, a similar response to Shiraz was observed. On day 0 and 6, *VvFLS1* expression the shaded berries, was at least 5-fold lower than in exposed berries and there were no detectable flavonols. After the removal of the boxes on day 0, *VvFLS1* expression in light induced berries increased by 2,600-fold on day 2 and then decreased by day 4 and day 6 although expression remained significantly higher than in exposed berries. Flavonols in light induced berries accumulated to levels greater than exposed berries by day 6. Over the 6-day sampling period, *VvFLS1* expression and flavonol accumulation in exposed berries remained unchanged.

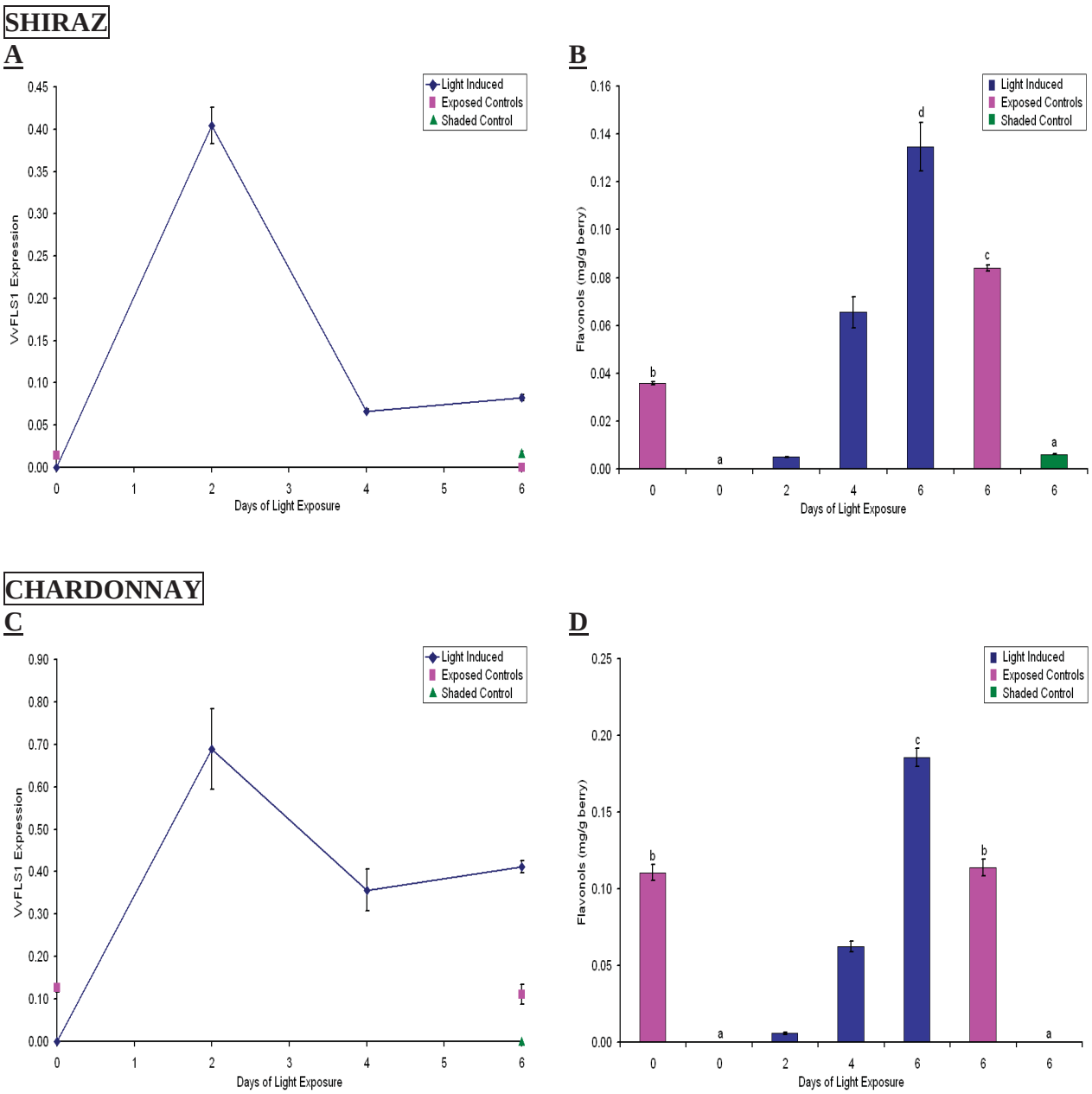


Figure 4.7 The influence of bunch light exposure, post-veraison, on *VvFLS1* expression & flavonol accumulation in Shiraz & Chardonnay berries. Boxes were applied to vines at budburst in the 2003-2004 season. Approximately 1.5weeks post veraison, 30 boxes were removed from vines & 100 berry skins randomly sampled (Light Induced (LI)) along with nearby control berries (Exposed Controls (EC)). LI samples were taken every 2nd day for 6 days, when another EC sample was taken. An additional shaded control (SC) sample was also taken on day 6. For each sample 100 berries were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates.

A,C Expression of *VvFLS1* relative *VvUBIQ1* expression in LI, EC & SC berry skins determined by RT-PCR.

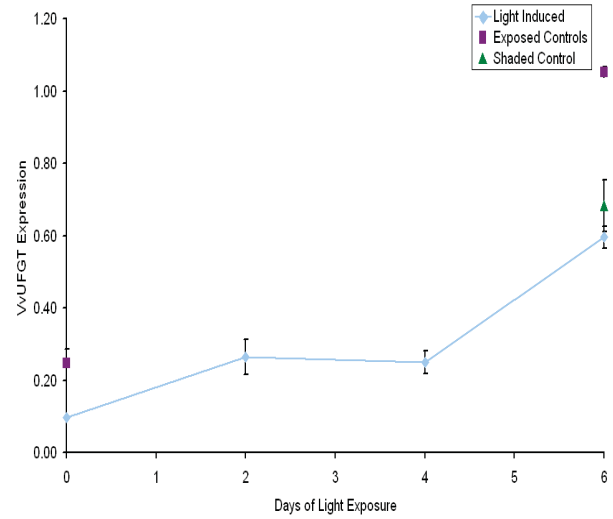
B,D Accumulation of total flavonols (expressed per g of berry) in LI, EC & SC berry skins determined by HPLC. Different letters indicate significant differences ($p<0.001$) between LI, EC & SC on days 0 & 6 determined by a 2-way ANOVA followed by a Fishers protected Least Significant Difference (LSD) test ($p=0.05$).

In Shiraz, shaded bunches were looser than exposed bunches and berries weighed approximately 0.1g less (**Table 4.6**). While shaded bunches looked less coloured than exposed bunches after removal of the boxes, both bunches appeared to accumulate anthocyanins over the 6 day sampling period, such that there was no noticeable difference by day 6 (**Figure 4.4**). In Chardonnay, shaded bunches had significantly less chlorophyll than exposed bunches. Berry weight in shaded bunches was significantly lower than in exposed bunches, 0.75g compared to 0.93g, respectively (**Table 4.6**). In both varieties, over the 6-day sampling period ^{o}Brix increased in exposed, shaded and light induced berries (**Figure 4.4**), as expected at this stage in grape berry development (Coombe 1987).

To compare with phenotypic observations in Shiraz, *VvUFGT* expression and anthocyanin accumulation was determined (**Figure 4.8**). On day 0 and day 6, *VvFLS1* expression in the shaded berries was around 2-fold lower than in exposed berries. There were also similar levels of anthocyanins detected in the shaded berry skins on day 0, however by day 6 exposed controls had a higher total anthocyanin content than shaded controls. Clearly, *VvUFGT* expression increased over the 6-day sampling period, in exposed (4-fold), shaded (7-fold) and light induced (6-fold) berries, as expected at this stage in development (Boss et al. 1996a). The increase in *VvUFGT* expression in berries corresponded with anthocyanin accumulation in exposed, shaded and light induced berries.

SHIRAZ

A



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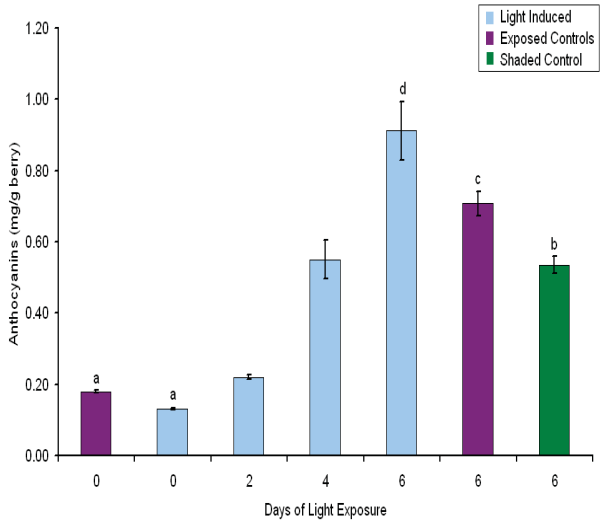


Figure 4.8 The influence of bunch light exposure, post-veraison, on *VvUFGT* expression & anthocyanin accumulation in Shiraz berries. Boxes were applied to vines at budburst in the 2003-2004 season. Approximately 1.5weeks prior to veraison, 30 boxes were removed from vines & 100 berry skins randomly sampled (Light Induced (LI)) along with nearby control berries (Exposed Controls (EC)). LI samples were taken every 2nd day for 6 days, when another EC sample was taken. An additional shaded control (SC) sample was also taken on day 6. For each sample 100 berries were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates.

A Expression of *VvUFGT* relative to *VvUBIQ1* expression in LI, EC & SC berry skins determined by RT-PCR.

B Accumulation of total anthocyanins (expressed per g of berry) in LI, EC & SC berry skins determined by HPLC. Different letters indicate significant differences ($p<0.001$) between LI, EC & SC on days 0 & 6 determined by a 2-way ANOVA followed by a Fishers protected Least Significant Difference (LSD) test ($p=0.05$).

4.3.2.4 Pre-harvest sampling

The last sampling time was approximately 2 weeks prior to harvest, when *VvFLS1* expression and flavonol accumulation was expected to be higher than at veraison (Downey et al. 2003b). The influence of bunch light exposure on *VvFLS1* expression and flavonol accumulation in Shiraz and Chardonnay berries, pre-harvest is shown in **Figure 4.9**.

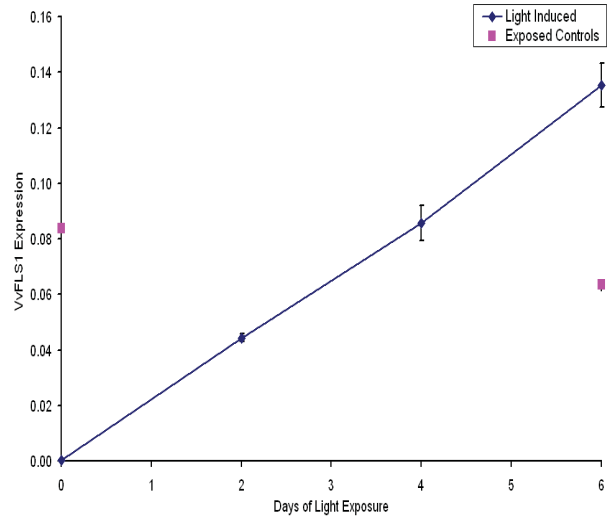
In Shiraz, on day 0, *VvFLS1* expression in the shaded berries was 270-fold lower than in exposed berries, corresponding with a 2-fold reduction in flavonol concentration. After the removal of the boxes on day 0, *VvFLS1* expression in light induced berries increased linearly, and by day 6 was 450-fold higher than exposed berries. Yet, in contrast to the results at earlier sampling times, there was no increase in flavonol concentration, with levels remaining less than 50% of exposed berries over the 6-day sampling period. Over the 6-day sampling period *VvFLS1* expression levels remained constant, while flavonol concentration slightly decreased by day 6.

In Chardonnay, on day 0, *VvFLS1* expression in the shaded berries was 160-fold lower than in exposed berries and there were no detectable flavonols. Following removal of the boxes on day 0, *VvFLS1* expression in light induced berries increased 50-fold on day 2. On day 4 *VvFLS1* expression had reached the highest being 360-fold greater than on day 0. By day 6, *VvFLS1* expression had reduced, to levels lower than exposed berries. The delay in *VvFLS1* expression (peak on day 4), corresponded with a slow increase in flavonol concentration over the 6 days; yet the level reached on day 6 was still less than 50% in exposed berries. Over the 6-day sampling period, *VvFLS1* expression increased-2-fold, while flavonol concentration decreased by about 2-fold.

In Shiraz and Chardonnay the shaded bunches ripened similarly to exposed bunches with no significant difference in berry weight and ^{o}Brix (**Figure 4.4** & **Table 4.6**). Shaded Shiraz bunches were looser than exposed bunches and while it appears berry set may have also been affected (less berries per bunch) this was only observed on some bunches. Following the observations earlier in the season, shaded Chardonnay bunches had less chlorophyll than exposed bunches (**Figure 4.4**).

SHIRAZ

A



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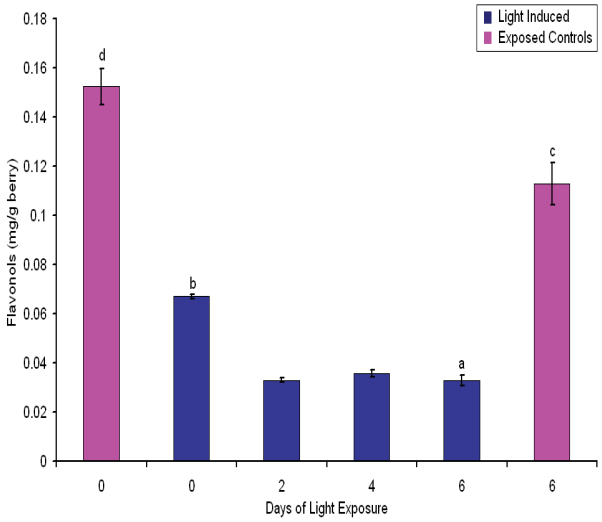
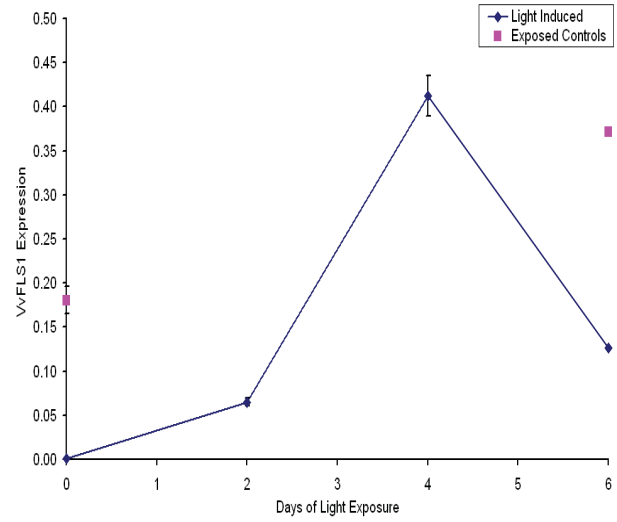


CHARDONNAY

C



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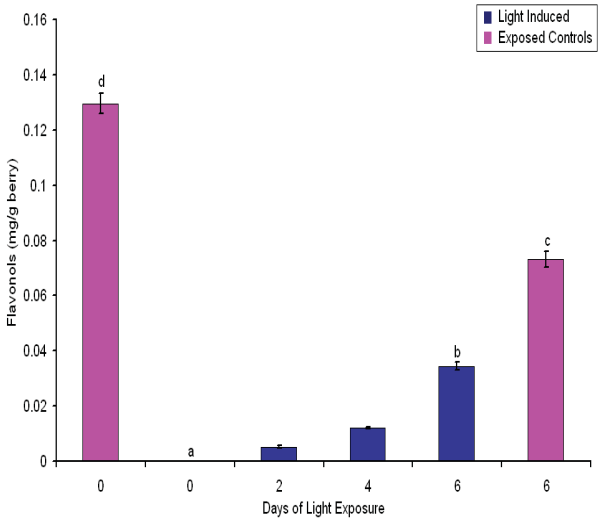


Figure 4.9 The influence of bunch light exposure, pre-harvest, on *VvFLS1* expression & flavonol accumulation in Shiraz & Chardonnay berries. Boxes were applied to vines at budburst in the 2003-2004 season. Approximately 2 weeks prior to harvest, 30 boxes were removed from vines & 100 berry skins randomly sampled (Light Induced (LI)) along with nearby control berries (Exposed Controls (EC)). LI samples were taken every 2nd day for 6 days, when another EC sample was taken. For each sample 100 berries were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates.

A,C Expression of *VvFLS1* relative to *VvUBIQ1* expression in LI & EC berry skins determined by RT-PCR.

B,D Accumulation of total flavonols (expressed per g of berry) in LI & EC berry skins determined by HPLC. Different letters indicate significant differences ($p<0.001$) between LI & EC on days 0 & 6 determined by a 2-way ANOVA followed by a Fishers protected Least Significant Difference (LSD) test ($p=0.05$).

The influence of bunch light exposure on *VvUFGT* expression and anthocyanin accumulation in Shiraz at harvest was also determined (**Figure 4.10**). On day 0, *VvUFGT* expression and anthocyanin accumulation in shaded berries was similar to exposed berries. After removal of the boxes on day 0, there was no significant increase in *VvUFGT* expression in light induced berries; rather there was a net decrease in expression by 1.5-fold on day 6. There was also a constant decrease in anthocyanin concentration over the 6 days, with similar levels in exposed and light induced berries by day 6.

SHIRAZ

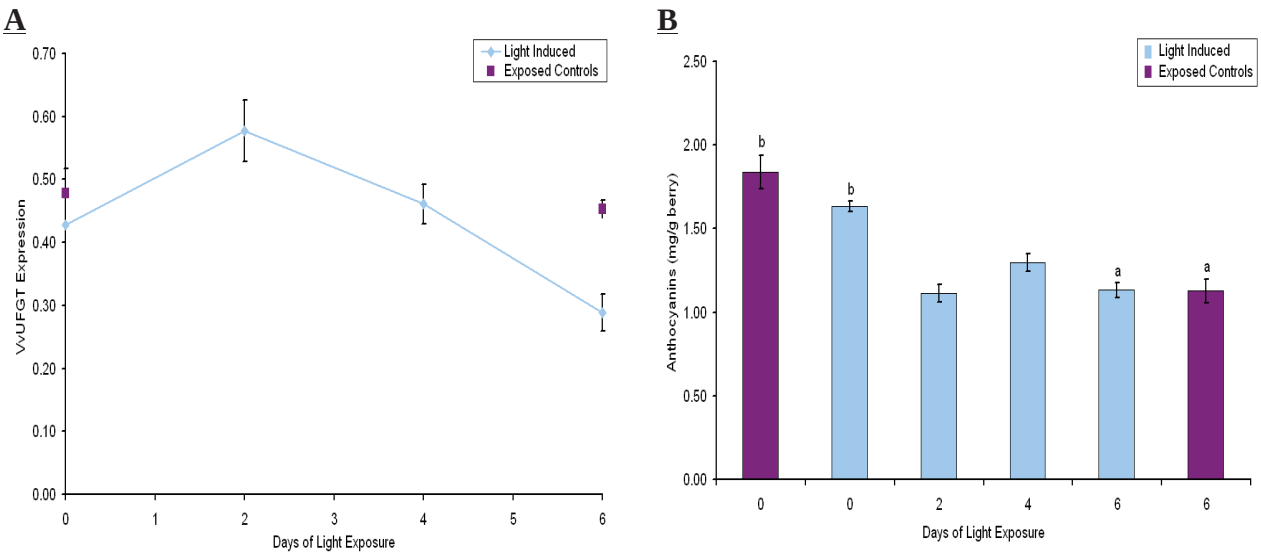


Figure 4.10 The influence of bunch light exposure, pre-harvest, on *VvUFGT* expression & anthocyanin accumulation in Shiraz berries. Boxes were applied to vines at budburst in the 2003-2004 season. Approximately 2 weeks prior to harvest, 30 boxes were removed from vines & 100 berry skins randomly sampled (Light Induced (LI)) along with nearby control berries (Exposed Controls (EC)). LI samples were taken every 2nd day for 6 days, when another EC sample was taken. For each sample 100 berries were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates.

A Expression of *VvUFGT* relative to *VvUBIQ1* expression in LI & EC berry skins determined by RT-PCR.

B Accumulation of total anthocyanins (expressed per g of berry) in LI & EC berry skins determined by HPLC. Different letters indicate significant differences ($p<0.001$) between LI & EC on days 0 & 6 determined by a 2-way ANOVA followed by a Fishers protected Least Significant Difference (LSD) test ($p=0.05$).

4.3.2.5 The influence of bunch light exposure on expression of other flavonoid genes (*VvLDOX* & *VvCHI*) during development

In addition to *VvFLS1* and *VvUFGT* expression, RT-PCR analysis was performed on other grapevine flavonoid pathway genes; *VvLDOX* and *VvCHI*, in Shiraz and Chardonnay, at all sampling times. **Table 4.7** shows summarised light induction data for *VvFLS1*, *VvLDOX* and *VvCHI* expression at different stages in development, while graphs are shown in **Appendix 4B.** Represented in **Table 4.7,** (unshaded) are the expression values for exposed (light=L) and shaded (dark=D) fruit (both on day 0) and light induced (LI), on the day at which expression peaked. Also shown (shaded) are the ratios of L: D indicating the influence of shading, LI: D indicating the level of induction and LI: L indicating the difference in expression between LI and L.

The developmental expression of *VvLDOX* and *VvCHI* was measured in exposed controls. In Shiraz, *VvLDOX* expression was high pre-flowering, but reduced by 21-fold by pre-veraison. Post-veraison, expression of *VvLDOX* increased, with higher levels measured pre-harvest. The developmental expression of *VvCHI* was similar to *VvLDOX,* despite lower expression levels for *VvCHI*. In Chardonnay, *VvLDOX* and *VvCHI* expression was high pre-flowering; however, in contrast to the expression pattern observed in Shiraz, the expression of *VvLDOX* and *VvCHI* remained low after veraison.

In Shiraz, at all sampling times, the L: D ratio representing the influence of shading treatment and the LI: D ratio representing the level of light induction for *VvFLS1* was higher than for *VvLDOX* and *VvCHI*. At all sampling times, the shading treatment appeared to have little to no effect on *VvLDOX* and *VvCHI* expression, the highest L: D ratio pre-flowering, with 7.8 and 2.6, respectively. Following the removal of the boxes there was some level of *VvLDOX* and *VvCHI* induction, the highest expression occurring on either day 2, 4 or 6 (see **Appendix 4B**). Pre-flowering, the level of *VvLDOX* and *VvCHI* induction matched that which the shading treatment imposed by day 6. At the pre-veraison and post-veraison stages of sampling, both genes were slightly induced, the highest LI: D ratio 8.8. It is important to note that post-veraison, *VvLDOX* and *VvCHI* expression in all samples (exposed, shaded, light induced) increased over the course of the 6 days (see **Appendix 4B**). The likely explanation for this is the phenological stage in development, where around veraison, large changes in cell metabolism occurs (Robinson and Davies 2000). Pre-harvest, the LI: D ratio for *VvLDOX* and *VvCHI* expression was 2.6 and 3.1, respectively.

Table 4.7 Summarised light induction data for *VvFLS1, VvLDOX* & *VvCHI* expression, in Shiraz & Chardonnay berries at different stages in development. RT-PCR analysis was used to determine expression levels of genes *VvFLS1*, *VvLDOX* & *VvCHI* normalised to *VvUBIQ1*. Represented in the table unshaded, are the expression values for exposed (**L**) & shaded (**D**) fruit on day 0. Light induced (**LI**) expression values (also unshaded) are on the day at which expression peaked, as indicated. Shaded values are the ratios of L: D expression (indicating the influence of shading), LI: D (indicating the level of induction) & LI: L (indicating the difference in expression between LI:L). **Appendix 4B** shows detailed graphs of the influence of bunch light exposure on expression of *VvLDOX* & *VvCHI* during development.

		Shiraz						Chardonnay					
Time of sampling	**Sample**	**Day**	***VvFLS1***	**Day**	***VvLDOX***	**Day**	***VvCHI***	**Day**	***VvFLS1***	**Day**	***VvLDOX***	**Day**	***VvCHI***
Pre-flowering	**Exposed L**	0	0.44	0	3.20	0	0.40	0	0.18	0	2.16	0	0.34
	Shaded D	0	0.04	0	0.41	0	0.15	0	0.14	0	0.03	0	0.01
	Light Induced LI	2	0.51	6	3.23	6	0.36	6	0.42	4	1.22	4	0.20
	L : D	**11.30**		**7.80**		**2.67**		**1.31**		**72.00**		**34.00**	
	LI : L	**1.17**		**1.01**		**0.90**		**2.34**		**0.56**		**0.59**	
	LI : D	**13.19**		**7.88**		**2.40**		**3.06**		**40.60**		**20.07**	
Pre-veraison	**Exposed L**	0	0.005	0	0.15	0	0.04	Pre-veraison sampling not performed in Chardonnay					
	Shaded D	0	0.002	0	0.16	0	0.03						
	Light Induced LI	2	0.18	2	0.33	2	0.11						
	L : D	**2.74**		**0.94**		**1.33**							
	LI : L	**38.28**		**2.20**		**2.75**							
	LI : D	**104.97**		**2.06**		**3.67**							
Post-veraison	**Exposed L**	0	0.01	0	1.68	0	0.29	0	0.13	0	0.21	0	0.06
	Shaded D	0	0.00001	0	0.97	0	0.15	0	0.0002	0	0.15	0	0.04
	Light Induced LI	2	0.40	6	5.47	6	1.33	2	0.69	6	0.45	4	0.13
	L : D	**1419.35**		**1.73**		**1.93**		**491.87**		**1.40**		**1.41**	
	LI : L	**28.20**		**3.26**		**4.59**		**5.44**		**2.13**		**2.44**	
	LI : D	**40024.18**		**5.64**		**8.87**		**2677.00**		**2.98**		**3.44**	
Pre-harvest	**Exposed L**	0	0.08	0	1.80	0	0.84	0	0.18	0	0.10	0	0.10
	Shaded D	0	0.0003	0	0.89	0	0.62	0	0.001	0	0.70	0	0.08
	Light Induced LI	6	0.14	2	2.31	6	1.95	4	0.41	4	0.14	4	0.19
	L : D	**275.60**		**2.02**		**1.35**		**160.59**		**0.14**		**1.33**	
	LI : L	**1.62**		**1.28**		**2.32**		**2.28**		**1.42**		**1.89**	
	LI : D	**445.84**		**2.60**		**3.15**		**366.84**		**0.20**		**2.51**	

On the whole, *VvLDOX* and *VvCHI* gene expression patterns observed in Shiraz were similar to Chardonnay. In Chardonnay berries, the L:D and LI:D ratios for *VvFLS1* were significantly higher than for *VvLDOX* and *VvCHI* (**Table 4.7**). Post-veraison and pre-harvest, the influence of shading on *VvLDOX* and *VvCHI* expression was low (L:D ratio <1.40) and the level of light induction was also small (LI:D ratio <3.4). However, at the pre-flowering stage of sampling, an atypical response to light induction was observed where, in contrast to the results observed at later stages in development and parallel experiments conducted in Shiraz, the effect of shading and subsequent response to light exposure was more pronounced for *VvLDOX* and *VvCHI* compared to *VvFLS1*, as represented by higher L: D and LI: D ratios.

4.3.3 Experiment 3- Hourly light induction in Shiraz berries, pre-veraison

To determine *VvFLS1* expression and flavonol accumulation over a shorter timeframe, an additional light induction experiment was performed in Shiraz berries in the 2004-2005 season. **Figure 4.11** shows the hourly influence of bunch light exposure on *VvFLS1* expression and flavonol accumulation in Shiraz berries, pre-veraison. The results observed are broadly similar to the previous years data (2003-2004), at that stage in development (pre-veraison). On day 0, *VvFLS1* expression in the shaded inflorescences was 18.5-fold lower than the exposed controls and no flavonols were detected. Three hours after the boxes were removed, *VvFLS1* expression had started to increase and after 11 hrs of exposure, expression had increased by 360-fold. The next day (day 1), *VvFLS1* expression levels had started to reduce and by day 2, *VvFLS1* expression levels were 70-fold greater than on day 0. By day 6, *VvFLS1* expression in light induced samples remained higher than in the exposed controls. Corresponding with an increase in *VvFLS1* expression, flavonol concentration increased, with flavonols detected after 6 hrs light exposure. However, by day 6, flavonol concentration in light induced fruit was still less than exposed control fruit.

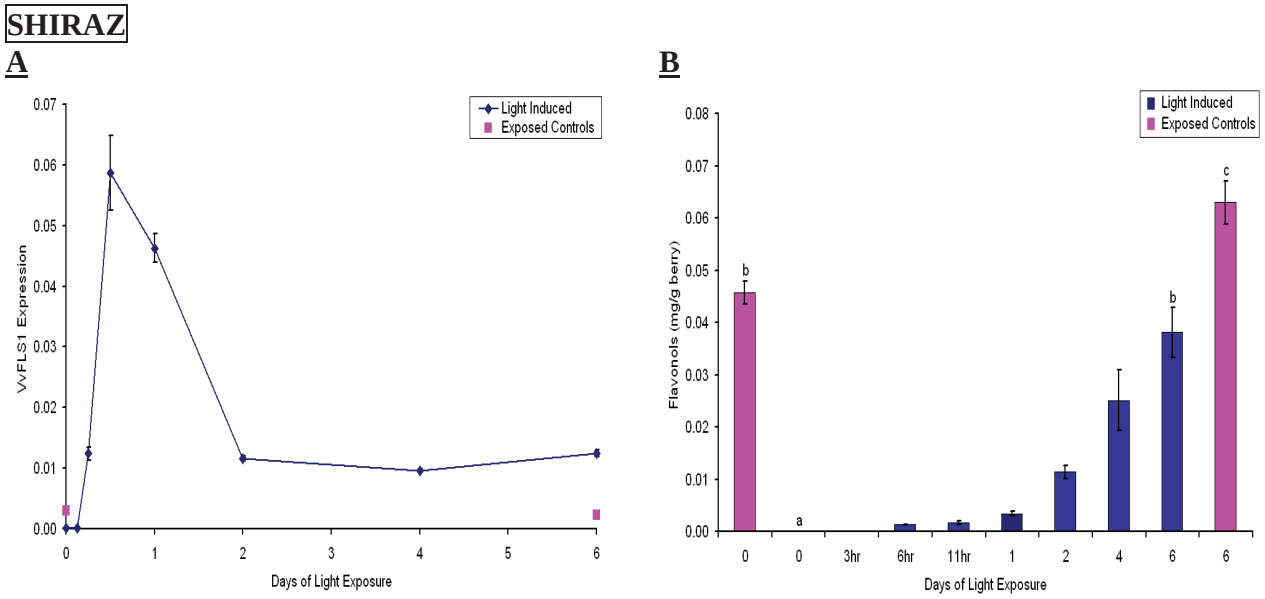


Figure 4.11 The (hourly) influence of bunch light exposure, pre-veraison, on *VvFLS1* expression & flavonol accumulation in Shiraz berries. Boxes were applied to vines at when shoots had ~8 leaves separated in the 2004-2005 season. Approximately, 2 weeks prior to veraison, starting at 9:00 am, 50 boxes were removed from vines & 100 berry skins randomly sampled (Light Induced (LI)) along with nearby control berries (Exposed Controls (EC)), day 0. LI samples continued to be sampled 3, 6, & 11 hrs after induction, followed by samples on day 1, 2, 4 & 6 (when another EC sample was taken). For each sample 100 berries were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates.

A Expression of *VvFLS1* relative to *VvUBIQ1* expression in LI & EC berry skins determined by RT-PCR.

B Accumulation of total flavonols (expressed per g of berry) in LI & EC berry skins determined by HPLC. Only the quercetin glycosides were detected in berry skins, which are summed to total flavonols. Different letters indicate significant differences ($p<0.001$) between LI & EC on days 0 & 6 determined by a 2-way ANOVA followed by a Fishers protected Least Significant Difference (LSD) test ($p=0.05$).

4.3.4 The diurnal pattern of *VvFLS1* gene expression in Shiraz fruit at different stages during development

In the 2003-2004 season, at the Coombe Vineyard, the diurnal expression of *VvFLS1*, *VvUFGT* and *VvLDOX* genes was investigated at 3 different sampling times during Shiraz berry development (post-flowering, post-veraison and pre-harvest). The first sampling time was approximately 1 week post-flowering. The diurnal pattern of *VvFLS1* expression in Shiraz inflorescences, recorded over 40 hrs is shown in **Figure 4.12**. Clearly, both temperature and light measurements reflected a day/night cycle. Expression of *VvFLS1* was high at this stage in development and appeared to follow a diurnal pattern of expression, as on both days, at 13:00 (when light exposure was at its greatest) *VvFLS1* expression was at its highest. Also, at times when it was dark, 05:00, 21:00, 01:00, *VvFLS1* expression was low. For the two peaks observed (at 13:00), *VvFLS1* expression increased (from 05:00) 3.1-fold and 1.8-fold, respectively.

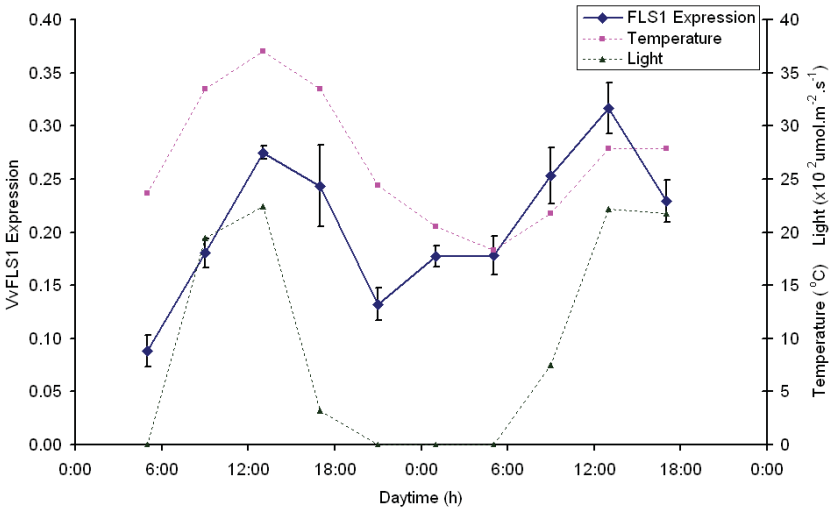


Figure 4.12 The diurnal pattern of *VvFLS1* expression in Shiraz, post-flowering. One week after flowering, starting at 05:00, 15 inflorescences were randomly sampled from vines. Inflorescences continued to be samples every 4 hr, for a total of 40 hr. For each sample inflorescences were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates. Expression of *VvFLS1* is relative to the expression of *VvUBIQ1* determined by RT-PCR. Temperature (°C) & light (PAR µmol.m^{-2}s^{-2}) was also recorded every 4 hr.

The second sampling time was approximately 1-week post veraison. **Figure 4.13** shows the diurnal pattern of *VvFLS1* expression in Shiraz berries post-veraison, recorded over 40 hr. At this stage in development *VvFLS1* expression was lower than at post-flowering, as expected (Downey et al. 2003b). *VvFLS1* expression was highest later in the afternoon on both days at 17:00, and was lowest overnight, at 01:00 and 05:00. The increase in *VvFLS1* expression, observed on day 1 was 1.2-fold (from 05:00) and on day 2 was 3.7-fold.

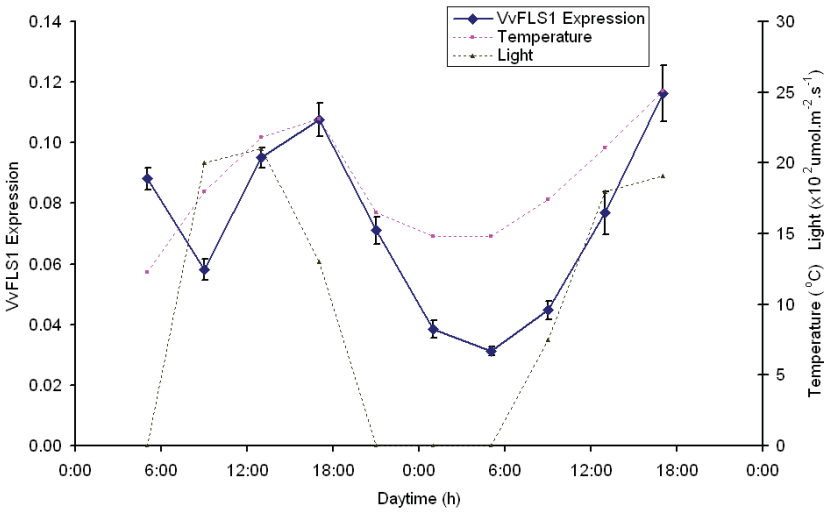


Figure 4.13 The diurnal pattern of *VvFLS1* expression in Shiraz, post-veraison. One week after veraison, starting at 05:00, 100 berries were randomly sampled from Shiraz vines. Berries continued to be samples every 4 hr, for a total of 40 hr. For each sample berry skins were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates. Expression of *VvFLS1* is relative to the expression of *VvUBIQ1* determined by RT-PCR. Temperature (°C) & light (PAR µmol.m^{-2}s^{-2}) was also recorded every 4 hr.

The last sampling time was approximately 1 week before harvest. **Figure 4.14** shows the diurnal pattern of *VvFLS1* expression in Shiraz berries pre-harvest, recorded over 40 hr. As expected at this stage in development, there was a high level of *VvFLS1* expression. *VvFLS1* expression was highest in the afternoon (17:00) and was low at 05:00, on both days. On the second day, there was a decrease in *VvFLS1* expression at 13:00; however by 17:00 levels were high, similar to levels on the previous day at the same time. The initial increase in *VvFLS1* expression, observed on day 1 at 17:00, (from 05:00) was 3.2-fold and on day 2 was 2.5-fold.

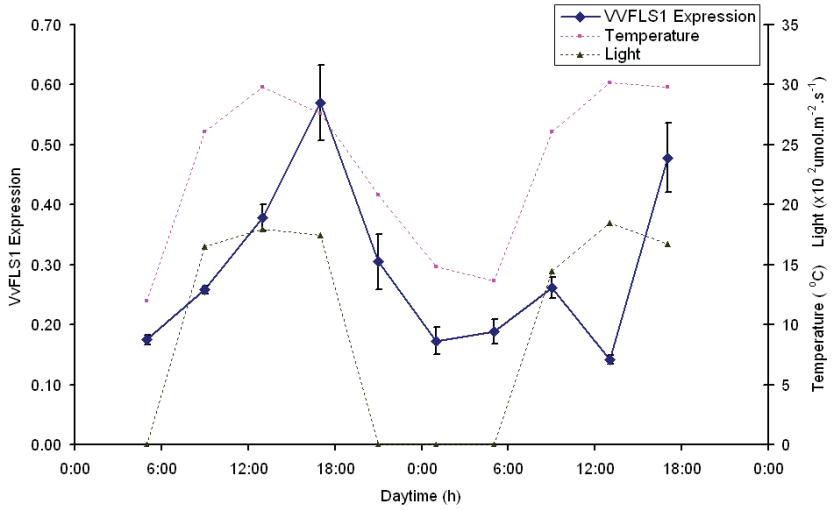


Figure 4.14 The diurnal pattern of *VvFLS1* expression in Shiraz, pre-harvest. One week before harvest, starting at 05:00, 100 berries were randomly sampled from Shiraz vines. Berries continued to be samples every 4 hr, for a total of 40 hr. For each sample the berry skins were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates. Expression of *VvFLS1* is relative to the expression of *VvUBIQ1* determined by RT-PCR. Temperature (°C) & light (PAR µmol.m^{-2}s^{-2}) was also recorded every 4 hr.

The diurnal expression of *VvLDOX* and *VvUFGT* are shown in **Figure 4.15**. The overall level of *VvLDOX* expression, at each sampling time was relatively similar, with pre-harvest samples having slightly higher expression levels. *VvLDOX* expression post-flowering, appeared to follow a diurnal pattern of expression, as on both days at 13:00 expression was highest whereas at 05:00, 21:00, 01:00 expression was low. For the two peaks observed (at 13:00), *VvLDOX* expression increased (from 05:00) 3.5-fold and 5.3-fold, respectively. For the later sampling times, *VvLDOX* expression did not appear to follow a diurnal pattern of expression. Post-veraison, *VvLDOX* expression remained relatively constant over 40 hr while pre-harvest, *VvLDOX* expression was low at times when it was light (9:00 and 1:00) on the second day.

Clearly, *VvUFGT* expression was higher at harvest than at post-veraison as expected for this stage in development (Boss et al. 1996a; Downey et al. 2004). It appeared that *VvUFGT* did not follow a diurnal pattern of expression. Similar to the post-veraison samples in Shiraz, *VvUFGT* expression remained relatively constant for the 40 hr. Also, pre-harvest on the second day, *VvUFGT* expression was low at times when it was light, particularly early in the morning.

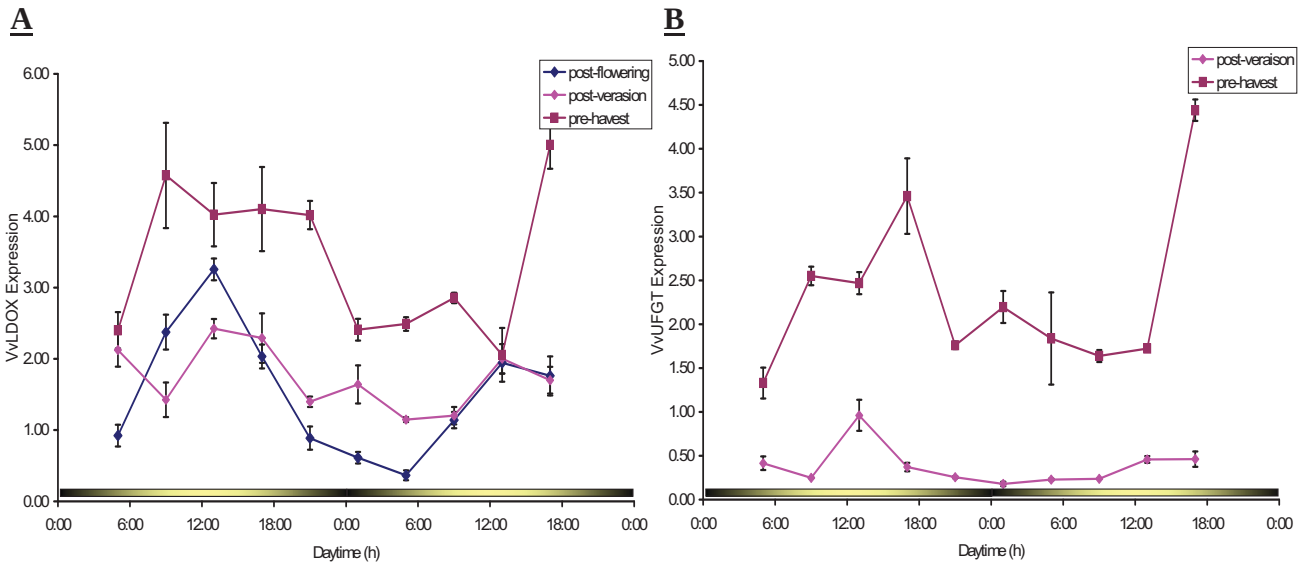


Figure 4.15 The diurnal pattern of *VvLDOX* & *VvUFGT* expression in Shiraz, during development. At three stages in development (post-flowering, post-veraison & pre-harvest), starting at 05:00, 100 berries were randomly sampled from Shiraz vines. Berries continued to be samples every 4 hr, for a total of 40 hr. For each sample 100 berry skins were pooled together. Error bars represent the standard error (±SEM) for three analytical replicates. A coloured bar at the base of the graph indicates the day/night cycle.

A Expression of *VvLDOX* relative to the expression of *VvUBIQ1* determined by RT-PCR.

B Expression of *VvUFGT* relative to the expression of the *VvUBIQ1* determined by RT-PCR.

4.4 Discussion

4.4.1 Expression of *VvFLS1* & accumulation of flavonols during grape berry development

The developmental expression of *VvFLS1* and accumulation of flavonols was observed in Shiraz and Chardonnay fruit, through analysis of the exposed controls. In both varieties, at pre-flowering and pre-harvest sampling stages, there were high levels of *VvFLS1* expression compared to samples taken around veraison. Corresponding with these periods of increased *VvFLS1* expression around flowering, the fruit had relatively high concentration of flavonols, which declined as the berries grew. There was an increase in flavonols per berry during ripening coinciding with elevated *VvFLS1* expression. These results confirm previous observations by Downey et al. (2003b). Furthermore, *VvFLS1* expression and flavonol content in Shiraz and Chardonnay leaves were greater than in other berry tissues (i.e. inflorescences, berry skins), as also reported by Downey et al. (2003b).

In all tissues analysed (inflorescences, leaves and berry skins), the quercetin glycosides were the predominant flavonols detected in Shiraz and Chardonnay, as also reported in other studies (Castillo-Munoz et al. 2007; Cheynier and Rigaud 1986; Cortell and Kennedy 2006; Downey 2002; Haselgrove et al. 2000; Price et al. 1995). However, because several flavonols were measured the total flavonol content was around 2-fold higher than reported by Downey et al. (2003b), yet when the quercetin-glycosides were plotted alone values were similar (data not shown).

Many of unknown flavonol compounds detected in grape berries from different cultivars have recently been identified (Castillo-Munoz et al. 2007; Mattivi et al. 2006), which has allowed the total flavonol composition during grape berry development to be determined in this investigation (data not shown). In Shiraz and Chardonnay inflorescences and leaves the glycosides of quercetin, myricetin, kaempferol and isorhamnetin were detected, with the proportion of quercetin derivatives (including isorhamnetin glycosides) making up ~90% of the total flavonols detected. During Chardonnay berry ripening (i.e. post-veraison and pre-harvest) myricetin and kaempferol glycosides could not be detected. In contrast, most of the flavonol compounds were detected in Shiraz skins after berry ripening (post-veraison and pre-harvest), and at the pre-harvest sampling time the proportion of quercetin derivatives around 65%, followed by myricetin-glycosides (~25%).

The differences in flavonol composition between Shiraz and Chardonnay and during grape berry development is in agreement with changes in expression of the flavonoid 3'-hyrdroxylase (*VvF3'H*) and flavonoid 3',5'-hydroxylase (*VvF3'5'H*) genes. A schematic diagram of the flavonoid biosynthetic pathway separated according the *VvF3'H* and *VvF3'5'H* genes is shown in **Figure 4.16**. Recently, it was shown in Shiraz that *VvF3'H* was expressed in grapes before flowering, when 3'4 hydroxylated flavonols are made (i.e. quercetin derivatives)(Bogs et al. 2006). It was also shown that in berry skins that expression of the *VvF3'H* and *VvF3'5'H* genes was low at the onset of ripening (veraison) but increased after veraison concomitant with the accumulation of 3'4 hydroxylated (quercetin derivatives) and 3'4'5'hydroxylated (myricetin derivatives) flavonols (Bogs et al. 2006). Therefore, the change in the proportion of quercetin and myricetin derivatives observed in this study, from early in development through to ripening in Shiraz corresponds with the work by Bogs et al. (2006). Similarly, Bogs et al. (2006) also showed that expression of *VvF3'5H* in Chardonnay remained relatively low throughout berry ripening, consistent with no significant amounts of myricetin detected during berry development. In this investigation myricetin derivatives were not detected in berry skins during ripening, which is again, in agreement with observations by Bogs et al. (2006).

In addition to *VvFLS1* expression and flavonol accumulation, other flavonoid biosynthetic genes were also investigated including *VvCHI, VvLDOX* and *VvUFGT*. Anthocyanin content was also measured in Shiraz samples. In Shiraz after veraison, *VvUFGT* expression and anthocyanin content was high which has also been reported by (Boss et al. 1996a; Downey et al. 2004). While at the pre-harvest sampling time there was a slight decrease in *VvUFGT* expression and anthocyanin accumulation over the 6 day period (**Figure 4.10**), overall levels were similar to those reported by Downey et al. (2004).

In both varieties, the expression of *VvCHI* and *VvLDOX* genes around flowering was high compared to later sampling times (**Table 4.8**). The increased expression of these early flavonoid pathway genes early in development is suggested to coincide with synthesis of flavonols and tannins (Bogs et al. 2005; Boss et al. 1996a), which may protect tissue from light and UV-damage and also protect the developing fruit from being eaten prior to seed maturation. After veraison, the pattern of *VvCHI* and *VvLDOX* expression differed between varieties. In Shiraz, expression of *VvLDOX* and *VvCHI* genes remained high, while in Chardonnay levels were much lower. In Shiraz, this increase in gene expression is suggested to be due to the onset of anthocyanin synthesis (Bogs et al. 2005; Boss et al.

1996a). These results confirm the differential regulation of the flavonoid biosynthetic pathway during development (Bogs et al. 2005; Boss et al. 1996a; Downey et al. 2003b) and show the differences in gene expression during development between red and white grape cultivars.

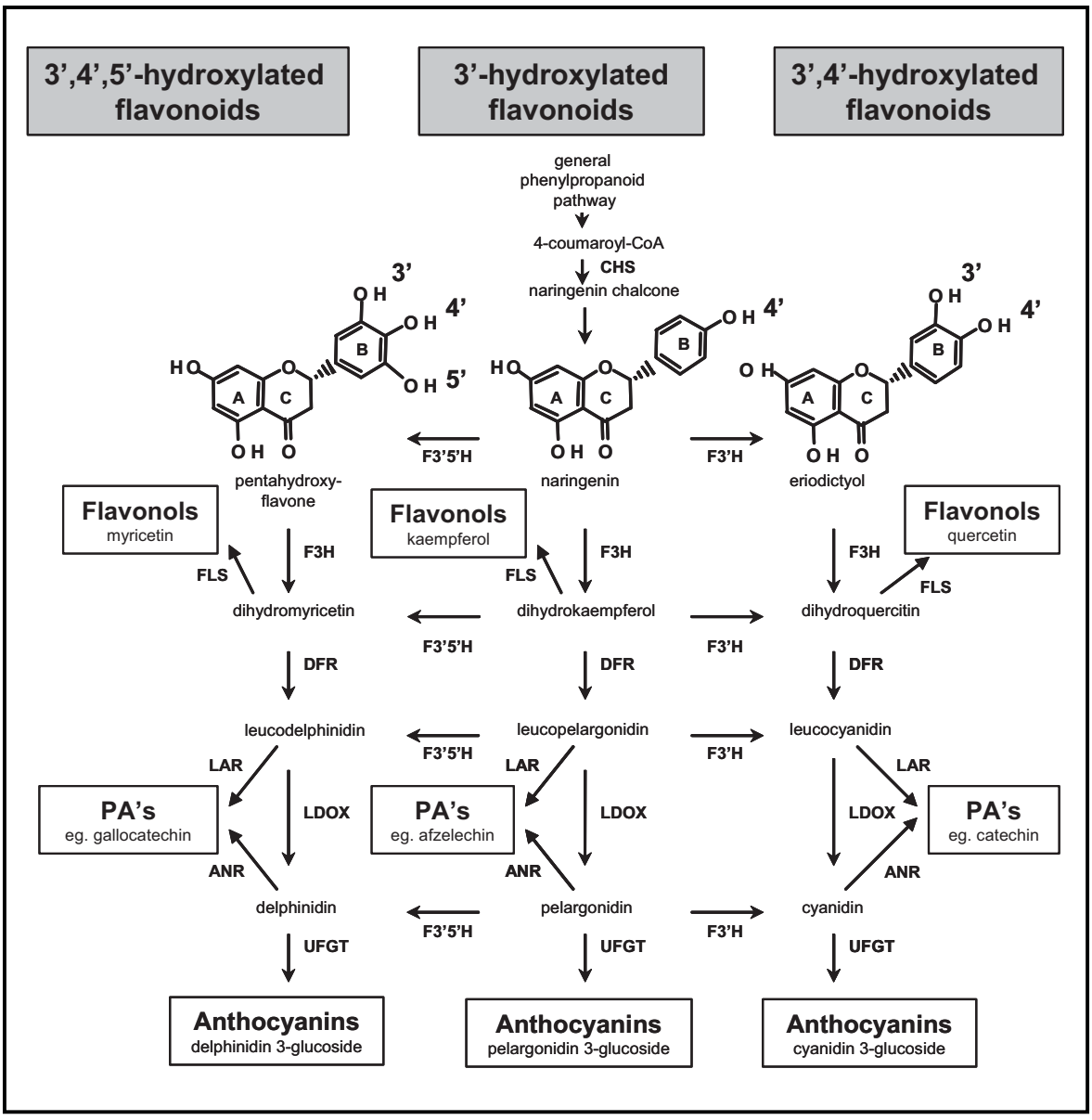


Figure 4.16 Schematic representation of the flavonoid biosynthetic pathway. Enzymes involved in the pathway shown are: CHS, chalcone synthase; CHI, chalcone isomerase; F3'H, flavonoid-3'-hydroxylase; F3'5'H, flavonoid-3',5'-hydroxylase; F3H, flavanone-3β-hydroxylase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin dioxygenase; FLS, flavonol synthase; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase; UFGT, UDP-glycose:flavonoid-3-O-glycosyltransferase. Examples for the different hydroxylation patterns of the flavonoid B-ring are given for naringenin (4'-hydroxylated), eriodictyol (3',4'-hydroxylated) & pentahydroxyflavone (3',4',5'-hydroxylated) (Bogs et al. 2006).

4.4.2 The effect of shading on berry development, *VvFLS1* expression & flavonol accumulation

Shading slightly affected Shiraz and Chardonnay bunch architecture. Early in development, shaded inflorescences had elongated stems with extended rachis laterals, the effect more pronounced in Shiraz than Chardonnay (**Figure 4.4**). Subsequently, throughout development shaded Shiraz bunches were looser than exposed controls. These observations are likely to be due to a small amount of reflected light entering the box and the inflorescences reaching towards the light. Downey et al. (2004) concedes that reflected light may enter the boxes; however the light intensity was found to be significantly less than direct light.

Despite the changes in bunch architecture, shading had no major effect on berry development. Pre-veraison and post-veraison, berry weight in shaded fruit was less than exposed controls; however by harvest, shaded and exposed berry weight was similar (**Table 4.6**), as also observed by Downey et al. (2004). Furthermore, sugar accumulation throughout development was similar in shaded and exposed bunches, consistent with previous observations (Cortell and Kennedy 2006; Downey et al. 2004). These results support the idea that photosynthesis was not compromised by the shading treatment as the leaves around shaded berries still had good exposure to sunlight.

In the field, the effectiveness of the shading treatment was confirmed by the visual observation that, at stages before veraison, shaded Shiraz and Chardonnay fruit was pale green colour compared dark green in exposed control fruit. This was also observed by Downey et al. (2004) who measured total chlorophyll content in shaded and exposed berries and showed that after berry set shaded berries contained a much lower concentration of chlorophyll compared to exposed controls. Chlorophyll synthesis is light induced (Raven et al. 1992) so shaded fruit would be expected to have less chlorophyll.

Table 4.8 shows summarized light induction data (*VvFLS1* expression and flavonol accumulation) in Shiraz and Chardonnay fruit at four separate times during development; pre-flowering, pre-veraison, post veraison and pre-harvest, in three different growing seasons. Represented in **Table 4.8** are the ratios of L: D (indicating the influence of shading) and LI: D (indicating the level of induction). It should be noted that caution should be taken when interpreting RT-PCR results, taking into consideration the magnitude of change in gene expression, where changes in gene expression around 2-3 may only reflect variation in the level of gene expression.

Table 4.8 Summarized light induction data (*VvFLS1* expression and flavonol concentration) in Shiraz & Chardonnay fruit, at different stages during development. At different stages in development, boxes were removed from fruit (day 0), & shaded (D) & exposed (L) control fruit sampled. Shaded fruit (now-light induced (LI)) continued to be sampled every 2nd day for 6 days. The change in *VvFLS1* expression & flavonol accumulation are represented as a ratio. The level of shading is represented by L: D ratio (expressed on day 0) & the level of light induction is represented by LI: D ratio (represented by the day of highest expression/accumulation). For *VvFLS1* expression the day of highest expression is indicated; however for flavonol accumulation this was always on day 6. Blank cells indicate data not available.

Time of sampling	Experiment Number	Sample Ratio		Ratio of *VvFLS1* expression		Ratio of flavonol concentration	
			Day of highest exp	Shiraz	Chardonnay	Shiraz	Chardonnay
Pre-flowering (Inflorescences)	2	L:D	2	11.3	1.3	3.9	2.4
		LI:D		13.2	2.6	3.9	2.7
Pre-flowering (Leaves)	2	L:D	2	7.3	3.9	33.7	36.3
		LI:D		5.2	9.6	23.8	19.4
Pre-veraison	1	L:D	2		110.7		6.6
		LI:D			2290.0		8.2
Pre-veraison	2	L:D	2	2.7		32.3	
		LI:D		105.0		35.6	
Pre-veraison	3	L:D	11hr	18.5		0.046 : 0	
		LI:D		359.1		0.038 : 0	
Post-veraison	2	L:D	2	1419.4	491.9	0.036 : 0	0.110 : 0
		LI:D		40024.2	2677.0	0.135 : 0	0.186 : 0
Pre-harvest	2	L:D	6 / 4	275.6	160.6	2.3	0.130 : 0
		LI:D		445.8	366.8	0.49	0.034 : 0

Overall, there were significant reductions in *VvFLS1* expression and flavonol content in Shiraz and Chardonnay shaded fruit compared to exposed fruit (**Table 4.8**). Expression of *VvFLS1* in shaded inflorescences, leaves and ripening fruit was considerably less than exposed controls, as also observed by Downey et al. (2004). Corresponding with low *VvFLS1* expression in shaded fruit, flavonol concentration was also reduced, and at some stages in development no flavonols were detected. This result is consistent with previous observations in grapes (Cortell and Kennedy 2006; Downey et al. 2004; Haselgrove et al. 2000; Pereira et al. 2006; Price et al. 1995; Spayd et al. 2002) and in other fruit crops such as apple (Solovchenko and Schmitz-Eiberger 2003; Takos et al. 2006). Interestingly, when flavonols were detected in shaded fruit, flavonol composition was not greatly different than in exposed fruit. These results suggest that shading does not differentially affect the pathways leading to the synthesis of different types of flavonol compounds (i.e. change in *VvF3'H* and *VvF3'5'H* gene expression).

Early in development, *VvFLS1* expression and flavonol content was least affected by shading, compared to stages later in development, as indicated by low L: D ratios (**Table 4.8**). Again, this could be due to a small amount of reflected light entering the box (as discussed above). Alternatively, it cannot be ruled out that developmental regulation maybe overriding the influence of shading at this early stage in development. As the season progressed, shading appeared to have a greater effect on *VvFLS1* expression and flavonol accumulation. Post-veraison the decrease in *VvFLS1* expression due to shading was at least 490-fold, and no flavonols were detected, indicting the effectiveness of the shading treatment (**Table 4.8**). In shaded Shiraz berries at pre-harvest, despite significantly reduced *VvFLS1* expression, moderate levels of flavonols were detected (**Figure 4.9**), the reasons for which is unclear.

At most stages in development, shading had little to no effect on *VvUFGT, VvLDOX* and *VvCHI* gene expression with levels similar to exposed controls. On average the L:D ratio for *VvLDOX* and *VvCHI* expression, at all stages in development (excluding Chardonnay pre-flowering) was around 2.0 (**Table 4.7**). Expression of *VvUFGT* and anthocyanin content was similar in shaded and exposed fruit, confirming suggestions by Downey et al. (2004), that light is not an absolute requirement for anthocyanin synthesis in Shiraz berries. However, it is worth mentioning that the effect of shading on anthocyanin content does appear to be cultivar specific, with shading inducing significant reductions in anthocyanin concentration in varieties such as Cabernet Sauvignon or Pinot Noir (Cortell and Kennedy 2006; Downey and Krstic 2005; Jeong et al. 2004).

Bunch shading slightly effected anthocyanin composition of the fruit, decreasing the proportion of trihydroxylated anthocyanins (i.e. glycosides of delphinidin, petunidin and malvidin) relative to the dioxygenated anthocyanins (i.e. glycosides of peonidin and cyanidin). In this study bunch shading decreased the proportion of dioxygenated anthocyanins by 25% compared to exposed berries, similar to previous reports (Cortell and Kennedy 2006; Downey et al. 2004). This change in anthocyanin composition has been associated with differential activity of the flavonoid biosynthetic genes *VvF3'5'H* and *VvF3'H* in response to light (Cortell and Kennedy 2006; Downey et al. 2004). The fact that anthocyanin and flavonol composition appears to change differentially to different environmental conditions (i.e. shading) makes the *VvF3'5'H* and *VvF3'H* genes of particular interest. It has yet to be determined whether this response differs in different varieties or is also influenced by temperature and is therefore an area of future research (see **Chapter 6, Section 6.6.5**).

While *VvFLS1* expression is clearly reduced in shaded fruit, it remains unknown as to whether lower levels of flavonols are caused by reduced biosynthesis or by degradation. In an attempt to address this issue, as a side experiment in 2004-2005, boxes were applied to vines pre-flowering (when flavonol synthesis is active) and shaded and exposed inflorescences were sampled on days 1, 2, 4, 7 and 10, after which *VvFLS1* expression and flavonol accumulation was determined (data not shown). Expression of *VvFLS1* shaded fruit was 3-fold less than the exposed controls after 24 hours (day 1), but the concentration of flavonols was the same after 10 days. This suggests that flavonols are relatively stable and that degradation processes are slow.

4.4.3 The effect of light induction on *VvFLS1* expression & flavonol accumulation

In both varieties, at most sampling times, a similar 'light induction response' was observed for *VvFLS1* expression and flavonol accumulation in inflorescences, leaves and berries (**Table 4.8**). Following low *VvFLS1* expression levels in shaded fruit on day 0, *VvFLS1* expression peaked on day 2 to levels greater than the exposed controls. On day 4 and 6 expression levels reduced to levels similar to the exposed controls. Corresponding to *VvFLS1* expression, flavonol content gradually increased by day 6 to levels similar to the exposed controls.

The samples taken pre-flowering (inflorescences and leaves) showed the lowest level of *VvFLS1* induction (maximum by 13.2-fold) compared to later stages in development (**Table 4.8**). Again, these results could be related to issues associated with sampling at this early stage in development (as previously described). It is important to mention there was one set of samples, which stood out from the majority of the data. In Chardonnay, pre-flowering samples, the shading treatment and subsequent light induction had a significantly greater effect on *VvLDOX* and *VvCHI* expression compared to *VvFLS1* expression. The reason for this atypical pattern of gene expression remains unclear.

Typical light induction responses were observed at the stages around veraison. The most dramatic 'light induction response', in terms of magnitude, was post-veraison, with *VvFLS1* expression increasing by at least 2500-fold (**Figure 4.7**). This corresponded with the production of flavonols to levels greater than exposed controls. Pre-harvest, there were notable differences in the 'light induction response' observed between Chardonnay and Shiraz. In Chardonnay, the response was

delayed, with *VvFLS1* expression peaking on day 4, and flavonols slowly accumulating. In Shiraz berries pre-harvest, *VvFLS1* expression increased linearly by 445-fold despite no net increase in flavonol concentration (the implications of which are discussed below).

The delay in *VvFLS1* gene expression and accumulation of flavonols observed pre-harvest in Chardonnay (compared to other sampling times), suggests that *VvFLS1* synthesis may become less light responsive as the berries start to ripen. This response is similar to results observed by Bias et al. (2000) who investigated the UV-light induction of stilbene phytoalexin biosynthesis during grape berry development. Grape berries (inc. Shiraz and Chardonnay) at different stages in berry development, were exposed to UV light for 10 mins and incubated for 0-8 hrs then on day 1, 2 and 3, after which stilbene synthase gene expression (*VvSTS*) and accumulation of resveratrol was determined. At the last sampling time (pre-harvest) the rate and maximal level of *VvSTS* expression and resveratrol accumulation was reduced compared to stages earlier in development earlier weeks (Bias et al. 2000).

At almost all stages in development flavonol content in shaded fruit increased after exposure to light (the exception, Shiraz, pre-harvest). Generally, the concentration of individual flavonols in light induced fruit increased proportionally to each other and by day-6 flavonol composition in light induced fruit was similar to that in exposed controls. However, at the stages in development where flavonols were not detected in shaded fruit (i.e. around veraison), the quercetin glycosides were the first flavonol compounds to be induced by light exposure followed by accumulation of some of the minor flavonols.

A 'light induction response' was not apparent for *VvLDOX, VvCHI* and *VvUFGT* genes and anthocyanins accumulated similar to exposed controls in Shiraz. However, there was a small change in expression of some genes in response to light (~3-fold). This is likely to be due to the fact that the removal of the boxes results in a dramatic dark-light change, which is likely to be perceived by the fruit as a large abiotic 'stress'. In response to a 'stress', many genes are up-regulated, including some of the flavonoid pathway genes, which have known roles in the UV-protection of tissues (Shirley 1996). The results observed in this investigation agree with suggestions by Downey et al. (2004) that light differentially regulates the expression of different genes in the flavonoid pathway, with *VvFLS1* clearly being much more responsive to light than the other flavonoid pathway genes.

The hourly light induction experiment, performed pre-veraison, allowed the timing of 'light induction response' to be narrowed, from day 2. It was shown that *VvFLS1* expression and flavonol accumulation in berries was induced by light after 6 hrs of exposure with the peak in *VvFLS1* expression occurring after 11 hrs (**Figure 4.11**). There was a slight delay in production of flavonols (i.e. *VvFLS1* expression increased after 3 hours, flavonols accumulated after 6 hours), which may be explained by the need for *de novo* protein synthesis during the light signal transduction process. This experiment indicates that most of the gene activity may in fact have been overlooked in the major light indication experiment (**Section 4.3.2**), where the earliest sampling time was on day 2. Nevertheless, these results strongly reiterate the highly light-dependent nature of flavonol synthesis.

While the data set described in this investigation contains some minor variations in timing (time of peak induction) and magnitude (fold-increase) the similarity at different stages in development and across seasons, establishes a convincing pattern for the light induction of *VvFLS1* synthesis and flavonol accumulation in fruit throughout development. This is in sharp contrast to expression of other flavonoid genes *VvUFGT, VvLDOX* and *VvCHI*, which were not greatly affected by shading or light exposure.

For flavonols to be accumulated in different grape tissues it is likely there are two key requirements; the first, involves the general activation of one or more of the 'early' genes in the flavonoid biosynthetic pathway prior to the flavonol branch point (eg. *VvPAL, VvCHS, VvCHI, VvF3H*), the second, involves activation of the enzyme responsible for the conversion of dihydroflavonols to flavonols (eg. VvFLS). It was demonstrated in this investigation that light affects these two regulatory processes differently. Expression of the 'early' pathway gene *VvCHI* was not greatly affected by light, however expression of the gene *VvFLS1* was found to be highly light responsive. This suggests that the *VvCHI* and *VvFLS* genes are not co-ordinately regulated for flavonol synthesis in grapes in response to light exposure. It also implies that the VvCHI enzymatic step is not rate limiting in the flavonoid biosynthetic pathway leading to flavonol synthesis. It is also worth noting that *VvLDOX* and *VvUFGT* expression and anthocyanin accumulation in Shiraz berries was largely unperturbed by exposure to light (**Figure 8** & **Figure 10**). This suggests that the 'late' pathway flux is not diverted away from anthocyanin production at the expense of flavonol production and that there must be enough substrate available to allow synthesis of both anthocyanins and flavonols at the same time in response to light exposure.

The light-responsiveness of the flavonoid biosynthetic pathway in grapevine appears to be different to other plant species. For example, in *Arabidopsis*, seedlings, plants and protoplasts the co-regulation of *AtCHS, AtCHI, AtF3H*, *AtFLS* genes has been shown in response to light (Hartmann et al. 2005; Kubasek et al. 1998; Kubasek et al. 1992; Pelletier et al. 1999; Pelletier et al. 1997). Also, recently the effect of light exposure on the flavonoid biosynthetic genes *(MdCHS, MdCHI, MdF3H, MdDFR, MdLDOX, MdUFGT, MdFLS, MdANR* and *MdLAR)* in apple was investigated (Takos et al. 2006). Light excluding bags were applied to fruit early in development and removed (around harvest) and fruit sampled on days 0,1, 2, 3, 4, 6 and 13 after exposure to light. It was shown that most of the 'shared genes' responsible for anthocyanin synthesis were up-regulated in response to light, while genes responsible for tannin synthesis (*MdANR/MdLAR*) remained unchanged. Takos et al. (2006) also showed *MdFLS1* expression increased in response to light exposure, peaking on day 2 by 140-fold, similar to levels of induction observed for *VvFLS1* in this investigation.

These studies in other plant species, together with the data presented in this study, suggest that light differentially regulates the expression of different genes in the flavonoid pathway in different plant species. It appears that there are light responsive regulatory mechanisms that are 'common', activating of most flavonoid biosynthetic pathway genes or 'specific', activating particular genes of the flavonoid biosynthetic pathway, either co-ordinately or individually. They also indicate the divergent mechanisms of flavonoid regulation in different plant species (Davies and Schwinn 2003b).

Work by Takos et al. (2006) also correlated the increased expression of the flavonoid genes in apple in response to light with the accumulation of anthocyanins. However, increased *MdFLS1* gene expression did not correspond to flavonol accumulation, which remained unchanged. This is similar to the result observed in this investigation pre-harvest, in Shiraz, where *VvFLS1* expression increased but there was no net increase in flavonol content. These results indicate regulation at the post-transcriptional level and there are two possible explanations for this result both which include substrate competition between the anthocyanin and flavonol pathways (Takos et al. 2006). The first, involves substrate (dihydroflavonols) competition by DFR, which would divert metabolism towards anthocyanin synthesis. Competition for substrates between anthocyanins and flavonol synthesis has been shown to occur in other plant species (Davies et al. 2003a; Holton et al. 1993; Martens et al. 2001; Nielsen et al. 2002), yet while this may explain the response pre-harvest, this was not observed post-veraison, when anthocyanins were also being synthesised.

The second level of substrate competition involves the glycosylation of quercetin, which provides stability to flavonol compounds. While the enzyme responsible for flavonol glycosylation (i.e. flavonol glycosyl-transferase (FGT)) in grapevines has not been determined, Ford et al. (1998a) showed the enzyme VvUFGT can catalyse the glycosylation of both cyanidin and quercetin. Therefore, substrate specificity could perhaps favour the glycosylation of anthocyanins over flavonols. However, this response would have also been observed post-veraison, which it was not. Consequently the results observed pre-harvest remains unclear.

4.4.3.1 Summary

This investigation clearly demonstrated the developmental expression of *VvFLS1, VvUFGT, VvLDOX* and *VvCHI* genes and the accumulation of anthocyanins and flavonols in Shiraz and Chardonnay fruit, confirming the differential regulation of the flavonoid biosynthetic pathway during grape berry development (Bogs et al. 2005; Boss et al. 1996a; Downey et al. 2003b). The results also show that *VvFLS1* gene expression and flavonol accumulation was highly light-dependent, compared to expression of other flavonoid biosynthetic genes (*VvCHI, VvLDOX* and *VvUFGT*), and in agreement with Downey et al. (2004), suggests light differentially regulates the expression of different genes in the flavonoid pathway, with *VvFLS1* being a branch pathway gene that is highly light-inducible.

Furthermore, the results presented in this investigation, indicate the light-responsive nature of *VvFLS1* expression and flavonol accumulation in Shiraz and Chardonnay fruit, throughout development. In general, it was shown that *VvFLS1* synthesis and flavonol accumulation was able to be induced by light by at least 100-fold, after 2 days of exposure. Most importantly, *VvFLS1* synthesis was able to be light induced at times when flavonols are not normally being synthesised (around veraison). This suggests light exposure can override the developmental control of *VvFLS1* expression and flavonol accumulation. It also indicates the complexity involved in *VvFLS1* gene regulation in grapes, during development and in response to light.

4.4.4 The diurnal pattern of *VvFLS1, VvUFGT* & *VvLDOX* gene expression during development

Plants are exposed to changing environmental conditions, one of the most dramatic being the daily alternation between light and darkness. The day/night cycling of gene expression is called a diurnal rhythm and is achieved primarily by two mechanisms: first by light and second by a free internal running circadian clock (Schaffer et al. 2001). Diurnal and circadian regulation of gene expression is crucial for coordinating the metabolic and physiological functions of plants, including sucrose/starch metabolism, nutrient acquisition, photosynthesis, oxidative stress, cold response and cell wall production (reviewed in McClung (2001)). Given that flavonol synthesis and accumulation is highly light regulated, with the ability to override the developmental expression and accumulation in grapes, the potential for changes in *VvFLS1* gene expression during a day/night cycle was investigated. Expression of *VvLDOX* and *VvUFGT* genes was also investigated.

At each sampling date, the expression level of each gene at a particular stage in development was as expected. For example, *VvFLS1* expression (high post-flowering, low post veraison and high pre-harvest), *VvUFGT* expression (low post-veraison and high pre-harvest) and *VvLDOX* expression (relatively high post-flowering, post-veraison and pre-harvest) levels were as reported by Bogs et al. (2005), Boss et al. (1996a) and Downey et al. (2003b).

It is important to note that the maximum variation in *VvFLS1*, *VvLDOX* and *VvUFGT* gene expression at each stage in development during the 40-hour period was around 3-fold. Considering the semi-quantitative nature of RT-PCR data, it still appears that *VvFLS1* gene expression follows a diurnal pattern of regulation, with higher expression at times when it was light **(Figure 4.12**, **Figure 4.13** & **Figure 4.14)**. However, it is important to note that *VvFLS1* expression was not absolute (i.e. there was still significant expression in the dark), perhaps indicating *VvFLS1* expression occurs after reaching a certain light threshold. At all stages in development, *VvFLS1* expression generally peaked during or at the end of the light period and was lowest at the end of the night. Also, the change in *VvFLS1* expression was generally more pronounced than *VvLDOX* and *VvUFGT* gene expression.

Recently, 30-50% of genes expressed in *Arabidopsis* were shown to be diurnally regulated, the changes in gene expression ranging from 2 to >300-fold (Blasing et al. 2005). While some

flavonoid genes showed diurnal changes (individual genes not described), genes involved in sucrose and starch metabolism, nutrient acquisition and redox regulation were especially dominant. The most frequent times for the maximum expression of these genes was either at the end of night or towards the end of the day (Blasing et al. 2005).

This expression pattern differs from a free-running circadian cycle where most genes peak during the subjective day or night. A comprehensive examination of clock-controlled genes in *Arabidopsis* found that 6% of 8000 genes examined exhibited circadian changes in steady state mRNA levels (Harmer et al. 2000). Included in this list were all of the key phenylpropanoid genes (except *AtCHI*), including; At*FLS1, AtUFGT* and *AtLDOX*, which were shown to be co-ordinately regulated to peak before dawn. It was also shown that a MYB transcription factor, *AtPAP1,* which has known involvement in activation of flavonoid genes, was also under circadian regulation. Harmer et al. (2000) suggested the circadian regulation of the flavonoid genes may orchestrate the production of photo-protective pigments early in the day with *AtPAP1* acting as a master regulator of these genes. It is also worth noting that a highly conserved promoter motif (AAATATCT), referred to as the 'EVENING element', was identified in 31 genes displaying circadian regulation, including *AtFLS1* (Harmer et al. 2000). Promoter sequence analysis (see **Chapter 5**) indicated this element was not present in the grapevine *VvFLS1* promoter sequence (data not shown).

It was also recently shown in *Anthurium andraeanum (*anthocyanin coloured lily), that *AmDFR* transcript levels were high at dawn and dusk and low at noon, suggesting diurnal regulation (Collette et al. 2004). However, expression of *AmCHS, AmF3H and AmANS* (~*VvLDOX*) remained unchanged. The EVENING element was not identified in the DFR promoter sequence (Collette et al. 2004). While the authors were unable to suggest a possible explanation as to the physiological function of the diurnal variation in *AmDFR* (not observed in the other genes), they reiterated the divergent pattern of flavonoid regulation in different plant species when making comparisons to *Arabidopsis*.

Post-flowering, *VvLDOX* expression appeared to be diurnally regulated when it was light. However after veraison, *VvLDOX* and *VvUFGT* gene expression remained relatively constant during the 24 hour cycle **(Figure 4.15)**. These results suggest some level of developmental regulation in diurnal changes of *VvLDOX* gene expression. This result was not unexpected, considering the sensitivity of the inflorescences and the activity of most of the flavonoid genes at this stage in development (as

previously mentioned). Also, at stages in development after veraison, the berries are less sensitive to environmental conditions and the accumulation of flavonoids is likely to occur over several days. Therefore, rapid fluctuations in the genes controlling these compounds over a few hours seem unlikely at these later stages in development.

Before drawing any conclusions about the diurnal regulation of *VvFLS* expression it is important to point out some limitations of the data. First, the magnitude of change in gene expression was low (around 3-fold), which can sometimes be considered normal variation in gene expression in RT-PCR analysis; second, that *VvFLS1* expression was not absolute, indicating perhaps a light threshold exists after which *VvFLS1* expression occurs; third, flavonol concentration was not determined, so the effect of post-transcriptional changes remains unknown; and fourth, while *VvFLS1* expression appears to be light regulated (immediate response to light), it cannot be ruled out that changes may be due to the previous days light condition. To separate the effects of circadian and diurnal rhythms, grapevines (trained to a 12 hr light/12 hr dark cycle) should be exposed to prolong periods of light or dark. Nevertheless, the fact that *VvFLS1* expression has been shown to be light responsive in grape berries after three hours and flavonols accumulated after 6 hrs (**Figure 4.11**) suggests diurnal regulation may occur.

4.4.4.1 Summary

This investigation revealed the response of *VvFLS1*, *VvLDOX* and *VvUFGT* gene expression to diurnal day/night cycles. *VvFLS1* gene expression appeared to be diurnally regulated, throughout development, with increased expression at times when it was light, peaking in the afternoon. This corresponds with the light-dependent synthesis of *VvFLS1* expression. *VvLDOX* expression was diurnally regulated around flowering, but not after veraison. Diurnal regulation was not observed for *VvUFGT* expression, which generally remained unchanged to day/night cycles.

With the development of microarray technology, analysis of the genes involved in diurnal and circadian regulation has become more efficient and effective. For example, in 2001, 11% of genes in *Arabidopsis* were reported to show diurnal regulation, while in 2005, this number increased to 30-50% (Blasing et al. 2005; Schaffer et al. 2001). This is one of the first investigations into the diurnal regulation of genes involved in the flavonoid pathway in grapes. While it appears *VvFLS1* is diurnally regulated, further experiments are required to confirm this pattern of expression.

4.5 Conclusion

Together, the light induction and the diurnal experiments, clearly demonstrate the light-dependent and responsive nature of *VvFLS1* expression and flavonol accumulation in Shiraz and Chardonnay fruit throughout development. While the magnitude of gene expression varied at different stages in development, it was apparent that *VvFLS1* expression and flavonol accumulation was able to respond to changes in light, within 12 hrs.

Flavonols appear to be a good indicator of bunch exposure to light, in agreement with previous studies. Therefore, the manipulation of flavonol levels in grape berries through canopy management should be relatively straightforward. An example of manipulation in the vineyard may include lifting wires, leaf plucking or shoot thinning at particular times for increased bunch exposure to light (Smart and Robinson 1991), which may have an effect on flavonol synthesis and wine quality. Also, although flavonols are not recognised as contributing to wine flavour, they may be an indicator of other light dependent compounds that can influence wine flavour and aroma compounds that potentially could improve wine quality.

One major finding of this investigation was that *VvFLS1* synthesis was able to be light induced at times when flavonols are not normally being synthesised, suggesting light exposure can override the developmental control of *VvFLS1* expression flavonol accumulation. This indicates the complexity involved in flavonol synthase (*VvFLS1*) gene regulation in grapevines, where there is a mechanism overriding the coordinate expression of flavonol genes during development in response to light.

Flavonoid metabolism is largely controlled by transcriptional gene regulation in plants (Davies and Schwinn 2003b). Recently in grape berries the transcriptional control of the anthocyanin gene (i.e. *VvUFGT*) and tannin genes (*VvLAR1* and *VvANR*) has been shown to be controlled by the *VvMYBA1/VvMYBA2* and *MYBPA1* genes, respectively (Bogs et al. 2007; Kobayashi et al. 2002; Walker et al. 2007). However, the transcriptional control of flavonols in grapevines remains unknown. In order to understand the basis of transcriptional control of flavonol formation in grape berries, we investigated the molecular mechanisms of *VvFLS1* gene regulation, as detailed in **Chapter 5**.

CHAPTER 5

The molecular mechanisms of flavonol gene regulation in grapevines

5.1 Introduction

Anthocyanins, tannins, and flavonols are synthesized via the flavonoid pathway, which consists of a number of enzymatic steps that each catalyse a sequential reaction in the pathway (**Chapter 1, Figure 1.3**). In grapevine, synthesis of these compounds occurs in different parts of the berry, at different developmental stages of the berry and synthesis of each flavonoid is regulated in response to different environmental cues, for example, light exposure.

Regulation of flavonoid synthesis occurs mainly via the coordinated transcriptional control of the structural genes by the interaction of DNA-binding MYB transcription factor proteins, basic helix-loop-helix (bHLH) proteins and WD40 proteins (Schwinn and Davies 2004; Winkel-Shirley 2001). In *Arabidopsis* transcriptional regulators for anthocyanin and tannin synthesis have been identified (Borevitz et al. 2000; Nesi et al. 2001; Ramsay et al. 2003; Walker et al. 1999; Zhang et al. 2003; Zimmermann et al. 2004). In grapevine, two MYB proteins (VvMYBA1 and VvMYBA2) have been shown to regulate anthocyanin synthesis (Kobayashi et al. 2002; Kobayashi et al. 2005) and recently a MYB protein (VvMYBPA1) was identified which regulated tannin synthesis early in berry development and in seeds (Bogs et al. 2007). Additionally a MYB transcription factor (VvMYB5a) has been shown to induce synthesis of anthocyanins, tannins, flavonols and lignin when expressed in tobacco (Deluc et al. 2006).

Presently, no transcriptional regulator(s) specific for flavonol biosynthesis in grapevines have been isolated. However, a MYB transcription factor protein (AtMYB12) was recently identified as a specific activator of flavonol biosynthesis in *Arabidopsis* (Mehrtens et al. 2005). Although the response of AtMYB12 to light has not yet been determined, it was shown to bind to a

specific DNA sequence, called the MRE (MYB recognition element), which comprises part of a light regulatory unit (LRU) in the promoter of target genes (Mehrtens et al. 2005).

In **Chapter 4,** the complexity involved in flavonol gene regulation in grapevines was highlighted, where it was suggested that expression of *VvFLS1* is controlled by a specific transcriptional regulator(s) which may be able to operate independently of the developmental control of the main flavonoid pathway and also in response to light. Therefore, in order to understand the basis of transcriptional control of flavonol synthesis in grapevines, the molecular mechanisms of flavonol biosynthesis was investigated.

5.2 Methods

Methods detailed in this section are in summarised form, and where possible have referred to a corresponding reference. General cloning and sequence analysis strategies are described in **Chapter 2, Section 2.3**.

5.2.1 Isolating & cloning *VvFLS1* promoter fragment(s) into the luciferase vector (pLUC)

To isolate the promoter of *VvFLS1*, the Universal Genome Walker^{TM} Kit (BD Biosciences, USA) was employed. The library was made by Dr. Jochen Bogs (CSIRO, Australia), using *Vitis vinifera* L. cv. Shiraz leaf genomic DNA as a template. The techniques used to isolate the promoter sequence(s) and clone promoter fragments into the luciferase vector pLUC are described by Bogs et al. (2007). Primers are listed in **Appendix 2C, Table 2C.4**.

5.2.2 Chardonnay suspension cell cultures

5.2.2.1 Growth conditions & preparation for assays

A suspension culture of Chardonnay cells (kindly donated by Debra M^{c}David (CSIRO, Australia) was used in transient assays for functional testing of the *VvFLS1* promoter (**Section 5.2.3)** and in the microarray experiment (**Section 5.2.4.2)**. The Chardonnay cell line was established in 2002 from Chardonnay petiole callus culture and is grown in liquid Grape Cormier (GC) medium, in the dark, at 26^{o}C and continuously shaken at 100 rpm **(Figure 5.1A)** (Do and Cormier 1990). For use in experiments, the cells were vacuum filtered onto filter paper discs and placed on plates containing GC media **(Figure 5.1B)**, as described Bogs et al. (2007) and Torregrosa et al. (2002). Prior to analysis, cells on disks were collected with a spatula, frozen in liquid N_2 and ground to a fine powder (liquid N_2).

5.2.2.2 Light induction experiments

The effect of light exposure on flavonoid content (particularly *VvFLS1* expression and flavonol accumulation) in Chardonnay cells was investigated. Chardonnay cell disks were placed into a plant tissue culture room under the following conditions: temperature 22-25^{o}C, light 240 $\mu mol.m^{-2}.s^{-1}$ (cool white), 16 hr light cycle. These conditions were considered adequate to achieve flavonol biosynthesis without effecting cell growth. Cells were harvested at the same time on each day (after receiving 13 hr light, 7 hr dark: 3 hr light) on days 0, 3, 7 and 10, along

with control plates, which were wrapped in Alfoil to prevent light exposure. HPLC analysis (anthocyanin, flavonol and tannin content) and RT-PCR experiments (*VvFLS1* gene expression) were performed on cells according to the methods in **Chapter 2**.

After the initial experiment (above) it was decided to narrow the timeframe of sampling, therefore the experiment was repeated, sampling 6 and 12 hr after the onset of light exposure, followed by daily samples on days 1, 2, 3, 6 and 8. Cells were analysed for *VvFLS1* gene expression and flavonol accumulation according to **Chapter 2**.

Figure 5.1 Chardonnay cells, in suspension culture & on disks contained on GC agar.

A The cells, established in 2002, are maintained in GC liquid media, in the dark, shaking until just before stationary phase of growth when cells are sub-cultured into new media.

B Cells at log phase are filtered onto Whatman1 discs & placed on GC-agar containing plates.

5.2.3 Transient assays testing the *VvFLS1* promoter(s)

A transient assay to functionally test the *VvFLS1* promoters (contained in the luciferase plasmid (pLUC)) was carried out according to the methods described in Bogs et al. (2007) and the references therein. In this system, filtered Chardonnay cells (**Section 5.2.2**) were bombarded with constructs containing potential transcription factors and the *VvFLS1* promoter, fused to a luciferase reporter vector. After 48 hr incubation in the dark, cells were harvested and luciferase activity measured as an indicator of *VvFLS1* promoter activity.

Constructs used in bombardments included: the luciferase vectors containing promoters (*VvFLS1* promoter1:pLUC, *VvFLS1* promoter2:pLUC), vectors containing MYB transcription factors (*VvMYBPA1*:pART7 (Bogs et al. 2007), *VvMYBF*:pART7 (unpublished, Bogs 2006), *VvMYBA2*:pART7 (Walker et al. 2007) and a bHLH transcription factor (*AtEGL3*:pFF19 (Ramsay et al. 2003). Also, the full length *AtMYB12* sequence (At2g47460, (Mehrtens et al. 2005) was isolated from *Arabidopsis* genomic DNA (kindly donated by Dr Felix Jaffe (CSRIO, Australia)) and cloned into the vector, pART7.

5.2.4 Identifying transcriptional regulators of *VvFLS1*

5.2.4.1 Database searching for AtMYB12 homologs & RT-PCR expression analysis of potential transcription factor candidate genes

In 2003 a grapevine EST sequence database (TIGR) became publicly accessible at http://www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=grape. BLAST analysis of AtMYB12, in this database, listed various grape EST sequences, of which six were selected for expression analysis, based on their sequence homology to AtMYB12. Primers were designed to these candidate genes (see **Appendix 2C, Table 2C.2**) and RT-PCR analysis was performed according to methods described in **Chapter 2.** Expression of the candidate genes was normalised to the expression of *VvUBIQ1* (TC32075). Two cDNA series were used to test candidate genes:

1. 3-day light induced Chardonnay cells (and dark controls) (see **Section 5.2.2**)
2. Shiraz grape berry developmental series as described by Downey et al. (2003a); Pre-flowering (~ 1 week (9-11-00)), veraison (11-1-01), pre-harvest (~1 week (8-3-01)).

5.2.4.2 Microarray analysis

5.2.4.2.1 Plant material

Three replicate plates containing filtered Chardonnay cell disks were exposed to light for 24 hrs, along with dark controls, as described **Section 5.2.2.** *VvFLS1* expression and flavonol accumulation was determined by RT-PCR and HPLC, respectively, according to methods described in **Chapter 2**.

5.2.4.2.2 Data analysis

Total RNA was extracted from light-exposed and dark-control Chardonnay cells, according to the methods described in **Chapter 2**, and two replicates of each treatment were sent to AGRF (Australian Genome Research Facility, Australia). Microarray analysis involved hybridisations using the Affy-1-cycle labelling technique. The *Vitis vinifera* L. cv. Cabernet Sauvignon cDNA microarray slide (kindly donated by Dr Mark R. Thomas (CSIRO, Australia) contained approximately 16,000 unique sequences, (part of the NCBI grapevine database http://www.ncbi.nlm.nih.gov/). The microarray procedures and *Vitis vinifera* slide information are available on the Affymetrix website (http://www.affymetrix.com).

The raw data was analysed in Avadis (V4) (Strand Life Sciences, USA), with kind assistance from Pat Iocco (CSIRO, Australia). The probe level was corrected using a normalisation method (GC-RMA), which accounts for technical bias in a sample. The data was then log transformed (base 2) which compresses the data, allowing large differences to stand out. Following this,

differential expression analysis (DEA) was performed on each gene, resulting in the following headings:

-Corrected p value. An unpaired t-test was conducted on the technical replicates; the correction type, Benjamini-Hochberg. The corrected p value indicates which genes significantly changed in response to light, where $p<0.001$ is highly significant and ns is not significant.

-Rank. These values are closely associated with the corrected p value, ranging from 0-16,601.

-Absolute value. These values are used to indicate the difference in gene expression between the light and dark samples (i.e. L-D).

-Regulation. Indicates whether genes were up/down regulated in response to light. This is also quantified as the ratio of L/D (additional calculation-no correction), where values >1.0 indicated up-regulation in response to light.

5.2.4.2.3 RT-PCR expression analysis of potential transcription factor candidate genes

From a list of unigenes represented in the microarray analysis, seven potential grape EST sequences were selected as potential candidates as transcriptional regulators of *VvFLS1*. Primers were designed to these candidate genes (**Appendix 2C, Table 2C.3**) and RT-PCR analysis was performed according to methods described in **Chapter 2.** Expression of the candidate genes was normalised to the expression of *VvUBIQ1* (TC32075). Two cDNA series were used to test candidate genes:

1. 24 hr light induced Chardonnay cells (and dark controls) **(Section 5.2.4.2)**
2. The light induction experiment (**Chapter 4**). Samples used included Shiraz, pre-flowering, light induced (D2), shaded (D0) and exposed (D0).

5.3 Results

5.3.1 Cloning & sequence analysis of *VvFLS1* promoter fragment(s)

In 2003, BioNavigator database searches revealed two partial grapevine (*Vitis vinifera* L. cv. Shiraz) *VvFLS* gene sequences; *VvFLS1* (AY257978-797 bp) and *VvFLS2* (AY257978-706 bp) (Downey et al. 2003b). It has been shown that expression of *VvFLS2* is low throughout berry development compared to *VvFLS1* (Downey et al. 2003b), therefore, the promoter of *VvFLS1* was selected for isolation and characterisation.

BLAST analysis of *VvFLS1* in the TIGR database identified a full-length grape (*Vitis vinifera* L. cv. Cabernet Sauvignon) EST sequence (TC46143-1293 bp). Sequence alignment of *VvFLS1* to TC46143 showed 93% sequence identity at the nucleotide level and 100% at the protein level (data not shown). The 265 aa VvFLS1 protein sequence had similarity to FLS sequences from *Arabidopsis* (AtFLS1 U84258), 66% identity and apple (MdFLS1 AF119095), 72% identity.

The initial attempt to isolate the *VvFLS1* promoter fragment from the Shiraz genomic library by genomic walking amplified a ~1200 bp fragment which contained an intron in the coding region of the *VvFLS1* gene. Primers were designed to regions flanking this intron (see **Appendix 2C, Table 2C.4**) and PCR cloning techniques were used to sequence the complete intron. Sequence analysis revealed the intron was 1176 bp in length starting at position 468 bp (from start codon).

Using genomic walking primers, designed upstream of the intron, an 811 bp *VvFLS1* promoter fragment was isolated (***VvFLS1* promoter1**). Regions of sequence identity between the coding region of *VvFLS1* and the *VvFLS1* promoter1 fragment indicated that the appropriate promoter fragment had been obtained. However, when attempts were made to re-isolate this *VvFLS1* promoter1 sequence from genomic DNA (for functional testing), a second *VvFLS1* promoter sequence was isolated **(*VvFLS1* promoter2**).

The original *VvFLS1* promoter sequence isolated from the genomic walking library (*VvFLS1* promoter1) aligned with the second *VvFLS1* promoter sequence isolated from Shiraz genomic DNA (*VvFLS1* promoter2) and showed 99% identity (**Figure 5.2**). The *VvFLS1* promoter2 sequence contained an additional ATC and ATG repeat and there were 4 random base pair mismatches. While several attempts (though PCR cloning strategies) were made to determine whether these sequence differences resulted from PCR errors, it was concluded that both sequences are most likely present in the grapevine genome. Subsequently both *VvFLS1* promoter fragments were functionally tested in the luciferase assay.

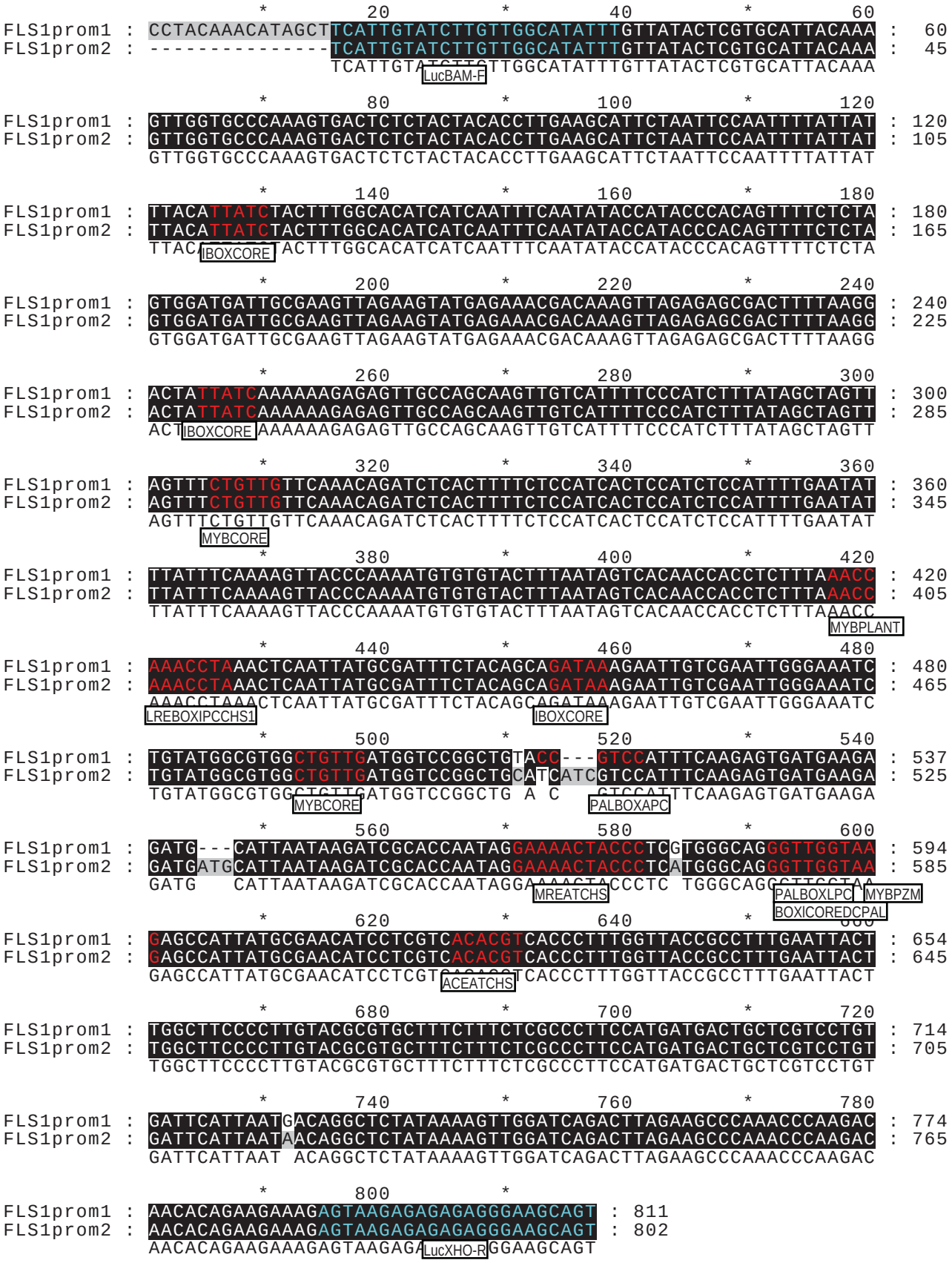


Figure 5.2 Nucleotide sequence alignment of the *VvFLS1* promoter fragments identified by PCR. The genomic sequence of *VvFLS1* was identified by genomic walking techniques. Two promoter fragments (~800 bp) were isolated: *VvFLS1* promoter1 (from the genomic walking library) & *VvFLS1* promoter2 (from genomic DNA). These promoter sequences were aligned with the ClustralW algorithm (Thompson et al. 1994) & displayed by GenDoc**.** The *VvFLS1* open reading frame starts at –812 bp. Dashes indicate gaps. Primers used for PCR are highlighted in blue. Putative DNA binding motifs identified by PLACE (Higo et al. 1999) are highlighted in red.

To identify DNA binding motifs/elements located in the *VvFLS1* promoter sequences, sequence analysis was undertaken using the PLACE (plant DNA cis-elements) database (http://www.dna.affrc.go.jp/htdocs/PLACE (Higo et al. 1999). DNA binding motifs located in *VvFLS1* promoter1 (811 bp) are listed in **Table 5.1** and shown in **Figure 5.2**.

Both promoters contained motifs characteristic for (TATA) box and (CAAT) boxes found in plant genomic sequences (Shirsat et al. 1989; Tjaden et al. 1995). There were also several other putative regulatory motifs located in the promoter sequences including those involved in responses to gibberellic acid (Ogawa et al. 2003), ethylene (Montgomery et al. 1993) and absisic acid (ABA)(Abe et al. 2003; Busk and Pages 1998; Gubler et al. 1995; Nakashima et al. 2006).

Numerous putative light responsive motifs were identified in both promoters. Many of these motifs are involved in regulation of genes involved in photosynthesis including: chlorophyll a/b binding proteins (Reyes et al. 2004), photosystem I proteins (Nakamura et al. 2002) and the chloroplast enzyme, GAP-DH (Chan et al. 2001). There were also motifs that are generally located in light regulated genes, including, GT-elements (Zhou 1999), I-boxes (Martinez-Hernandez et al. 2002; Terzaghi and Cashmore 1995) and phytochrome A binding sites (Jiao et al. 2005). Also located in the *VvFLS1* promoter sequences was a circadian control motif, which was identified in the tomato promoter involved in phytochrome A mediated gene expression (Piechulla et al. 1998).

Motifs conserved in the promoter of PAL genes (first step into the phenylpropanoid pathway) PALBOXAPC, PALBOXLPC, BOXLCOREDPAL were identified in the *VvFLS1* promoter sequence. These motifs have been associated with the UV-light responsiveness of PAL expression in suspension cultures of parsley and carrot (Logemann et al. 1995; Maeda et al. 2005). Interestingly, the *VvFLS1* promoter2 sequence lacked the PALBOXAPC motif, which was disrupted by the ATC repeat.

There were several MYB binding motifs located in both promoters, most of which had some association with the flavonoid biosynthetic pathway including; MYBCORE, MYBPLANT and MYBPZM (Grotewold et al. 1994; Solano et al. 1995; Tamagone et al. 1998) (**Table 5.1**). These MYB binding motifs were also found to be present in most of the grapevine flavonoid biosynthetic genes including *VvCHI* (976 bp, accession AM25948), *VvCHS* (656 bp, Trace database (http://www.ncbi.nlm.nih.gov/Traces/trace.cgi?)), *VvF3'5'H* (1136 bp, accession AM259482), *VvLDOX* (2484 bp, accession AF290432), *VvANR* (1034 bp accession AM259484), *VvLAR1* (1342 bp, accession AM259481), and *VvUFGT* (1684 bp, accession AY955269) (data not shown).

Both *VvFLS1* promoters contained motifs, which are part of a putative light regulatory unit (LRU). The LRU typically comprises a MYB recognition element (MRE) and an ACGT-containing element (ACE)(Feldrugge et al. 1997). These DNA binding motifs have been shown to be involved in mediating the induction of the flavonoid genes in response to light in *Arabidopsis* plants and parsley suspension cells (Feldbrugge et al. 1997; Hartmann et al. 1998; Schulze-Lefert et al. 1989). Two MRE motifs and one ACGT site were identified in the *VvFLS1* promoters, the LREBOXIIPCCHS1, MREATCHS and ACEATCHS, respectively. The presence of the LRU elements in the *VvFLS1* promoter sequences, along with the core flavonoid MYB binding motifs and the light regulated motifs were a good indication that the *VvFLS1* promoter sequence had been obtained, and further justified functional testing of these sequences in transient assays.

Table 5.1 Key motifs identified within the *VvFLS1* promoter1 sequence (811 bp) after analysis in the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE (Higo et al. 1999). Listed alongside each motif name includes: the motif number (assigned by PLACE), the location in the *VvFLS1* promoter sequence (from the start codon (ATG)), the motif sequence, the role (may be putative), the species it was identified in & corresponding reference. (*) Indicates motif not located in *VvFLS1* promoter2 sequence. (partial) indicates that only part of the motif was homologous to the promoter sequence. (+/-) Indicates same motif, in the inverted direction. These motifs are also indicated on the *VvFLS1* promoter sequence(s) shown in **Figure 5.2**.

Motif Name (Alphabetical)	Number	Location	Motif	Keywords/ Role	Species	References
IBOXCORE	S000199	-354 (+) -563 (-) -682 (-)	GATAA	I BOX, light regulation, leaf, shoot, general light regulation	All	(Terzaghi and Cashmore 1995)
MYBCORE	S000176	-314 (+) -501 (+)	CNGTTR	MYB, dehydration, stress, flavonoid biosynthesis, leaf, shoot	At, Ph	(Solano et al. 1995)
MYBPLANT	S000167	-388(+)	MACCWAMC	MYB flower, phenylpropanoid, lignin, leaf, shoot	At, Zm, Pc, Ps	(Tamagone et al. 1998)
MYBPZM	S000179	-221 (-)	CCWACC	MYB, P gene, seed, flavonoid biosynthesis	Zm	(Grotewold et al. 1994)
MREATCHS (partial)	S000356	-230 (-)	TCTA**ACCTACC**A	MYB, CHS, Light, UV-A, UV-B, leaf, Shoot, ACE, MRE	At	(Hartmann et al. 2005)
ACEATCHS (partial)	S000355	-186 (+)	G**ACACGT**AGA	bZIP, MYB, CHS, Light, UV-A, UV-B, leaf, Shoot, ACE, MRE	At	(Hartmann et al. 2005)
LREBOXIPCCHS1 (partial)	S000302	-384 (+)	**AACCTAA**CCT	MYB, CHS, light, Box I, Leaf, shoot, LRE, MRE	Pc	(Feldbrugge et al. 1997)
BOXLCOREDCPAL	S000492	-220 (-)	ACCWWCC	MYB, PAL UV-B, elicitor	Dc	(Maeda et al. 2005)
PALBOXAPC*	S000137	-294 (+)	CCGTCC	Box A, PAL	Pc	(Logemann et al. 1995)
PALBOXLPC	S000138	-216 (-)	YCYYACCWACC	Box L, PAL	Pc	(Logemann et al. 1995)

5.3.2 Functional analysis of *VvFLS1* promoter using a transient assay

To functionally test the *VvFLS1* promoters, a transient expression system using the Chardonnay grape cell suspension culture was employed. AtMYB12 (At2g47460) has been shown to be a

specific transcriptional regulator of flavonol biosynthesis in *Arabidopsis* (Mehrtens et al. 2005), and therefore appeared a likely candidate for activation of the *VvFLS1* promoter(s). Grapevine transcription factors used to test the activation of *VvFLS1* promoters included a anthocyanin regulator; VvMYBA2 (Kobayashi et al. 2002), a tannin regulator; VvMYBPA (Bogs et al. 2007) and VvMYBF. *VvMYBF* is a putative transcription factor, which has a gene expression pattern similar to *VvFLS1* gene expression during development (Bogs 2006). However, in RT-PCR experiments performed with a light/dark series (i.e. Chardonnay cell culture system, 24 hrs) *VvMYBF* had low gene expression in both the light and dark (data not shown).

The system used to investigate whether the *VvFLS1* promoters could be activated by different transcription factors included a transient expression method using grape cell culture and the dual-luciferase assay system. In this system, the co-transfection of effectors (transcription factors) and dual-luciferase reporter plasmids allows quantification of promoter activity by measuring firefly luciferase activity (promoter of interest cloned into pLUC), which is normalised by measuring *Renilla reniformis* luciferase activity (Horstmann et al. 2004).

While AtMYB12 has been shown to act independently of a bHLH co-factor (Mehrtens et al. 2005), the MYB transcription factors, VvMYBPA2 and VvMYBPA1 require a bHLH for activation (Bogs et al. 2007; Walker et al. 2007). Therefore, a plasmid encoding a bHLH protein from *Arabidopsis* (AtEGL3) (Ramsay et al. 2003) was co-bombardment with constructs (unless specified without (w/o), only for AtMYB12. Additionally, it is important to note that the WD40 protein (also required for transcriptional activation) is not transiently expressed in these experiments, as in cell cultures this protein has found to be non-limiting (Walker 2006a).

Figure 5.3 shows the transient assays carried out with the two *VvFLS1* promoters to compare the activation capacities of transcription factors: AtMYB12, VvMYBF, VvMYBA and VvMYBPA1. Both *VvFLS1* promoter constructs displayed a similar pattern of activation, however *VvFLS1* promoter1 was more strongly activated than *VvFLS1* promoter2. AtMYB12 appeared to activate both promoters and the level of activation was higher when the bHLH was absent. There was little to no activation of the *VvFLS1* promoters by the other transcription factors VvMYBA, VvMYBPA1 and VvMYBF, when compared to the *VvFLS1* promoter activity (alone) without a transcription factor. While these results show the *VvFLS1* promoters are activated by AtMYB12, the level of activation is low compared to other studies using this type of reporter systems (Bogs et al. 2007; Takos et al. 2007). Therefore further investigations were warranted into searching for transcriptional regulators of flavonol biosynthesis in grapes.

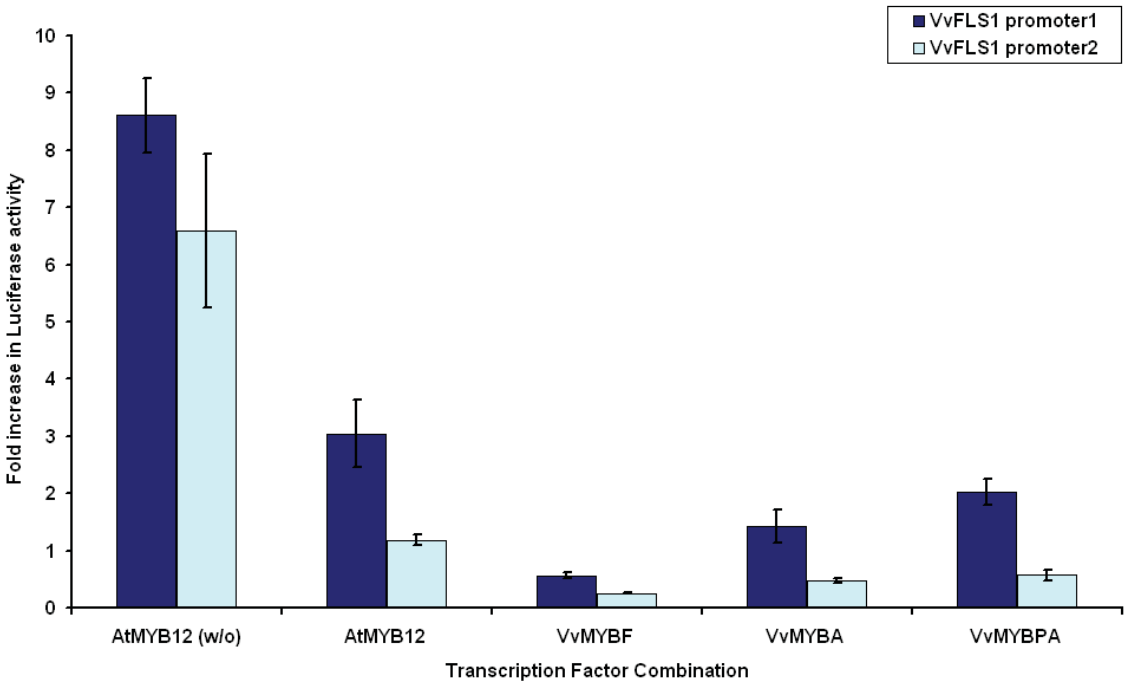


Figure 5.3 Transient assays on the *VvFLS1* promoter sequence(s) to compare the activation capacities of transcription factors AtMYB12, VvMYBF, VvMYBA, VvMYBPA. The MYB transcription factors used for transfection of the Chardonnay grape cell cultures are indicated. AtEGL3, a bHLH protein was co-bombarded with each transcription factor, unless specified ((w/o) without AtEGL3, AtMYB12). Data is the mean of the ratio of *P. pyralis* LUC activity to *R. formis* activity normalised to *VvFLS1* promoter activity (alone) without transcription factors. Thus, fold-increases <1 indicates the *VvFLS1* promoter activity (alone) is greater than with the transcription factor co-expressed**.** Error bars represent the standard error (±SEM) of three bombardments from three separate experiments.

5.3.3 Light induction of Chardonnay cell cultures

With flavonol biosynthesis clearly able to be light induced in Shiraz and Chardonnay bunches (Downey et al. 2004), **Chapter 4**), the effect of light exposure on flavonoid content (particularly *VvFLS1* expression and flavonol accumulation) in Chardonnay cells was investigated. The Chardonnay cells, normally grown in the dark, had no detectable anthocyanins, flavonols or hydroxycinnamates **(Table 5.2**). Expression of *VvFLS1* was also very low in these dark-grown cells **(Figure 5.4)**. A small amount of tannins was detected in cells after hydrolysis, 1.44 mg/g (predominantly epigallocatechin, catechin, epicatechin), however no free monomers were detected.

Table 5.2 Flavonoid composition of dark-grown Chardonnay cells & after three days exposure to light. The composition of anthocyanins, flavonols, hydroxycinnamates & tannins was determined by HPLC. Mean values are expressed mg/g tissue & in brackets is the standard error (±SEM) for three analytical replicates.

Flavonoid composition	Dark	Light
Anthocyanins	0	0
Flavonols	0	0.013 (±0.0005)
Hydroxycinnamates	0	0
Tannins	1.40 (±0.013)	1.45 (±0.017)

The initial light induction of Chardonnay cells was performed after 3, 7 and 10 days exposure to light (**Figure 5.4**). After exposure to light, *VvFLS1* expression rapidly increased by 5700-fold on day 3 and by day 7 levels had started to reduce. This increase in *VvFLS1* expression coincided with an accumulation of flavonols (the quercetin-glycosides only), where by day 7 levels had reached 0.020mg/g tissue. No anthocyanins or hydroxycinnamates were detected after exposure to light, and tannin concentration remained relatively unchanged (**Table 5.2**). There was no obvious phenotypic effect of light exposure on the Chardonnay cells compared to those in kept in the dark, after 10 days exposure.

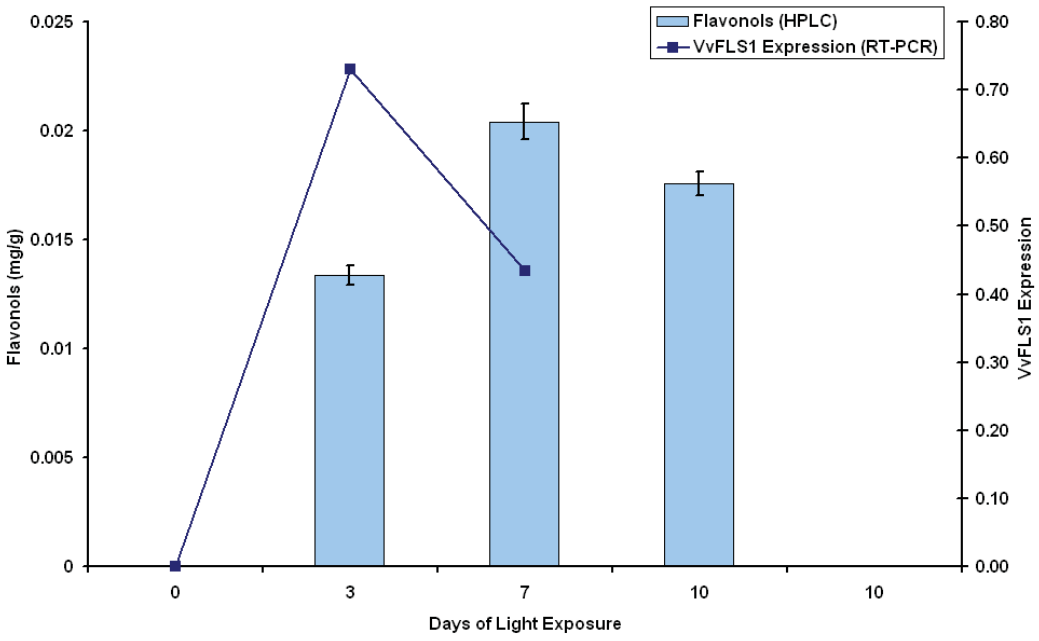


Figure 5.4 Light induction in Chardonnay cell suspension culture; *VvFLS1* expression & flavonol accumulation. Cells were filtered onto paper & exposed to light for 3, 7, & 10 days. Expression of *VvFLS1* is relative to *VvUBIQ1* expression in light induced cells on day 0, 3 & 7 determined by RT-PCR. Accumulation of total flavonols (expressed per g tissue) in light induced cells determined by HPLC. Only quercetin glycosides were detected in cells, which were summed to total flavonols. A dark control sample was also taken on day 10. Error bars represent the standard error (±SEM) for three analytical replicates.

To narrow the time-frame of *VvFLS1* expression, the experiment was repeated and Chardonnay cells were sampled after 6 hrs and 12 hrs on the initial day of exposure followed by sampling on day 1, 2, 3, 6 and 8 (**Figure 5.5**). As expected, in the dark there was little *VvFLS1* expression and no detectable flavonols and this did not change over the 8-day sampling period. After exposure to light, *VvFLS1* expression started to increase and after 24 hours (day 1) levels had increased by 14500-fold from day 0. By day 8, in light induced cells, *VvFLS1* expression was the highest, with expression levels significantly greater than in the dark. Corresponding with an increase in *VvFLS1* expression, flavonols were detected after 2 days light exposure, and by day 6 flavonol concentration was 0.025 mg/g. These results indicate flavonol synthesis in the Chardonnay cells is induced by light and thus provides a good test system for evaluating regulation of this part of the pathway.

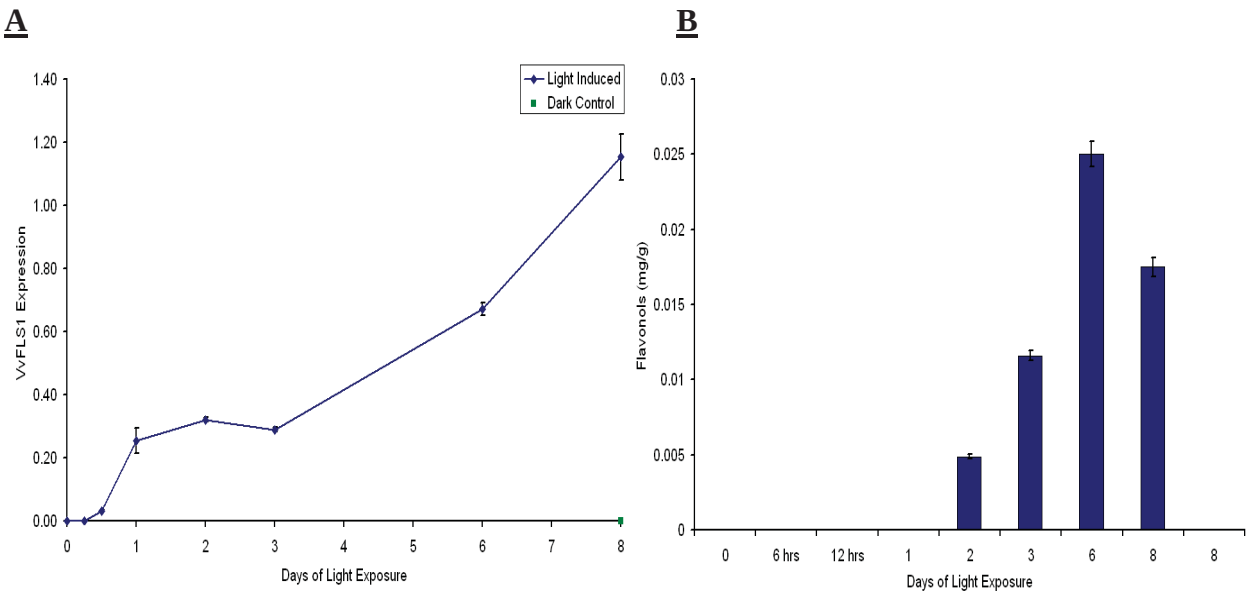


Figure 5.5 Light induction in Chardonnay cell suspension culture; *VvFLS1* expression & flavonol accumulation. Cells were filtered onto paper & exposed to light & sampled every 6 hours after induction, followed by samples on day 1, 2, 3, 6 & 8 (when dark control sample was taken). Error bars represent the standard error (±SEM) of three analytical replicates.

A Expression of *VvFLS1* relative to *VvUBIQ1* expression in light induced cells as determined by RT-PCR.

B Accumulation of total flavonols (expressed per g tissue) in light induced cells determined by HPLC. Only quercetin glycosides were detected in cells, which were summed to total flavonols.

5.3.4 Identifying transcriptional regulators of *VvFLS1*

5.3.4.1 Database searching for AtMYB12 homologs & RT-PCR expression of potential candidate genes

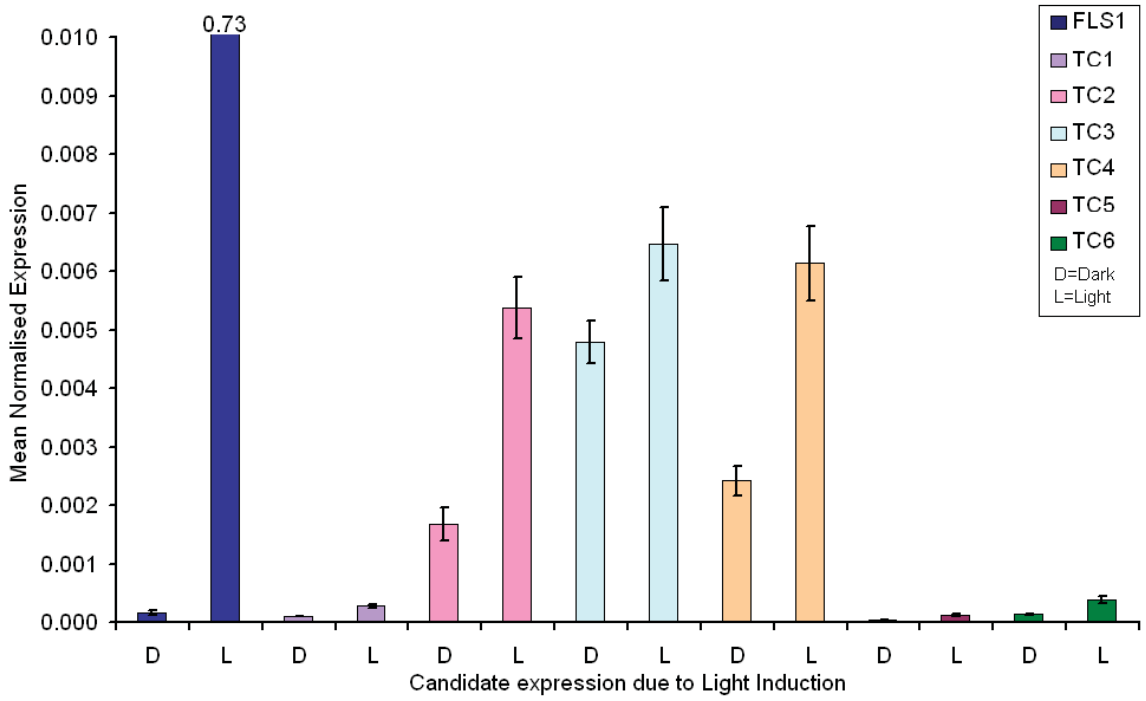
As AtMYB12 was shown to activate the *VvFLS1* promoters (**Section 5.3.2**), this transcription factor appeared the most likely target to use for isolation of potential grapevine homologs. The AtMYB12 protein sequence was used to identify grape homologs in the TIGR EST-database. There are over 100 members of the MYB family protein family in *Arabidopsis*, which are highly conserved in their N-terminus region (with an R2R3 repeat required for the interaction of bHLH)(Stracke et al. 2001). Therefore, initial BLAST searches were performed with the C-terminal region of AtMYB12, however this did not reveal any candidate proteins. Repeated BLAST analysis with the full length AtMYB12 (371 aa) identified a variety of potential grape EST sequences. **Table 5.3** shows the top six EST sequences with 45-70% identity to AtMYB12, which were selected for RT-PCR expression analysis. At the time of this analysis, none of these EST sequences were characterised in grapevine, however in 2006, the putative functions of TC1 (TC46393) and TC2 (TC41702) were revealed, both of which were suggested to have roles in flavonoid regulation (Bogs et al. 2007; Deluc et al. 2006).

Table 5.3 Putative AtMYB12 homologs identified by BLAST analysis in the grapevine TIGR EST database. Six candidate gene sequences were selected for RT-PCR gene expression analysis. A TC (Tentative Consensus) number was assigned to each gene & the TIGR TC, the % identity to AtMYB12 & gene title are also shown. In bold indicates the putative gene role.

Assigned TC #	TIGR TC #	% Identity	Putative role
TC1	TC46393	62	*Similar to* UP|Q40920 (Q40920) **MYB-like transcriptional factor** MBF1, *partial (34%)*. (Bogs et al. 2007) subsequently published this sequence as: MYBPA1 (AM259485) involved in tannin regulation in grapevines
TC2	TC41702	58	(Q6Q789) **MYB transcription factor**, *complete.* (Deluc et al. 2006) subsequently published this sequence as: *MYB5a* (AY555190) involved in regulation of the flavonoid pathway early in development in grapevines
TC3	TC47032	68	*Homologue to* UP|Q9ATD1 (Q9ATD1) **GHMYB9**, *partial (74%)*
TC4	TC47565	45	*Homologue to* UP|Q6Q789 (Q6Q789) **Myb transcription factor,** *partial (49%)*
TC5	TC45624	69	*Similar to* UP|O49021 (O49021) **MYB-like DNA-binding domain protein** (Myb-like transcription factor 5), *partial (56%)*
TC6	TC44301	66	*Homologue to* UP|Q9XHV0 (Q9XHV0) AtMYB103 (**MYB transcription factor**), *partial (53%)*

To determine the expression pattern of the genes corresponding to the identified TC sequences, RT-PCR was performed with two cDNA series; the first, 3-day light exposure in Chardonnay cells (along with dark control) (see **Section 5.3.3)** and the second, during Shiraz berry development (Downey et al. 2003a). **Figure 5.6** shows *VvFLS1* expression in the light was clearly elevated compared to the dark, as expected (**Figure 5.4**). The candidate genes also showed an increase in expression in the light (the average change ~2.5-fold), however levels were significantly less than for *VvFLS1* expression. In response to developmental expression, *VvFLS1* showed the characteristic pattern of expression; high levels early and late in development (Downey et al. 2003b). None of the candidates matched the developmental pattern of *VvFLS1* expression. These results suggest none of the putative grapevine *AtMYB12* gene homologs are likely to be involved in the regulation of *VvFLS1* expression.

A



B

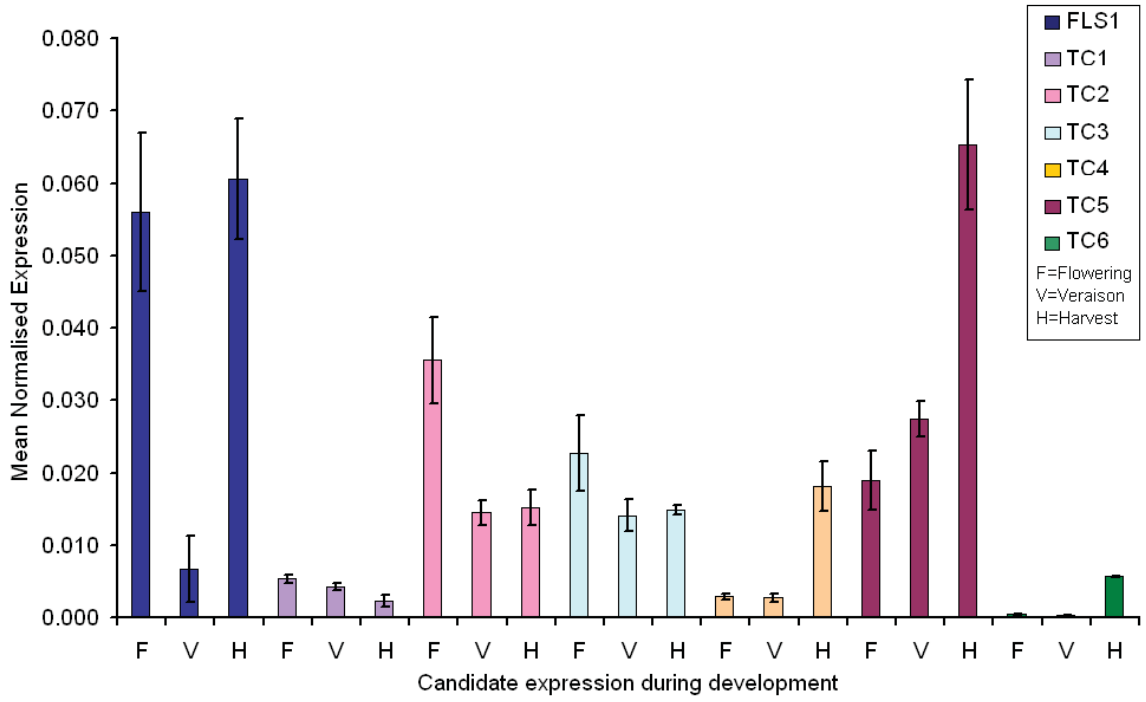


Figure 5.6 RT-PCR expression patterns for candidate (AtMYB12 homolog) genes

A Expression of *VvFLS1* & candidate genes (TC1, TC2 TC3, TC4, TC5, TC6 -see **Table 5.3**) in light exposed (3-days) chardonnay cells (D-dark control, L-light exposed). Data is expressed relative to the expression of *VvUBIQ1*. Error bars represent the standard error (±SEM) for three analytical replicates.

B Expression of *VvFLS1* & candidate genes (TC1, TC2 TC3, TC4, TC5, TC6 -see **Table 5.3**) during Shiraz berry development (Downey et al. 2003a): F-flowering 09-11-00, V-veraison 11-01-01, H-harvest 08-03-01). Data is expressed relative to the expression of *VvUBIQ1*. Error bars represent the standard error (±SEM) for three analytical replicates.

5.3.4.2 Microarray analysis.

To continue the search for candidate transcription factors that regulate the light induction of flavonol biosynthesis, microarray analysis was used. This technology utilises a large-scale approach for analysis and quantification of gene expression patterns by rapid screening of gene libraries. In this experiment, a grapevine library consisting of 16,602 grape EST sequences was screened.

5.3.4.2.1 Plant material

After narrowing down the time frame of *VvFLS1* expression in Chardonnay cells after light exposure (**Section 5.3.3)**, cells were sampled after 24 hrs exposure for microarray analysis. Chardonnay cells (in triplicate) were exposed to light for 1 day (24 hrs) along with dark controls. An additional sample was taken after 3 days exposure for determination of flavonol concentration. *VvFLS1* gene expression after 24 hrs light exposure increased by 840-fold (**Figure 5.7**) and flavonols had accumulated by day 3 (data not shown). The maximum level of *VvFLS1* expression after day 1 was similar to levels shown in **Figure 5.5**.

5.3.4.2.2 Data analysis

Technically, the microarray analysis was well executed with the analytical replicates tightly correlated (R^2=0.99) (data not shown). The complete microarray data set is available in **Appendix 5A (on CD),** which includes the 'raw' data (after normalisation GC-RMA method), the log transformed data and differential expression analysis (DEA) for each gene (as described in **Section 5.2.4.2.2**).

Table 5.4 shows the total number of genes that significantly changed in response to light, as sorted by the corrected p-value, and also indicates the number of genes that were up-regulated. Approximately 50% of genes contained on the microarray slide were significantly affected by light exposure (p<0.05). Of those genes, 70% were up-regulated in response to light. As the p value decreases (i.e. the change in gene expression becomes more significant) the total number of genes significantly decreases, yet the proportion of up-regulated genes to down-regulated genes remains relatively constant (60-70%).

Table 5.4 Microarray results: The total number of genes changed in response to light. An unpaired t-test was performed & a corrected p value was determined (correction type, Benjamini-Hochberg). The total number of genes in the microarray was 16,602. Indicated in the table is the number of genes which significantly changed in response to light exposure & those which were up-regulated. The order of significance ranges from ns (not significant) to p<0.0010 (highly significant).

	ns	p<0.05	p<0.02	p<0.01	P<0.0050	p<0.0010
Genes significantly changed	8622	7980	453	16	2	1
Genes up-regulated	4711	5655	296	12	1	0

A wide variety of genes were up-regulated in response to light exposure, including those involved in cell rescue and defence, metabolism (both primary and secondary), photosynthesis, protein packaging, protein synthesis, signal transduction, transport facilitation, transcriptional regulation and development. There were also many genes which function has yet to be assigned. **Table 5.5** lists the 15 unigenes that showed significant differences ($p<0.01$) in expression in response to light. It is worth noting that the top ranked unigene (0) has been removed as it comprises of irregular data, with a p value of 0. Headings listed in **Table 5.5** include the ranked values, absolute values (indicating the L-D difference), regulation (up/down) and the calculated L/D ratio (indicating the magnitude of light induction (i.e. fold-changes)). It is important to note, that when the data was sorted according to each of these calculated values, similar unigenes were observed at the top of the list (data not shown).

Table 5.5 Unigenes identified by microarray analysis showing significant differences ($p<0.01$) in gene expression after Chardonnay cells were exposed to light for 24 hrs. Table headings listed include: unigene number (#), gene title, average expression level (light & dark), corrected p value, rank, absolute value (difference L-D) & regulation (up or down). The magnitude of light induction (i.e. fold-change) is calculated as the L/D ratio. Indicated in bold are unigenes with roles in transcriptional regulation. Shaded unigenes are those in the flavonoid pathway. The top ranked unigene (0) is not shown.

Unigene #	Gene Title	Average Light	Average Dark	P value	Rank	Absolute Value	Regulation	(L/D) Ratio
Vvi.15689	Transcribed locus	6.90	2.67	0.003478	1	18.76	up	2.58
Vvi.14867	Transcribed locus	7.68	3.26	0.005107	3	21.40	up	2.36
Vvi.8350	Transcribed locus, weakly similar to NP_566290.1 **zinc finger** (GATA type) family protein [*Arabidopsis thaliana*]	8.21	9.35	0.005107	2	2.20	down	0.88
Vvi.1650	Cultivar Shiraz flavonol synthase	12.87	2.28	0.005241	4	1545.82	up	5.66
Vvi.13116	Transcribed locus, strongly similar to XP_482618.1 glyceraldehyde-3-phosphate dehydrogenase [Oryza sativa (japonica cultivar-group)]	5.67	2.89	0.005715	7	6.84	up	1.96
Vvi.117	CHS mRNA for chalcone synthase	13.26	3.28	0.005715	8	1009.05	up	4.04
Vvi.6974	Transcribed locus, strongly similar to NP_198446.3 **DNA-binding protein**, putative [*Arabidopsis thaliana*]	8.60	4.62	0.005715	6	15.71	up	1.86
Vvi.6857	Transcribed locus, weakly similar to XP_478692.1 chlorophyll a/b-binding protein [Oryza sativa (japonica cultivar-group)]	11.91	1.66	0.005715	5	1221.56	up	7.19
Vvi.9633	Transcribed locus, moderately similar to XP_476319.1 putative **DNA-binding protein** phosphatase 2C [Oryza sativa (japonica cultivar-group)]	2.80	2.52	0.006645	9	1.21	up	1.11
Vvi.2302	Transcribed locus, moderately similar to NP_199935.2 ubiquinol-cytochrome C chaperone family protein [*Arabidopsis thaliana*]	10.75	8.80	0.006645	10	3.85	up	1.22
Vvi.7710	Transcribed locus, moderately similar to NP_199326.1 Ras-related protein (RHA1) / small GTP-binding protein [*Arabidopsis thaliana*]	11.33	10.98	0.007847	12	1.27	up	1.03
	Unclassified	10.90	2.40	0.007847	11	361.13	up	4.54
Vvi.14511	Transcribed locus	9.06	10.78	0.008077	13	3.27	down	0.84
Vvi.7215	Transcribed locus, weakly similar to NP_173153.1 SOUL heme-binding family protein [*Arabidopsis thaliana*]	13.08	9.00	0.008077	14	16.99	up	1.45
Vvi.10229	Transcribed locus, weakly similar to NP_175145.2 homeobox-**leucine zipper transcription factor** family protein [*Arabidopsis thaliana*]	10.25	11.32	0.008938	15	2.10	down	0.91

Certain unigene classes were noticeably separated in **Table 5.5** including those related to photosynthesis (eg. chlorophyll a/b binding proteins, photosystem I proteins and the chloroplast protein (GAP-DH)), light regulated signal transduction processes (eg. ubiquinol-cytrochrome C chaperone proteins) and cell regulation processes (eg. RAS-related and GTP-binding proteins). The flavonoid pathway genes, *VvFLS1* and *VvCHI*, were also strongly up-regulated in response to light, ranked 4th and 8th, respectively. Additionally, it should be noted that the *VvUbuiquitin1* (*VvUBIQ1*) EST sequence TC32075 (unigene number Vvi.713) used for normalisation of data in all RT-PCR experiments, remained unchanged in response to light (data not shown).

Of the 15 unigenes of significance ($p<0.01$) (**Table 5.5**), 4 unigenes were identified with potential roles in transcriptional regulation. Two of these genes were down-regulated in response to light (Vvi.8350 and Vvi.10229) while the remaining two had putative roles in ATP/DNA binding (Vvi. 6974) and the DNA binding of protein phosphatases, involved in pathway signalling (Vvi. 9633). Therefore, searching continued at $p<0.05$ and candidates screened for high L/D ratios. Seven unigenes with homology to DNA binding transcription factors including, MYB, bHLH and bZIP proteins, were identified (**Table 5.6**). These unigenes (candidate genes) were subsequently analysed by RT-PCR gene expression analysis, to determine if they are potential transcriptional regulators of *VvFLS1* (see **Section 5.3.4.3.2.3**).

Table 5.6 Potential transcriptional regulator candidate genes identified by microarray analysis. Seven candidate gene sequences were selected for RT-PCR expression analysis. An assigned TC number was given to each candidate gene. Table headings listed include: unigene number (#), gene title, average expression level (light & dark), corrected p value, rank & absolute value (i.e. difference L-D). The magnitude of light induction (i.e. fold-change) is calculated as the L/D ratio. Indicated in bold is the key gene role.

Assigned TC#	TC#	Unigene #	Gene Title	Average Light	Average Dark	P value	Rank	Absolute value	(L/D) Ratio
TC7	39225	Vvi.10003	Transcribed locus, weakly similar to NP_201053.2 **myb family transcription factor** (MYB96) [*Arabidopsis thaliana*]	7.55	4.39	0.0226139	792	8.94	1.72
TC8	38623	Vvi.6975	Transcribed locus, weakly similar to NP_849568.1 **myb family transcription factor** [*Arabidopsis thaliana*]. LHY (late elongated hypocotyl) DNA binding transcription factor. Circadian clock associated	9.89	8.15	0.025953	1443	3.33	1.21
TC9	40540	Vvi.6616	Transcribed locus, weakly similar to NP_909959.1 **bZIP transcription factor protein** [Oryza sativa (japonica cultivar-group)]	8.38	6.02	0.0312679	3980	5.14	1.39
TC10	43962	Vvi.5444	Transcribed locus, weakly similar to NP_567548.1 pseudo-response regulator 2 (APRR2) (TOC2) [*Arabidopsis thaliana*]. **Transcription factor**.Circadian clock associated	6.28	2.53	0.0221245	588	13.47	2.48
TC11	45686	Vvi.1267	Transcribed locus, moderately similar to NP_922730.1 putative **Myb-related protein** [Oryza sativa (japonica cultivar-group)]	8.86	7.48	0.0272594	1770	2.60	1.18
TC12	49275	Vvi.3440	Transcribed locus, moderately similar to NP_568246.1 **DNA binding/transcription factor. bZIP protein** HY5 (HY5) [*Arabidopsis thaliana*]	10.06	5.39	0.0271022	1752	25.52	1.87
TC13	46999	Vvi.7854	Transcribed locus, weakly similar to UP_Q9DF41 **bHLH-WRPW transcription factor** [*Arabidopsis thaliana*]	11.39	2.81	0.0162863	104	381.75	4.05

Microarray analysis also indicated the light induction response of the flavonoid biosynthetic genes in grapevine (**Table 5.7**). Starting at the top of the flavonoid biosynthetic pathway (**Chapter 1, Figure 1.3**), two *VvPAL* homologs were identified, one of which was up-regulated in the light with a L/D ratio of 2.2 (although not significant). *VvCHS* was strongly up-regulated by light, however *VvCHI* and *VvF3H* remained unchanged. The *VvFLS* isoforms *VvFLS1* and *VvFLS2* (Downey et al. 2003b) were identified in the microarray data set. *VvFLS1* was strongly up-regulated in the light, while *VvFLS2* remained unchanged. Genes involved in the later part of the flavonoid biosynthetic pathway including, *VvDFR*, *VvLAR1/2*, *VvLDOX* and *VvUFGT* showed no significant response to light (as determined by significance), however the L/D ratio for *VvLDOX* was ~2.0, similar to that of the light inducible *VvPAL1* gene.

Table 5.7 Flavonoid gene expression in Chardonnay cells after exposed to light for 24h as determined by microarray analysis. Table headings listed include: unigene number (#), gene title, average expression level (light & dark), corrected p value, rank, absolute value (i.e. difference L-D) & regulation (up or down). The magnitude of light induction (i.e. fold-change) is calculated as the L/D ratio. Flavonoid gene names are indicated in bold.

Unigene #	Gene Title	Average Light	Average Dark	P value	Rank	Absolute Value	Regulation	(L/D) Ratio
Vvi.1950	Transcribed locus, moderately similar to NP_190894.1 phenylalanine ammonia-lyase 2 (**PAL2**) [*Arabidopsis thaliana*]	12.42	12.55	0.41995484	14393	1.09	down	0.989678
Vvi.5899	Transcribed locus, moderately similar to NP_181241.1 phenylalanine ammonia-lyase 1 (**PAL1**) [*Arabidopsis thaliana*]	11.15	4.96	0.027940005	2046	72.79	up	2.246522
Vvi.117	**CHS** mRNA for chalcone synthase	13.26	3.28	0.005715045	8	1009.05	up	4.042882
Vvi.124	**CHI** mRNA for chalcone isomerase	13.61	12.89	0.027719306	1865	1.65	up	1.056031
Vvi.128	**F3H** mRNA for flavanone 3-hydroxylase	5.77	6.73	0.030088216	3262	1.94	down	0.857363
Vvi.8746	Cultivar Shiraz flavonol synthase (**FLS2**)	2.16	2.10	0.029324556	2701	1.04	up	1.026423
Vvi.1650	Cultivar Shiraz flavonol synthase (**FLS1**)	12.87	2.28	0.005240533	4	1545.82	up	5.656478
Vvi.120	**DFR** mRNA for dihydroflavonol reductase	10.28	10.13	0.07568185	9745	1.11	up	1.015293
Vvi.6810	Putative leucoanthocyanidin reductase 2 (**LAR2** gene)	7.83	6.27	0.021805065	546	2.95	up	1.248802
Vvi.14896	Putative leucoanthocyanidin reductase 1 (**LAR1**) gene, lar1-1 allele)	2.43	2.37	0.033093527	4650	1.04	up	1.023016
Vvi.127	**LDOX** mRNA for leucoanthocyanidin dioxygenase	6.18	3.09	0.02540366	1277	8.48	up	1.997614
Vvi.17	**UFGT** mRNA for UDP glucose: flavonoid 3-O-glucosyltransferase	2.77	2.72	0.49507818	14789	1.04	up	1.018607

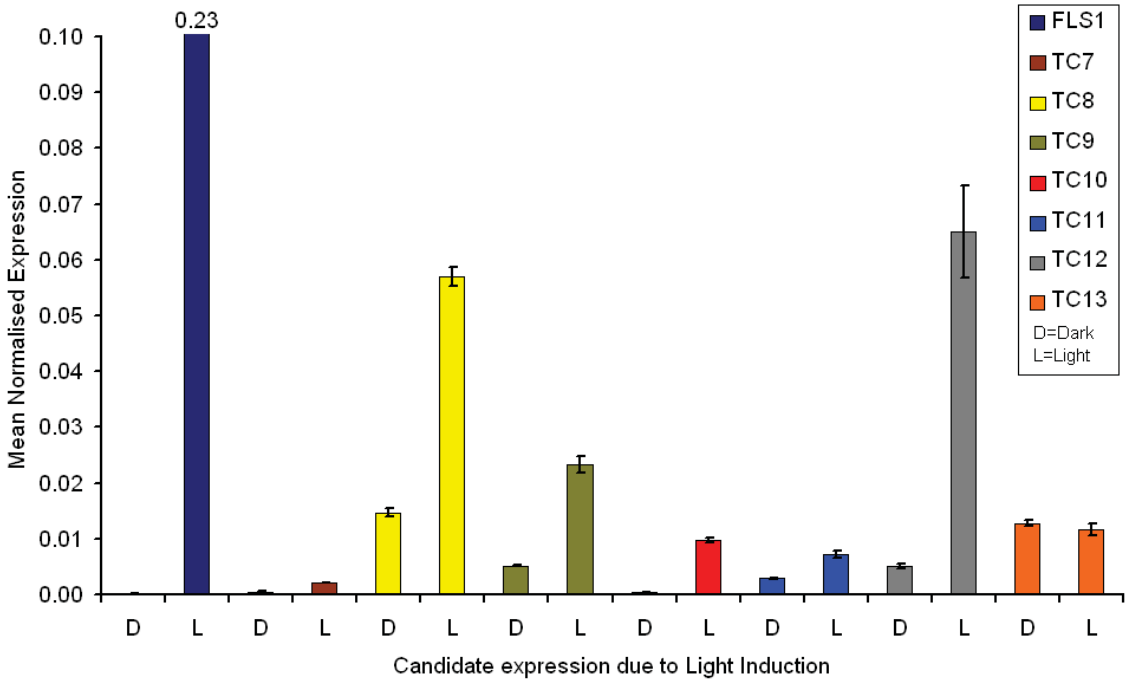
5.3.4.2.3 RT-PCR expression of potential transcription factor candidate genes

The seven transcription factor candidate genes identified by microarray analysis (as listed in **Table 5.6)** were selected for gene expression analysis by RT-PCR as potential transcriptional regulators of *VvFLS1* biosynthesis. Two cDNA series were used to test candidates; the first, the microarray experiment, where Chardonnay cells were exposed to light for 24 hrs (along with dark control) (**Section 5.2.4.2.1**) and the second, in Shiraz inflorescences, pre-flowering on day 0 (exposed control and shaded) and 2 days after exposure to light (light induced) (**Chapter 4)**.

Figure 5.7 shows that *VvFLS1* expression, in the light, was clearly elevated compared to in the dark (as expected, **Section 5.3.3**). The candidate genes (TC7 → TC12) also showed an increase in expression in the light (the change ranging from 2.5 to 20-fold) however, overall expression levels were significantly less than *VvFLS1* expression. Candidate gene TC13 did not increase in response to light, and therefore was not included for further analysis.

The pattern of *VvFLS1* expression, pre-flowering and in response to light was as expected (**Chapter 4**), with levels high in exposed controls (E), low in shaded (S) and high in light induced (L). Most of the candidate genes did not match this pattern of *VvFLS1* expression, however, there were two candidates (TC10 and TC12), which did appear to follow the pattern of expression. Candidate gene, TC10, is similar to AtTOC2, a transcriptional pseudo-response regulator, which has been shown to be involved in clock rhythms in *Arabidopsis* that is influenced by light and dark (McClung 2001). Candidate gene, TC12 had homology to AtHY5, a bZIP transcription factor protein that binds directly to the promoters of light inducible genes, promoting their expression and photomorphic development (Osterlund et al. 2000). Interestingly, AtHY5 has been shown to bind to the *AtCHS* gene in response to UV-B exposure in *Arabidopsis* seedlings (Ang et al. 1998). These candidates were further tested by RT-PCR analysis in additional light induction and developmental series as shown in **Appendix 5B**. Due to time constraints, these were not functionally tested in transient assays using the *VvFLS1* promoters.

A



B

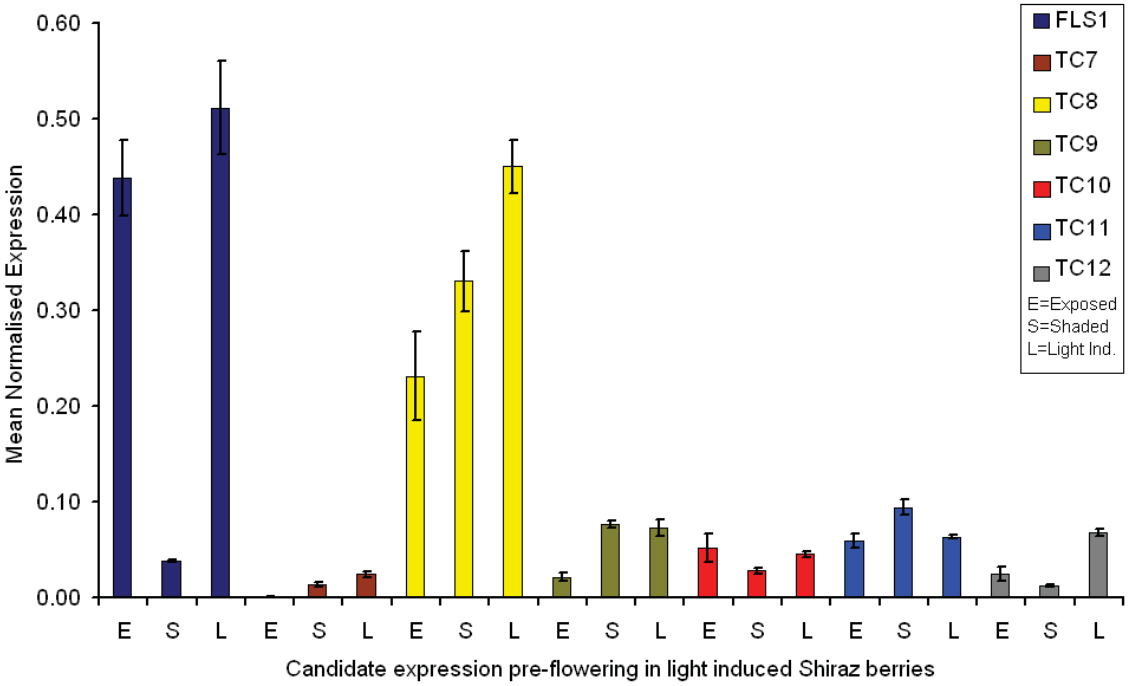


Figure 5.7 RT-PCR expression patterns for candidate (microarray) genes.

A Expression of *VvFLS1* & the candidate genes (TC7, TC8, TC9, TC10, TC11, TC12 –see **Table 5.6**) in light induced chardonnay cells after 24 hrs of exposure (D=Dark, L=Light). Data is expressed relative to the expression of *VvUBIQ1*. Error bars represent the standard error (±SEM) for three analytical replicates.

B Expression of *VvFLS1* & the candidate genes (TC7, TC8, TC9, TC10, TC11, TC12 –see **Table 5.6**) in Shiraz inflorescences, pre-flowering, upon light exposure for 2 days (as described in **Chapter 4**) (exposed fruit (E) (Day 0), shaded fruit (S) (Day 0), light induced fruit (L)(Day 2)). Data is expressed relative to the expression of *VvUBIQ1*. Error bars represent the standard error (±SEM) for three analytical replicates.

5.4 Discussion

Flavonols are important products of the flavonoid biosynthetic pathway, formed at different stages of grape berry development. Additionally, in grapevine, *VvFLS1* appears to be the only flavonoid pathway gene that is significantly up-regulated by light. It was suggested (in **Chapter 4**) that expression of *VvFLS1* is controlled by a specific transcriptional regulator(s) which may be able to operate independently of the developmental control (to the main flavonoid pathway) and also in response to light. Therefore, in order to understand the complexity of transcriptional control of flavonol synthesis in grapevines, the molecular mechanisms of *VvFLS1* gene regulation was investigated, with the aim to:

1) **Isolate the *VvFLS1* promoter and analyse the sequence for light regulatory units (LRU)**
2) **Develop a transient reporter assay for testing the *VvFLS1* promoter(s) activity**
3) **Identify candidate transcription factors genes and test the activation of *VvFLS1* promoter in the transient assay**

5.4.1 The light induction of flavonoid gene expression & accumulation in Chardonnay cell cultures

There are numerous investigations in grapevine cell cultures showing modification of the flavonoid biosynthetic pathway (particularly anthocyanins and tannins) in response to chemical elicitors (such as jasmonic acid) or limited nutrients (sucrose or phosphase) (Dedaldechamp and Uhel 1999; Dedaldechamp et al. 1995; Do and Cormier 1991; Feucht et al. 1999; Feucht et al. 1996; Larronde et al. 1998). In response to light, suspension cultures of *Vitis vinifera* L. cv. Gamay Fréaux (red cell line) have been shown to enhance anthocyanin and stilbene biosynthesis (Krasnow and Murphy 2004; Zhang et al. 2002). Additionally, in *Arabidopsis* protoplast cell cultures, *AtFLS1* gene expression has shown to be induced by light after 6 hrs, with maximum induction after 10 hrs (Hartmann et al. 2005).

In **Chapter 4**, it was clearly demonstrated that flavonol biosynthesis could be rapidly induced in bunches in response to light exposure. To understand the basis of transcriptional control of flavonol formation additional light induction experiments were performed. In the vineyard, experiments can be variable and can only be repeated on a seasonal basis. Therefore a light-inducible system, which was robust, could be routinely repeated in the laboratory, and where a large change in light inducible genes was required. It appeared obvious to trial a suspension culture of Chardonnay cells, which had all of the above benefits, however it remained unclear as to whether flavonols or any other flavonoids would be light inducible in these cells. Therefore,

the influence of light exposure on dark-grown Chardonnay cell cultures and flavonoid gene expression (and accumulation) was determined.

Dark-grown Chardonnay cells had low *VvFLS1* expression and no detectable flavonols, however, they could be rapidly induced by light to activate *VvFLS1* expression with the subsequent production of flavonols (**Figure 5.4** & **Figure 5.5**). Although there were slight differences in the light induction of *VvFLS1* expression it was demonstrated that after 12 hrs of light exposure, levels had increased (**Figure 5.5**). Corresponding with this increase in *VvFLS1* expression, flavonols were detected after light exposure on day 2, with the maximum observed around day 6 and 7. The delay in accumulation of flavonols compared to rapid induction of *VvFLS1* gene expression may be explained by the need for *de novo* protein synthesis during the light signal transduction process, as also observed in the hourly light induction experiment (**Chapter 4)**. These results confirm that flavonol biosynthesis is highly light responsive (as observed in **Chapter 4**) and demonstrate a light-inducible Chardonnay cell test system for flavonol biosynthesis.

The flavonoid composition of Chardonnay cells was determined by HPLC in the dark and after exposure to light for 3 days (**Table 5.2**). No anthocyanins or hydroxycinnamates were detected in dark or in light induced cells. Tannin content remained unchanged in the dark and light with levels similar to values reported by Decendit and Merillon (1996) in Gamay Fréaux (red cell line). This amount is approximately 30% of the amount of tannins detected in ripe Shiraz berry skins. No flavonols were detected in the Chardonnay cells in the dark, however after 3 day exposure to the light, concentration increased to 0.013mg/g tissue, which is approximately 50% less than the amount detected in ripe Chardonnay berry skins. In grape cell culture, considerable effort has been directed toward the improvement of anthocyanin and tannin biosynthesis for commercial applications (Zhang and Furusaki 1999). The experiments described in this investigation demonstrate a light inducible grapevine cell culture system, which may have the potential to be modified to allow rapid outputs of flavonols. Yet, it should be noted, that levels of flavonoids in the Chardonnay cell culture are still considerably less than those in Chardonnay grapes.

Microarray analysis of dark and light induced Chardonnay cells provided information on expression of a number of flavonoid pathway genes (**Table 5.7)**. Only *VvFLS1* and *VvCHS* genes were strongly up-regulated in the light, while most of the flavonoid biosynthetic genes (including *VvPAL, VvCHI, VvLDOX, VvUFGT, VvF3H, VvDFR* and *VvLAR1/2*) were not significantly affected by light, with expression remaining relatively stable. These results reiterate the highly light responsive nature of *VvFLS1* expression (**Chapter 4**).

The results also suggest that light differentially regulates the expression of genes of the flavonoid pathway. As discussed in **Chapter 4**, for flavonol biosynthesis to occur it is likely that there must be some level of co-activation of genes in the 'early' part of the pathway (eg. *VvPAL, VvCHS, VvCHI, VvF3H*). In *Arabidopsis* and apple the co-regulation of the 'early' pathway genes (*CHS, CHI, F3H*, *FLS*) has been shown in response to light for anthocyanin biosynthesis (Hartmann et al. 2005; Kubasek et al. 1998; Kubasek et al. 1992; Pelletier et al. 1999; Pelletier et al. 1997; Takos et al. 2006). However, in this investigation, it appears that essentially only *VvFLS1* and *VvCHS* are coordinated in response to light, particularly as there was only small increase in the expression level of *VvCHI* and expression of *VvF3H* actually decreased in response to light exposure. These differences in expression levels indicate that gene activity in the flavonoid biosynthetic pathway is complex, where it may be that some genes are activated that are necessary for the production of many (in the case of *VvCHS*) kinds of flavonoids whereas other genes are only activated for one subclass of flavonoids (like *VvFLS1*). These results also indicate that regulation may be different in different plant species. Nevertheless, in this investigation, the strong up-regulation of *VvCHS* and *VvFLS1* suggest these genes are coordinated in their response to light most likely for the rapid production of flavonols.

5.4.2 Cloning & sequence analysis of *VvFLS1*promoter fragment(s)

At the start of this investigation, there were only two published partial grapevine *VvFLS1* gene sequences, *VvFLS1* and *VvFLS2* from Shiraz (Downey et al. 2003b) and a partial grapevine EST sequence (TC24143) from Cabernet Sauvignon. As *VvFLS1* expression was shown to be significantly up-regulated by light (Downey et al. 2004); **Chapter 4)** the promoter sequence of this gene was targeted in grapevines.

Two *VvFLS1* promoter sequences were identified (*VvFLS1* promoter1 (811 bp) and *VvFLS1* promoter2 (817 bp), and while most of the sequence was homologous (99% nt identity), there were some minor changes and deletions (**Figure 5.2**). The reason for these two *VvFLS1* promoter sequences is unclear. During cloning of the *VvFLS1* promoter, an 1176 bp genomic intron was identified. The 1176 bp intron was in a similar position to one of the introns in *AtFLS1* however; the *AtFLS1* intron was only 72 bp in length (U84258 (Wisman et al. 1998)). Introns, are non-coding segments of DNA, which are spliced from the coding sequence during transcription and while their biological role remains largely unknown, they are thought to have some regulatory roles in cells (Campbell 1996).

In 2006, five genomic *VvFLS* sequences (***VvFLS1*-5cs**) from Cabernet Sauvignon were reported (Fujita et al. 2006). Two of these gene sequences, *VvFLS1cs* (AB086055) and *VvFLS4cs* (AB092591)) corresponded to *VvFLS1* and *VvFLS2*, respectively (Downey et al. 2003b), with ~98% identity at the protein level. The remaining *VvFLS* sequences, *VvFLS2cs* and *VvFLS3cs*, were weakly expressed around flowering with ~70-80% homology to *VvFLS1*, while *VvFLS5cs* was only 50% identical to *VvFLS1* (Fujita et al. 2006). The promoter sequence of *VvFLS4cs* was approximately the same length (812 bp) as *VvFLS1* promoter1 with 98% nt identity, and did not contain the additional ATC and ATG repeats as observed in *VvFLS1* promoter2 sequence. All of the *VvFLS1-5cs* gene sequences were shown to contain two introns (Fujita et al. 2006), one of which was in the same position and of similar length as that observed in *VvFLS1*. The work by Fujita et al. (2006) confirms the *VvFLS1* promoter1 sequence isolated from grapevines and also strongly suggests there is likely to be more than one intron in the *VvFLS1* gene (isolated from Shiraz).

The *VvFLS1* promoter sequences were analysed for the presence of key DNA binding sites using the PLACE database (**Table 5.1**). Both *VvFLS1* promoters contained similar motifs, and those commonly identified were involved in responding to environmental cues, particularly light. The response of light activated transcription has been extensively reviewed in Terzaghi and Cashmore (1995). The general theory adopted for light regulated transcription is that upon light perception, signals are transduced via intermediates to activate transcription factors which then bind to their associated sequences within the promoter regions of light regulated genes (Terzaghi and Cashmore 1995). The two predominant groups of genes that respond to light signals include those using light as source of information about the environment as well as a source of energy for photosynthesis. Various motifs from both these gene types were located in the *VvFLS1* promoter sequences, of particular interest IBOX motifs, which was present near key flavonoid DNA binding domains.

There were three motifs identified in the *VvFLS1* promoter1 sequence associated with the UV-light responsiveness of the flavonoid biosynthetic gene, *PAL*. PALBOXAPC and PALBOXLPC motifs were located in the promoter sequence of the *PcPAL* genes in parsley, which in suspension cells was found to be up-regulated in response to UV light exposure, the maximum occurring after 8 hrs (Logemann et al. 1995). Similarly, the BOXCOREDCPAL motif was located in the promoter of the carrot *DcPAL1* gene, which in suspension cells was found to be upregulated in response to UV light (Maeda et al. 2005). The two PALBOXPC motifs were not present in the promoters of the grapevine flavonoid biosynthetic genes (*VvCHS, VvCHI,*

VvF3'5H, VvLDOX, VvANR, VvLAR1, VvUFGT), unlike the BOXCOREDCPAL motif, which was located in each of these genes (data not shown). Also, sequence analysis of the two *VvFLS1* promoters also indicated the PALBOXAPC motif was not present in the *VvFLS1* promoter2 sequence.

There were several flavonoid MYB binding sites identified in the *VvFLS1* promoter sequences, including MYBCORE, MYBPLANT, MYBPZM (**Table 5.1**), which were also found in other promoters of grapevine flavonoid biosynthetic genes (Bogs et al. 2007). Whilst there are specific target recognition sites for different MYB proteins (Jin and Martin 1999) the frequency of these sites in the grapevine flavonoid biosynthetic genes suggests they may be common DNA MYB-binding motifs involved in the coordinated regulation of these genes.

Of particular interest was the fact that both *VvFLS1* promoters contained parts of a putative light regulatory unit (LRU) consisting of a MYB recognition element (MRE) and a nearby ACGT-containing element (ACE). These elements identified by Hartmann et al. (2005) have been found to be necessary for light responsiveness of the structural genes (*AtCHS, AtCHI, AtF3H* and *AtFLS1*) required for flavonol synthesis in *Arabidopsis*. The ACE elements are bound by bZIP factors or bHLH factors and the MRE elements are bound by MYB factors. Hartmann et al. (2005) also identified a third cis-acting element called an RRE element (R response element or MYC binding site) located in the promoter sequence of *AtCHS* which was shown to be a bHLH binding site not essential for light responsiveness. It was proposed that a combination of a MYB and bZIP factor confer light responsiveness while a MYB and bHLH factor confer tissue specificity (Hartmann et al. 2005).

LRU elements identified in the *VvFLS1* promoter sequences, included the *Arabidopsis* MRE (MREATCHS) and ACE (ACEATCHS) motifs (as described above, Hartmann et al. (2005)), as well as a MRE binding motif identified in the promoter of *PcCHS* in parsley (LREBOXIIPCCHS1) (Feldbrugge et al. 1997). While either a MRE or an ACE site was located in the promoters of the other flavonoid biosynthetic genes (*VvCHS, VvCHI, VvF3'5H, VvLDOX, VvANR, VvLAR1, VvUFGT*), the combination of both was only identified in the *VvFLS1* promoter sequence (data not shown).

It is important to note that the results of this type of *in silico* sequence analysis have to be experimentally investigated. It could be that the relative numbers of motifs or positioning of motifs might either allow or prevent a productive interaction of factors that need to contact each other for promoter activation. The presence of these binding sites suggests regulation by the

respective factors, however their actual implications in transcriptional regulation of *VvFLS1* expression have yet to be demonstrated.

5.4.3 Development of a transient reporter assay for *VvFLS1* promoter(s)

Activation of the *VvFLS1* promoters by MYB transcription factors was established using a transient expression method with grape cell culture and the dual-luciferase system. AtMYB12 was the primary transcription factor used to functionally test the *VvFLS1* promoter(s) in the transient assay. AtMYB12 has been shown to be a specific activator of flavonol biosynthetic pathway, activating *AtCHS* and *AtFLS* genes as its primary targets (Mehrtens et al. 2005). AtMYB12 has also shown to act via the MRE site (part of the LRU) in the promoters of these genes (Mehrtens et al. 2005).

Constructs containing *Arabidopsis* cDNAs encoding MYB factors have been shown to activate the grapevine flavonoid biosynthetic genes in this type of Chardonnay cell transient assay (Bogs et al. 2007; Walker 2006a). As controls, the *VvFLS1* promoter was also tested with different MYB transcription factors; including; VvMYBA2 (BAD18979) which activates *VvUFGT* controlling anthocyanin biosynthesis in grapes (Kobayashi et al. 2002; Walker et al. 2007), VvMYBPA (AM259485) which has been shown to activate the genes involved in tannin biosynthesis (*VvLAR1, VvANR*) in grapes (Bogs et al. 2007) and VvMYBF, a putative transcriptional regulator that has a matching developmental gene expression pattern to *VvFLS1* (Bogs 2006b).

The transient assays showed that AtMYB12 activated the grapevine *VvFLS1* promoter, whereas the transcription factors, VvMYBA2, VvMYBPA1 and VvMYBF did not (**Figure 5.3**). These results suggest the *VvFLS1* promoter sequence was isolated, and also indicate the development of a transient reporter assay for this gene. However, the level of activation of *VvFLS1* promoter by AtMYB12 was still relatively low (~7-fold) compared to other studies (Bogs et al. 2007; Takos et al. 2007; Walker et al. 2007). In *Arabidopsis* the *AtFLS1* promoter was activated by at least 1000-fold by AtMYB12 (Mehrtens et al. 2005) and in grapevine, activation of the tannin (*VvANR* and *VvLAR1*) and anthocyanin (*VvUFGT*) promoters by their respective MYB transcription factor has been shown to be by at least 30-fold (Bogs et al. 2007; Walker et al. 2007). It could be that AtMYB12 may not work well on the *VvFLS1* promoter, and that a grapevine MYB12 homolog would be more effective, if there is one. Nevertheless, these results support the notion of differential control of flavonol, anthocyanin and tannin biosynthesis pathway by specific transcription factors (as shown in *Arabidopsis*).

It is also important to note, that the *VvFLS1* promoter1 was more strongly activated than *VvFLS1* promoter2, perhaps due to the absence of the PALBOXAPC in the *VvFLS1* promoter2 sequence (as noted earlier). Also AtMYB12 did not require a bHLH (i.e. AtEGL3) for activation (as shown by Mehrtens et al. (2005)), rather the inclusion of this transcription factor in bombardment decreased *VvFLS1* promoter expression. This may be due to the AtEGL3 cDNA interacting with the promoter and preventing other regulatory co-factors from binding to and activating the promoter(s) of *VvFLS1*.

While AtMYB12 was shown to activate the promoters of *VvFLS1*, the level of activation was relatively low. Possible methods, which may improve the activation efficiency of the *VvFLS1* promoter sequence include, using a grapevine transcriptional regulator (as discussed above) and/or using longer *VvFLS1* promoter sequence (which may contain additional DNA binding motifs). It also cannot be excluded that additional MYB, bHLH, bZIP transcription factors may be involved in the regulation of flavonol synthesis and/or are involved in different combinations required for activation. Therefore searching should be expanded to include these types of transcriptional regulators, which should be functionally tested in different arrangements. Future experiments are discussed in detail in **Section 5.5.**

5.4.4 Identifying transcriptional regulators of *VvFLS1* & RT-PCR expression of transcription factor candidate genes

Although AtMYB12 was shown to activate the *VvFLS1* promoters, this was at low levels (**Section 5.3.2**). Therefore, two approaches were employed to search for possible grapevine transcription factor candidates that may regulate *VvFLS1* expression, including:

5.4.4.1 Database searching for AtMYB12 homologs in grapevine

Six putative candidate genes were identified after BLAST analysis of AtMYB12 in the grapevine EST database (**Table 5.3**). At the time of analysis, most of these candidate genes were assigned putative MYB like-DNA binding transcription factor roles, however, subsequent publications have revealed, TC1 (TC46393) is an transcriptional regulator of tannin synthesis in grapevines, assigned MYBPA1 (Bogs et al. 2007) and TC2 (TC41702) is able to regulate anthocyanins, tannins, flavonols and lignin when expressed in tobacco, assigned VvMYB5a (Deluc et al. 2006).

5.4.4.2 Microarray analysis for candidate transcription factors

Microarray analysis (made from light induced Chardonnay cells) did not reveal any obvious transcriptional regulator candidate genes at significance level $p<0.01$. However, when the level of significance was lowered ($p<0.05$), numerous transcriptional regulator candidate genes were identified. Seven candidate genes were selected based on their putative roles in transcriptional regulation, the gene-types included MYB, bHLH and bZIP proteins (**Table 5.6**).

It has been clearly demonstrated that *VvFLS1* expression has two distinct periods of expression, early in development (around flowering) and during ripening (towards harvest) and is also highly light responsive (Downey et al. 2003b; Downey et al. 2004; **Chapter 4** & **Chapter 5**). As regulation of most flavonoid biosynthetic genes is by MYB-type and bHLH transcription factors expression of transcriptional regulators controlling flavonol synthesis (i.e. candidate genes) should coincide with expression of *VvFLS1*.

Most of the candidates shown in **Figure 5.6** and **Figure 5.7** showed elevated expression levels in the light compared to the dark but the relative increase in the light was generally less than for *VvFLS1* expression. In response to developmental expression, the majority of the candidate genes did not match the pattern of *VvFLS1* expression, suggesting most of the candidate genes were unlikely targets for *VvFLS1* regulation. The exception, however, were two candidate genes TC10 and TC12 (identified by microarray analysis) which had similar patterns of gene expression to *VvFLS1* (see **Appendix 5B)**. Candidate gene, TC10 was similar to TOC2, a transcriptional pseudo-response regulator in *Arabidopsis*. TOC1 and TOC2 genes have suggested roles as components of an oscillator involved in clock rhythms, which are able to are influenced by the light and dark (McClung 2001).

Candidate gene TC12 had homology to AtHY5, a bZIP transcription factor that binds directly to the promoters of light inducible genes, promoting their expression and photomorphic development (Osterlund et al. 2000). AtHY5 is suggested to be involved in responses to phytochromes and photoreceptors (Ang et al. 1998). Interestingly, this transcription factor acts antagonistically with AtCOP1 (a RING finger protein with WD-40 repeats) (Osterlund et al. 2000). It has been shown in tomato that down regulation of *AtHY5* gene expression results in defects in light responses including reduced chlorophyll/carotenoid expression (Liu et al. 2004). In contrast, repression of *AtCOP1* gene expression resulted in plants with elevated levels of fruit carotenoid levels (Liu et al. 2004). Additionally, AtHY5 has been shown to bind to *AtCHS* in response to UV-B exposure in *Arabidopsis* (Ang et al. 1998) however recently this interaction was proposed to also involve an interacting partner(s) that would concurrently be induced by UV-B (Oravecz et al. 2006).

Unfortunately due to time constrains, these candidate genes were not functionally tested, and are therefore suggested to be good targets for future experiments (see **Section 5.6**). While logical attempts (via database searching for AtMYB12 homologs and microarray analysis) were made to isolate potential transcription factor candidates, reasons why candidates were not readily identified (by these two methods) may include:

a) MYB proteins are highly conserved proteins, of which there are over 100 family members in *Arabidopsis* (Stracke et al. 2001). Consequently, database searching for homologous sequences has a high probability of identifying a wide range of these proteins. It should also be noted that homology isn't necessarily a good predictor of function. These concerns on the effectiveness of data base searching was demonstrated in this investigation after yielding no obvious grapevine homologues after BLAST analysis of AtMYB12 (**Table 5.3**), rather VvMYBPA1 a transcriptional regulatory of the tannins pathway in grapes was identified (Bogs et al. 2007).

b) The time of sampling material used for microarray analysis. In this study, Chardonnay cells were exposure to light for 24 hr, after which *VvFLS1* expression had increased by 838-fold. Previous experiments had indicted that *VvFLS1* expression remained high up until day 8 (**Figure 5.5**). It is generally considered in transcription cascade events, that expression of the transcription factor precedes expression of the corresponding structural gene, and that these transcripts are 'short' lived and 'long' lived, respectively (Jin and Martin 1999; Walker 2006b). Therefore the capacity to identify a candidate transcription factor for *VvFLS1* is based on the expression pattern of *VvFLS1* and to anticipate the expression of the preceding transcription factor. It may be that the transcription factors that regulate *VvFLS1* are only expressed transiently and at low levels making it difficult to identify by conventional screening methods. An example of this has been recently shown in carrot cell cultures where the transcriptional regulator of DcPAL, *DcMYB1*, was observed to reach a maximum 2 hr after UV-B irradiation (after which expression levels were significantly reduced), followed by the induction of the *DcPAL1* gene 1 hr after the maximum (Maeda et al. 2005). This data indicates how tightly regulated the timing and activation is of MYB transcription factors and the corresponding structural genes. Therefore, it cannot be ruled out in this investigation that perhaps the expression peak of the transcriptional regulator of *VvFLS1* has been overlooked, in sampling after 24 hr.

c) The transcription factor sequence may not be in the TIGR database or on the microarray slide. Transcription factors are usually expressed at lower levels than the structural genes they activate and only when required ('short' lived) (Jin and Martin 1999). As a consequence,

cloning strategies used to generate the libraries for the database/microarray slide, may not detect these transcripts and isolation of the transcriptional regulators for *VvFLS1* by these methods would prove unsuccessful. It is also worth noting, that the method of candidate isolation and screening used in this investigation (i.e. matching RT-PCR expression patterns with *VvFLS1*) is a correlative measure only and to be certain of the role of these transcriptional regulator candidate genes they would each have to be cloned and functionally tested with the *VvFLS1* promoters.

5.4.5 Transcriptional regulation of *VvFLS1*

Transcriptional regulation of the flavonoid biosynthetic pathway has been largely characterized in *Arabidopsis*. The MYB proteins AtPAP1 and AtPAP2 regulate anthocyanin synthesis by activation of the flavonoid pathway genes (*AtCHS, AtF3H, AtDFR, AtLDOX, AtUFGT*) (Borevitz et al. 2000; Tohge et al. 2005). The MYB protein AtTT2 regulates tannin synthesis by activation of *AtDFR, AtLDOX* and *AtANR* (Borevitz et al. 2000; Nesi et al. 2001) and recently the MYB protein AtMYB12 was identified as a flavonol specific regulator activating *AtCHS* and *AtFLS* (Mehrtens et al. 2005).

While *Arabidopsis* has been an excellent model in delineating this fundamental basis of flavonoid synthesis and gene regulation (Winkel-Shirley 2001), it produces a dry fruit (similar to cereals), which does not normally accumulate anthocyanins. Recently, in apple, a MYB transcription factor (MdMYB1) was identified, which functioned to co-ordinately regulate the genes involved in the anthocyanin pathway from *MdCHS* to *MdUFGT* (excluding *MdCHI*) (Takos et al. 2007), in an action similar to AtPAP1 in *Arabidopsis* (Tohge et al. 2005). Anthocyanin regulation by MYB proteins have also been reported in crop plants such as pepper and strawberry (Aharoni et al. 2001; Borovsky et al. 2004).

Only recently, in grapevines has the MYB transcription factors responsible for activation of anthocyanin and tannin biosynthesis been identified. Two MYB transcription factors, VvMYBA1 and VvMYBA2, have been shown to regulate *VvUFGT* and anthocyanin biosynthesis in red berries (Kobayashi et al. 2002; Kobayashi et al. 2005; Walker et al. 2007). The MYB transcription factor VvMYBPA1 has been shown to activate the promoters of *VvLAR* and *VvANR*, but not *VvUFGT,* indicating regulation of the tannin pathway (Bogs et al. 2007). A MYB transcription factor VvMYB5a has also been shown to affect metabolism of anthocyanins, tannins, flavonols and lignin when expressed in tobacco, suggesting it controls different branches of the flavonoid biosynthetic pathway (Deluc et al. 2006). Presently, transcriptional regulation of flavonol biosynthesis in grapevines remains unknown, although attempts were made to isolate one in this investigation.

It has been extensively shown in this investigation that flavonol biosynthesis is highly light responsive**,** and that exposure to light is able to override the developmental control of flavonol biosynthesis in grapes (**Chapter 4** & **Chapter 5)**. Therefore it was suggested that flavonol biosynthesis may be controlled by a specific transcriptional regulator(s) which may be able to operate independently of the developmental control (to the main flavonoid pathway) and also in response to light.

In *Arabidopsis* and apple the MYBs, *AtPAP1* and *MdMAB1* have been shown to be responsive to light for anthocyanin biosynthesis (Takos et al. 2007; Vanderauwera et al. 2005). In contrast, it has yet to be established in grapevines, whether the anthocyanin pathway is light responsive as regulated by *VvMYBA1/VvMYBA2* gene expression, however, considering that anthocyanin synthesis is unperturbed by shading and subsequent light exposure (**Chapter 4**) indicates it is unlikely they are regulated by light. However, there is some variability in the response of grapevine cultivars to light (Cortell and Kennedy 2006; Downey and Krstic 2005; Jeong et al. 2004), which may complicate the regulatory mechanisms of anthocyanin accumulation. Furthermore, it is unlikely that the expression of the grapevine tannin regulator (*VvMYBPA1*) is light responsive, as tannin regulation in grapevines appears to be largely unaffected by light (Downey et al. 2004). This would be similar to expression of the tannin regulator *AtTT2*, which does not change in response to light (Vanderauwera et al. 2005).

While it has not been determined if AtMYB12 is influenced by light (Mehrtens et al. 2005), the tight light inducible co-regulation of *AtCHS* and *AtFLS* observed by Hartmann et al. (2005) indicates an attractive model of AtMYB12 as a master switch for the accumulation of flavonols in repose to light. In this investigation, microarray analysis showed that *VvFLS1* and *VvCHS* gene expression were strongly up-regulated by light compared to the other flavonoid pathway genes (**Table 5.7**) and that the *VvFLS1* promoter contained light regulatory motifs, similar to the *AtFLS1* promoter sequence **(Table 5.1**). Furthermore, the flavonol specific regulator AtMYB12 was shown to activate the *VvFLS1* promoters (**Figure 5.3**). Therefore, the observation that in both grapevine and *Arabidopsis* that *FLS1* and *CHS* genes are highly light inducible (compared to the other flavonoid pathway genes), together with the fact that AtMYB12 activated the *VvFLS1* promoters, suggests a similar mechanism of light regulation of the flavonol biosynthetic pathway may exist.

5.4.6 Summary

Despite efforts made in this investigation, the actual mechanism of *VvFLS1* transcriptional regulation still remains unknown. While similarity in the transcriptional control of flavonol biosynthesis to *Arabidopsis* has been proposed, the fact that regulation of the anthocyanin pathway is different should not be disregarded, and indicates that transcriptional regulation of different pathways of the flavonoid biosynthetic pathway may be different in different plant species. It could be that there are two MYB transcription factors regulating flavonol biosynthesis in grapevine; one during development and/or another in response to light. Alternatively, it cannot be ruled out that there may be other regulatory factors such as bHLH or bZIP proteins that may be involved in the regulation of flavonol synthesis.

This investigation suggests a high level of complexity of regulation of flavonol synthesis in grapevines, including developmental and light regulation by several transcription factors. Significant progress has been made in this area of research including:

1. Isolation of the two *VvFLS1* promoter sequences which contained the presence of an light regulatory units (LRU)
2. Development of a transient reporter assay for testing *VvFLS1* promoter activity, as shown by activation of both *VvFLS1* promoters by AtMYB12
3. Development of a robust light inducible test system, derived from Chardonnay cell cultures
4. Identification of two putative candidate transcription factors genes which have yet to be functionally tested by microarray analysis

However, it is clear that further experiments are required to elucidate regulation of flavonol biosynthesis, as listed below in **Future Directions** (**Section 5.5).**

5.5 Future directions

While this investigation did not isolate a grapevine transcriptional regulator of the *VvFLS1* gene, it made substantial progress in this area of research. Future directions suggested to continue this investigation include isolating additional *VvFLS1* promoter sequence and isolating transcriptional regulators of *VvFLS1*.

5.5.1 Isolating additional *VvFLS1* promoter sequence

It is generally considered that between 1-2Kb is the suggested amount of genomic sequence upstream of the coding region of plant genes, required for promoter analysis (Seki et al. 2002). In this investigation, 800 bp of upstream sequence was obtained and this region contained key DNA binding motifs such as the LRU. Thus, it was considered worthwhile to attempt functional testing with this promoter fragment. However, it was recognised that additional regulatory elements may exist upstream from this region.

In 2006, after the experiments described here were completed, access to a grape genome-sequencing database (*Vitis vinifera* L. cv. Pinot Noir) was kindly arranged by Dr Riccardo Velasco (Istituto Agrario San Michele all'Adige, Italy). BLAST analysis of *VvFLS1* in this database, revealed a 9.2kb genomic fragment (referred to as ***VvFLS1pn***). The majority of this sequence was the upstream genomic sequence (5075 bp) and when aligned to *VvFLS1* promoter1, showed 98% nt identity in the overlapping sequence. *VvFLS1pn* also contained two introns, in the same position and of similar length to *VvFLS4cs*. An alignment of the current grapevine *VvFLS1* genomic sequences; *VvFLS1* (2079 bp, coding sequence of AY257978; (Downey et al. 2003b), intron and promoter1 genomic sequence (this experiment)), *VvFLS4cs* (3729 bp, AB092591; (Fujita et al. 2006)) and *VvFLS1pn* (9283 bp, not published), is shown in **Appendix 5C (on CD).**

With access to the additional *VvFLS1* upstream genomic sequence, *VvFLS1pn* (5075 bp) was analysed using the PLACE (plant DNA cis-elements) database (Higo et al. 1999). There was no obvious clustering of motifs related to flavonoid biosynthesis and/or light regulated transcription in the first 3 Kb of the *VvFLS1pn* promoter sequence. However, -2 Kb from the start codon (ATG) several significant DNA binding motifs were located (shown in **Appendix 5C**). Of particular interest, was the presence of an RRE site (EBOXBNNAPA), located ~1170 bp upstream of the start codon (ATG). The RRE comprises of part of the LRU in the *Arabidopsis AtCHS* promoter and is involved in the regulation of the flavonoid genes in response to light,

acting synergistically and independently of a functional ACE site (Hartmann et al. 2005). In the *VvFLS1pn* promoter sequence, this RRE is in close proximity to key motifs including an IBOX, MYBPZM and an ACE site.

Initiation of DNA transcription occurs only after successful recognition of the appropriate transcription factor(s) to the specific motif contained in the promoter sequence (Weisshaar and Jenkins 1998). With this in mind, along with the added advantage of now knowing more than 5Kb of the *VvFLS1* promoter sequence (and DNA binding domains contained therein), functional assay should be repeated with longer promoter sequence, which may ultimately improve the activation of these promoter sequences. Furthermore, while part of the *VvCHS* promoter sequence was identified, there were no LRU motifs identified, most likely due limited genomic sequence (i.e. 656 bp). The co-regulation of *AtFLS1* and *AtCHS* genes in response to light and the fact that AtMYB12 has shown to activate these genes in *Arabidopsis* (Hartmann et al. 2005; Mehrtens et al. 2005) warrants isolation of the *VvCHS* promoter in grapevine and subsequent functional analysis.

5.5.2 Isolating transcriptional factor candidate genes

Two grapevine candidate genes (TC10 and TC12) were identified in this investigation as potential transcriptional regulators of *VvFLS1* based on RT-PCR expression pattern, however these were not functionally tested. Therefore, they are suggested to be the next likely candidates to test in transient assays, particularly TC12, as it has homology to AtHY5 a bZIP transcription factor protein shown to influence carotenoid content in tomato plants (Liu et al. 2004).

Although this investigation was primarily searching for MYB transcription factors, the influence bHLH, bZIP transcription factors involved in the regulation of flavonol synthesis in grapes cannot be excluded. Thus, further searching for transcriptional regulators should not be limited to only one of these transcriptional factor families. Furthermore, different combinations of transcription factors should also be trialed in repeated transient assays. While in this investigation, the co-bombardment of AtEGL3 (a bHLH protein) down regulated *VvFLS1* promoter activation with AtMYB12, it cannot excluded that there may be different combinations of MYB, bHLH, bZIP transcription factors involved in the regulation of flavonol synthesis. Hartmann et al. (2005) demonstrates the complexity that could be involved regulation of the flavonoid biosynthetic pathway, where DNA binding domains of different combinations of MYBs, bHLH and bZIP proteins determines, light responsiveness and/or tissue specificity.

The identity of a transcription factor mediating light induction of *VvFLS1* in grapevines has yet to be determined. With limited knowledge of the number of transcription factors present in grapevine, combined with the fact that these genes are highly homologous and typically expressed at lower levels and at different times, elucidation of the gene(s) responsible for the light induction of *VvFLS1* expression in grapevines is likely to be a challenge. Nevertheless, this investigation has established groundwork that will make future investigations easier to identify transcriptional regulators of flavonol synthesis in grapevines.

CHAPTER 6

Conclusions & future directions

6.1 General introduction

Anthocyanins, tannins and flavonols contribute to grape and wine quality by influencing the colour and mouthfeel of red wine. These compounds are synthesized in different parts of the berry, during different stages of berry development (Downey et al. 2003a; Downey et al. 2003b) and are influenced by environmental and viticultural factors such as temperature and light exposure (Downey et al. 2006; Downey et al. 2004; Mori et al. 2007; Spayd et al. 2002). At the start of this investigation, Sas and Lim (2003) from Hardy Wine Company (HWC) highlighted the need for better indicators of grape and wine quality, particularly in cool viticultural regions. Consequently, one aim of this study was to investigate the relationship between the different products of the flavonoid biosynthetic pathway (anthocyanins, flavonols and tannins) and determine their role in grape and wine quality. This study also aimed to investigate how changes in bunch light exposure at different stages in grape berry development influences the levels of these flavonoids in grapes. This work followed on from previous research showing shading had little effect on Shiraz berry development, ripening, anthocyanin and tannin accumulation, but significantly decreased flavonol biosynthesis (Downey et al. 2004). Lastly, with a focus on the light induced expression of flavonol biosynthesis in grapevines, this study sought to investigate the molecular mechanisms controlling flavonol synthesis.

6.2 The flavonoid composition of Shiraz grapes from a warm & cool climate region & their role in grape & wine quality

The first part of this study aimed to investigate the relationship between the flavonoid compounds in grapes from a warm and cool climate and the impact on grape and wine quality. Flavonoid composition (anthocyanins, flavonols and tannins) in Shiraz grapes from a range of warm (Riverland) and cool (McLaren Vale) climate vineyards was determined by HPLC and spectrophotometric methods in two seasons. This allowed the flavonoid content and composition to be compared in these different climatic regions and also enabled the relationship between the different flavonoid compounds to be determined. Additionally, the relationship between fruit composition and grape and wine quality was investigated.

In both years, the grapes from warm and cool climates formed two distinct data sets based on flavonoid composition. In the warm region total anthocyanin and flavonol content was lower than in the cool region, however tannin content was similar in both regions. Furthermore, there was a higher proportion of anthocyanin malvidin derivatives and coumaroyl-glucosides derivatives in the warm region compared to the cool region. It was suggested that temperature, rather than light exposure, may be the climatic factor altering anthocyanin composition and that these types of compounds are likely to be more stable and/or resistant to degradation at higher temperatures compared to other anthocyanin compounds (Downey et al. 2004; Mori et al. 2007; Spayd et al. 2002). However, whether this change in anthocyanin composition is due to anthocyanin degradation and/or inhibition of anthocyanin biosynthesis remains to be elucidated.

A correlation between anthocyanins and flavonols in both the warm and cool climate samples in both years was observed, however those from the warm region always had lower anthocyanin for a given level of flavonol. As expected, the level of tannin in the seeds was greater than in skin for all samples. In both years, there was a weak correlation between anthocyanin levels in the skin and skin tannins, but no relationship with seed tannins. These results suggest there is some co-ordination in the synthesis of anthocyanins, flavonols and skin tannins. This observation was noted as surprising considering differences in temporal expression during development and in response to different environmental cues. The influence of transcriptional and post-transcriptional mechanisms of flavonoid regulation must also be considered. Although significant progress has been made in

understanding flavonoid genes involved transcriptional control, there is much to be learnt regarding post-transcriptional processes.

This investigation also found that many of the historical measures of grape quality including TSS, berry weight and colour were not good indicators of grape and wine quality. Moreover those, which traditionally are not measured including grape tannins and flavonols, also did not correlate with grape and wine quality. Interestingly, there was some correlation between grape and wine quality and some of the quantitative vineyard measurements in the cool region indicating the usefulness of vineyard assessments. The two regions clearly separated based on yield and the levels of total anthocyanins were inversely related to yield, although the correlations were weak. This suggests high yields and colour levels are to some extent achievable.

In wines from the cool region, grape colour did not invariably correlate with wine colour. There was also a non-linear relationship between wine anthocyanins and final wine grade, indicating a maximum in anthocyanin content at some intermediate level of wine quality. It was suggested that the presence of other wine constituents might be influencing the 'quality' allocation as determined by the winemaker. Overall the lack of strong correlations between grape composition, vineyard attributes and grape and wine quality was suggested to be due to the fact that 'quality' is largely subjective and that vineyards are highly variable. A better understanding of vineyard variability and how to manage and sample fruit by grape growers, together with quantitative chemical information that can be provided to winemakers throughout the winemaking process, is desirable to ensure production of 'quality' wines.

6.3 The influence of bunch light exposure on flavonol synthesis in Shiraz & Chardonnay grapes during berry development

The second part of this study was to investigate the influence of bunch light exposure on flavonol synthesis in Shiraz and Chardonnay grapes during berry development. Light-excluding boxes were applied to bunches at budburst and boxes were subsequently removed at four sampling times: flowering, pre-veraison, veraison and harvest. At each sampling time, berry skins were sampled when the boxes were removed and then every second day (light induced), along with exposed controls for one week. Flavonol accumulation and *VvFLS1* gene expression was determined by HPLC and RT-PCR, respectively.

At all four sampling times, flavonol accumulation and *VvFLS1* expression in the boxed fruit was significantly less than in bunches exposed to light, confirming observations by Cortell and Kennedy (2006) and Downey et al. (2004). On removal of boxes at flowering, pre-veraison and veraison, flavonols accumulated to levels similar to that of the exposed control fruit over a period of 4-6 days. There was a significant increase in *VvFLS1* expression 2 days after exposure to light in parallel with the accumulation of flavonols. At harvest, in Chardonnay, *VvFLS1* expression peaked by day 4, while in Shiraz *VvFLS1* expression increased linearly and was highest at day 6. In contrast to the results for the earlier sampling times, the total amount of flavonols accumulated at harvest was less than 50% of exposed controls in Chardonnay and Shiraz grapes. These results show that flavonols are able to be induced by bunch light exposure at different times during berry development, including times when flavonols are not normally being synthesised. This suggests bunch light exposure can override the developmental control of flavonol accumulation.

It was also shown that total anthocyanin content was similar in shaded and exposed fruit, however there were effects of shading on anthocyanin composition. Shaded fruit had a lower proportion of tri-hydroxylated anthocyanins relative to di-hydroxylated anthocyanins. It was suggested that this change in anthocyanin composition is due to changes in the activity of the flavonoid 3'-hydroxylase (VvF3'H) and flavonoid 3',5'-hydroxylase (VvF3'5'H) enzymes, as also suggested by Downey et al. (2003b) and Cortell and Kennedy (2006). Expression of upstream and downstream genes *VvCHI, VvLDOX* and *VvUFGT* did not greatly increase in response to light. It was suggested that light differentially regulates the expression of different genes in the flavonoid pathway, with *VvFLS1* being a branch pathway gene that is highly light inducible.

In the diurnal experiments it appeared that *VvFLS1* expression followed a diurnal pattern of gene expression peaking in the afternoon when it was light. This corresponds with the light-dependent synthesis of *VvFLS1* expression. However, there were several limitations noted these diurnal experiments including, the low magnitude of gene expression, the influence of unknown post-transcriptional changes and separation of diurnal and circadian rhythms. To resolve some of these limitations future experiments would test different day/night lengths and to utilise microarray technology.

6.4 The molecular mechanisms of flavonol gene regulation in grapevines

To further investigate the light induced expression of *VvFLS1* in grapevines, the molecular mechanism of transcriptional control was investigated. The promoter of Shiraz *VvFLS1* was cloned by genomic walking. Its sequence was analysed and a putative MYB responsive element (MRE) and several light responsive elements (LRE) were identified. In the promoter of the *Arabidopsis AtFLS1* gene, the MRE site is activated by the transcription factor, AtMYB12 (At2g47460), resulting in increased *AtFLS1* expression and flavonol accumulation.

Identification of potential transcription factor regulators of the *VvFLS1* promoter, was achieved by two techniques. The first involved BLAST sequence search analysis in a grapevine expression (EST) database with AtMYB12. Six potential transcription factors were identified, although none of these appeared to be the direct grapevine homolog of AtMYB12. The second technique involved using DNA microarray technology to identify candidate transcription factors that were up-regulated in Chardonnay cell suspension cultures exposed to light for 24 hr compared to dark grown controls. This microarray analysis identified an additional seven potential transcription factors. Primers were made to all thirteen potential transcription factor candidates were tested by RT-PCR to determine the pattern of expression in the light opposed to dark and during grape berry development. While most candidates showed a similar expression pattern to *VvFLS1* in the light, only two candidates were selected for further isolation and characterisation. Unfortunately, these were not functionally tested with the *VvFLS1* promoter due to time constraints.

To functionally test the *VvFLS1* promoter(s), a transient assay was developed in Chardonnay suspension cells. Cells were bombarded with constructs containing potential transcription factors and the *VvFLS1* promoter(s), fused to a luciferase reporter vector. After 48hrs incubation in the dark, cells were harvested and luciferase activity measured as an indicator of *VvFLS1* promoter activity. Of the different transcription factors tested with the *VvFLS1* promoter(s) including VvMYBPA (tannin regulator), VvMYBA (anthocyanin regulator), the highest luciferase activity was observed using AtMYB12. While this result shows activation of the *VvFLS1* promoter by AtMYB12, further functional analysis with other transcription factors is required, for more complete understanding of the *VvFLS1* promoter and its transcriptional control.

6.5 Relevance of current findings to the Australian grape & wine industry

The results presented in the first part of this investigation are directly relevant to the future of the Australian grape and wine industry. Winery payment structures to grapegrowers are increasingly being linked to fruit quality specifications, such as grape colour (Barnett 2004; Bevin 2005; Swinburn 2003). In this investigation, no 'new' indicators of grape and wine quality were identified, such as flavonols or tannins, however the results clearly showed discrepancies in using grape colour to stream fruit.

What the industry requires is a quality measure that is objective (i.e. fruit composition), consistent (i.e. season to season) and universal (i.e. transferable across regions). Consideration must be given in regards to the size of the winery (and the tonnes of fruit arriving at the weighbridge at harvest) and the targeted wine styles, and it needs to be relatively rapid and inexpensive. All regions will require skilled representatives to take measurements and if possible the winemaker(s) should inspect all vineyards. It is important to note that the objective quality measure is unlikely to be a single constituent, and it is suggested a staged approach is required, that considers different aspects of 'quality', for example 1st stage (TSS, pH, TA), 2nd stage (colour, tannins), 3rd stage (flavour and aroma compounds). It is clear that objective measures of fruit and wine quality will remain an important area for the wine industry.

The second part of this investigation showed the light responsiveness of flavonol biosynthesis during grape berry development. While use of light-proof boxes is not expected in a commercial setting, the results presented suggest that the timing of current practices that expose fruit to sunlight may be of importance in achieving higher levels of flavonols. Practices that are currently used in the industry to increase light interception to the bunch, include lifting wires, leaf plucking or shoot thinning (Smart and Robinson 1991).

Flavonols appear to be a good indicator of bunch exposure to light (Cortell and Kennedy 2006; Downey et al. 2004; Haselgrove et al. 2000; Price et al. 1995; Spayd et al. 2002). Although they are not recognised as contributing to flavour of wines, they may be an indicator of other light dependent compounds such as wine flavour and aroma compounds, including the C_{13}-norisoprenoids and

carotenoids (Bureau et al. 2000; Gerdes et al. 2000; Hashizume and Samuta 1999; Marias et al. 1992; Razungles et al. 1998). Recently, it was highlighted by Hardy Wine Company (HWC) that they are still looking for quantitative measures for bunch light exposure, with the idea that this factor closely associates with grape and wine quality (Sas 2007).

This anecdotal observation was recently confirmed, after the influence of shading on wine quality was determined (Ristic et al. 2007). In wines, it was found that shaded fruit had a lower wine colour density, total phenolics, anthocyanins and tannins compared to exposed fruit. Analysis of potential flavour compounds indicated that wine made from shaded fruit had decreased levels of the C_{13}-norisoprenoids, ß-damascenone and 1,2,6-trimethyl-1,2-dihydronaphthalene (TDN). Sensory analysis indicated that the wines made from shaded fruit were lower in astringency, fruit flavour and flavour persistence. These results suggest that a high degree of shading results in unfavourable grape composition and wine properties (Ristic et al. 2007).

The third part of this investigation was to isolate transcriptional regulator(s) of the flavonol branch pathway and although it would appear that this type of research has no obvious and immediate relevance to the Australian grape and wine industry, this type of research is performed with consideration for long-term goals. Flavonoids have important health benefits (Baldi et al. 1995; Hertog and Hollman 1996; Lairon and Amiot 1999; McDonald et al. 1998; Plumb et al. 1998; Sato et al. 1996), therefore it makes these compounds an attractive target for genetic engineering strategies aimed at producing products with increased nutritional value. Transgenic approaches to increase flavonoid production may include manipulation of structural genes and/or manipulation of regulatory genes, of which examples exist in other crop species such as tomatoes, potatoes and kiwifruit (reviewed in Dixon and Steele (1999), Dixon et al. (2005), Schijlen et al. (2004) and Schwinn and Davies (2004)). Presently, there is still much to be learned about the regulation of the flavonoid biosynthetic pathway in plants and in grapevine, however outcomes from this research may eventually be used to improve the flavonoid composition of grape berries through genetic manipulation technologies.

6.6 Suggestions for future research

A number of key issues have arisen from the results of the current investigation and are potential areas for future research.

6.6.1 The influence of temperature, independent of light, on the flavonoid content & composition of grapes

Temperature and light are closely intertwined climatic factors that can influence overall vine growth and berry composition (Downey et al. 2006). Historically, it has been difficult to separate the effects of light and temperature, however recently with the development of light excluding boxes (Downey et al. 2004), the influence of light exposure on grape bunches, independent of changes in temperature or vine structure, has been demonstrated (Cortell and Kennedy 2006; Downey et al. 2006; Ristic et al. 2007; **Chapter 4**).

Presently, there are limited reports on the effect of temperature, independent of light on the synthesis of flavonoid compounds. Generally, the extant literature suggests that temperature has a greater influence on anthocyanin biosynthesis than light, with reduced anthocyanin content in hot climates and changes in anthocyanin composition with high temperatures (Downey et al. 2006). This phenomenon was observed in this investigation with changes in anthocyanin content and composition in the warm region compared to the cool (**Chapter 3**). However, it remains unknown whether this change in anthocyanin content and composition is due to anthocyanin degradation and/or inhibition of anthocyanin biosynthesis whereby higher temperatures may cause changes in enzyme kinetics or gene expression (Downey et al. 2006; Mori et al. 2007; Mori et al. 2005; Spayd et al. 2002; Yamane et al. 2006). Furthermore, there is very little information regarding the impact of temperature on flavonol or tannin biosynthesis, and as such, further investigation into the effect of temperature independent of light on flavonoid content and composition is warranted.

Recently at the 13th Australian Wine Industry Conference, a poster by Soar et al. (2007) was presented which investigated the use of three different experimental systems to modify grapevine canopy and/or bunch temperature. The different heating strategies included a whole vine heating chamber, under-vine tents and grape bunch blowers based on the concept by Tarara et al. (2000). While the impact on fruit and vine phenology and composition was not determined, the use of these experimental systems to achieve changes in bunch temperature (alone) appears a positive approach to unravelling the effect of temperature on flavonoid biosynthesis.

6.6.2 Post-transcriptional regulation of the flavonoid compounds in grapes

While the transcriptional control of the flavonoid biosynthetic genes remains an international research area of current focus, post-transcriptional regulation of the pathway has received much less attention. As part of a side experiment, it was highlighted that anthocyanins and tannins, but not flavonols were localised in anthocyanic vacuolar inclusion (AVI) bodies isolated from grape cell culture (**Chapter 3**). This is of some significance, as the role of anthocyanins, flavonols or tannins acting as co-pigments in grape berry cells has not been firmly established. It would be of interest to explore this observation in more detail and determine if there is a selective interaction between anthocyanin and tannin compounds in AVIs. The localisation of flavonols also needs to be determined. Once the relationship between the flavonoids and AVIs have been established, the information must then be transferred to grape cells (outside of culture), as it is likely there will be additional factors that could influence the levels of flavonoid compounds in grape berries such as skin thickness, cell size or cell wall properties.

There is little information regarding the glycosylation of flavonols in grapes. Flavonols in grape berry tissues are detected in the glycosylated form and as such it has been proposed that there must be a glycosylation enzyme responsible for flavonol glycosylation (i.e. a flavonol specific glycosyl-transferase (FGT)). A FGT has been identified in *Petunia,* (Miller et al. 2002; Miller et al. 1999; Vogt and Taylor 1995) and while the *Arabidopsis thaliana* genome sequencing investigation has putatively identified further FGTs, no sequence exists for any flavonol specific glycosyl-transferase in grapevine. Although the enzyme UFGT can glycosylate flavonols (Ford et al. 1998a), *VvUFGT* is not expressed when flavonols are made around flowering so there must be a separate FGT in grapevines.

Also, while the pattern of tannin synthesis and accumulation in Shiraz seeds and skins has been clearly established (Bogs et al. 2005; Downey et al. 2003a), a number of areas still remain to be elucidated. These include the post-veraison decline in tannins, widely held to represent a decrease in extractability, due to binding with other cellular components (Amrani-Joutei et al. 1994; Cheynier et al. 1997; Downey et al. 2003a; Fournand et al. 2006; Gagne et al. 2006; Geny et al. 2003; Hazak et al. 2005; Kennedy et al. 2000a). Furthermore, the biochemistry behind tannin polymer formation remains unclear. Little is known about the processes of polymerisation in condensed tannins and there is debate whether the process of polymer formation is caused by chemical reactions or enzymatic processes (Bakker and Timberlake 1997; Downey 2002; Mateus et al. 2002; Schwinn and Davies 2004; Tanner 2004; Vidal et al. 2002; Xie and Dixon 2005).

Overall, it remains unclear how transcriptional control of flavonoid biosynthesis translates into control on a metabolic level and further research is required. Much of the enzymology of flavonoid metabolism still remains to be thoroughly investigated including enzyme structure, function and regulation. A better understanding of the mechanism(s) by which flavonoids are modified, how the transport of these compounds occurs, and how their sequestration in vacuoles influences co-pigmentation associations, may provide a opportunity to better manipulate the type and amount of flavonoids extracted from cells during winemaking (see **Section 6.6.4**).

6.6.3 Improve vineyard sampling techniques & methods used to analyze grape composition

In this investigation, the lack of strong correlations between grape composition, vineyard attributes and grape and wine quality was discussed in relation to the fact that 'quality' is largely subjective and that vineyards are highly variable (**Chapter 3**). While subjectivity is unlikely to ever be removed from the allocation of wine quality, understanding vineyard variability and how to manage and sample fruit together with quantitative chemical analyses will provide the means for grapegrowers and winemakers to improve grape and wine quality.

Vineyard variability is a continuing challenge for the Australian grape and wine industry. Currently, many commercial vineyards use 'Precision Viticulture' a technology that aims to reduce vineyard variability. Precision Viticulture uses technologies such as Global Positioning Systems (GPS), remote sensing, and Geographical Information Systems (GIS) to link on site measurements (physical, chemical and biological) to specific locations in the vineyard (Bramley and Hamilton 2004; Hall et al. 2002). Spatial variations in topography, climatic conditions, physical and chemical characteristics of the soil and pests and diseases have been associated with variations in yield and fruit ripeness (Bramley and Hamilton 2004; Hall et al. 2002). Furthermore, the use of Precision Viticulture has shown relationships between the total phenolic content of grapes (i.e. colour and tannin content) and vine vigour (Cortell et al. 2005; Lamb et al. 2004). More research aimed at achieving a better understanding of vineyard variability is likely to assist grapegrower and winemakers in managing such variability via selective sampling and harvesting thereby producing better quality grapes and wines. However, consideration needs to be given regarding the time taken to scan and divide blocks according to different specifications. Currently, limitations with Precision Viticulture lie with a lack of rapid, simple and inexpensive analytical methods to analyse fruit (Cortell et al. 2005).

The next area of future research includes development of rapid measures of grape compounds particularly those, which could be used as indicators of 'quality'. In the last decade, total grape colour measurements have been routinely measured by the Australian wine industry by measuring the absorbance of grape extracts at 520 nm (Iland et al. 2000). The absorbance at 280 nm also provided a measure of 'total phenolics', which was commonly used as an indicator of tannin content, although it also included anthocyanins, flavonols or other simple phenolics.

An accurate, inexpensive and rapid measure of tannins in grapes and wine has long been sought by the grape and wine industry (Herderich and Smith 2005). Therefore, the development of spectrophotometric measurements of precipitatable tannins has evolved, using BSA or methyl-cellulose as the precipitating compound (Harbertson et al. 2002; Sarneckis et al. 2006). Recently these methods have been scaled down to micro-well formats for rapid analysis (Heredia et al. 2006; Mercurio et al. 2007a). Measuring grape and wine tannins may help a winemaker make decisions on when to press and what to blend. However this research must be combined with knowledge on the extraction of these compounds from different parts of the berry and what this means for wine colour intensity, stability and quality.

Many Australian wine companies, including HWC, have now adopted the use of Near-Infrared Spectroscopy (NIR) technology (Dambergs et al. 2003; Sas and Lim 2003). NIR techniques are based on a calibration developed between the intensity of absorbance values in the NIR spectrum and analytical results determined by standard ('reference') laboratory methods (Gishen and Dambergs 1998; Gishen et al. 2001). NIR is used to measure sugar/alcohol, acids, moisture content and more recently, colour (Bevin 2005). In the past, NIR calibrations were difficult to establish, but with a large amount of data now collected from different regions/seasons/cultivars, it has proven its usefulness as a rapid, non-destructive analysis. A good correlation between NIR and HPLC grape colour measurements was observed in this investigation, indicating the effectiveness of this method to predict grape anthocyanin concentration.

Recently, it was highlighted that NIR technology is being used to measure tannin content in grapes and wine (Cozzolino et al. 2004; Dambergs 2007; Skogerson et al. 2007). However these measurements are still largely considered provisional, as calibration points from different climatic regions are significantly variable and thus cannot be used for all grape samples. It would also be of interest to see if flavonols, or some of the pre-cursors for flavour and aroma compounds could also

be measured by NIR in grapes and wine, especially as some of these compounds are influenced by bunch light exposure, a climatic factor which HWC representatives have associated with grape and wine quality (Sas 2007).

6.6.4 Better understanding of the transfer of flavonoid compounds from fruit to wine during fermentation

In this investigation, it was found that grape colour did not invariably correlate with wine colour, the reasoning that wine is a very complex medium and/or there may have been different winemaking techniques applied to different batches of grapes (**Chapter 3**). This lack of correlation has been previously reported for anthocyanins (Francis et al. 2004; Mazza and Miniati 1993; Somers 1986; Somers and Evans 1977) but also recently for tannins (Adams 2007; Harbertson et al. 2002; Hazak et al. 2005). There are numerous studies that report the flavonoid content and composition in grapes or in finished wines particularly in relation to different fermentation practices (reviewed in Kennedy et al. (2006a) and Sacchi et al. (2005)). However, there are limited studies that have actually measured flavonoid composition during a 'typical' fermentation with a focus on the extraction from the different parts of the berry (i.e. seed and skin) and the stability of the different flavonoid classes. It could be that extraction of the flavonoids is influenced by skin thickness, cell size or cell wall properties whereby flavonoids may be binding to the insoluble matrix of the grape berry. This would influence the extraction of flavonoids from fruit during winemaking and hence translation into wines.

To explore the interactions between the flavonoid compounds extracted from different parts of the berry during fermentation, small-scale ferments would be considered necessary. Recently, Holt (2007) indicated use of 100 berry ferments of differing combinations of skin, seed and flesh to look at the extraction of the flavonoid compounds from these tissue types. The flavonoid composition of these berry ferments and corresponding wines have not yet been established (Holt 2007). Future experiments may include up-scaling the fermentation to enable translation into a commercial winemaking environment and determine the influence it may have on wine quality and sensory attributes. However, it should be noted methods to sample the ferment should be considered, particularly as it is extremely difficult to get a 'representative' sample of skin, seed and juice/wine from an active ferment. These sets of future experiments are likely to be a challenge.

6.6.5 Closer examination of the activity of the *VvF3'H* & *VvF3'5'H* genes in relation to flavonoid composition & in response to different environmental cues

Most of the known phenylpropanoid pathway genes encoding the flavonoid biosynthetic enzymes have been cloned in grapevine and their expression determined during berry development in red and white grapes (Bogs et al. 2005; Bogs et al. 2006; Boss et al. 1996a; Boss et al. 1996b; Boss et al. 1996c; Downey et al. 2003b; Ford et al. 1998a; Robinson and Davies 2000; Sparvoli et al. 1994). The accumulation of flavonoid end-products (anthocyanins, flavonols and tannins) has also been determined (Boss et al. 1996a; Boss et al. 1996b; Boss et al. 1996c; Downey et al. 2003a; Downey et al. 2003b; Kennedy et al. 2001; Kennedy et al. 2000a; Kennedy et al. 2000b).

In this investigation, the composition of anthocyanins and flavonols was shown to change in response to shading and during development, respectively (**Chapter 4**). These changes were associated with the activity of the flavonoid 3'-hyrdroxylase (*VvF3'H*) and flavonoid 3',5'-hydroxylase (*VvF3'5'H*) genes. The *VvF3'H* and *VvF3'5'H* genes were recently identified in grapevine and their expression pattern determined during Shiraz and Chardonnay grape berry development (Bogs et al. 2006) however, little is known about their response to different environmental conditions such as temperature or light exposure. It could be that the gene encoding *VvF3'5'H* is light sensitive and expression is down-regulated in low light conditions. This change in activity may also be dependent on the types flavonoid being accumulated.

Given the possibility of down-regulation of *VvF3'5'H* in the shade, the next immediate obvious experiments would be to determine if the reverse trend was observed under high light conditions (Downey 2002). The Chardonnay cell culture exposed to light (**Chapter 5**) would be a potential test-system for this purpose. Also the heating systems described by Soar et al. (2007) could be of some use to determine whether temperature influenced *VvF3'5'H* and *VvF3'H* gene expression. With these gene sequences now cloned in grapevine (Bogs et al. 2006), these experiments could be conducted in the immediate future.

6.6.6 The influence of light quantity & quality on flavonol biosynthesis

As shown in a variety of different vine, bunch and cell experiments, flavonol biosynthesis in grape tissues is highly light responsive (Downey et al. 2004; Haselgrove et al. 2000; Pereira et al. 2006; Price et al. 1995; Spayd et al. 2002) (**Chapter 4** & **Chapter 5**). The idea that bunch light exposure

is an indicator of grape and wine quality is widely regarded in the industry (Sas 2007), however it is likely that there is some intermediate level of fruit light exposure to produce optimal 'quality', with over-exposed or densely shading giving fruit negative quality characteristics. It remains unclear whether high-light conditions (i.e. above 'control' level) would increase flavonol biosynthesis in different tissues. It would also be of interest to determine the influence of light quality, which could be achieved by excluding different wavelengths (Jordan 2004). In petunia leaves, flavonol content and composition has been shown to change in response to different levels of UV-B light (low, ambient and high)(Ryan et al. 2002).

With the development of the robust light inducible test system, derived from Chardonnay cell cultures, the exclusion of different light types is a readily achievable experiment that could be performed. Furthermore, considerable effort has been recently directed toward the improvement of anthocyanin and/or tannin biosynthesis derived from grape cell cultures, because of the apparent health benefits (Zhang and Furusaki 1999). Accordingly, the light inducible Chardonnay cells used in this investigation (**Chapter 5**) may have potential to be modified to allow rapid outputs of flavonols. It may also be a good system to look at the synthesis of other flavour & aroma pathways in response to light.

6.6.7 Isolation of the transcriptional regulator of flavonol biosynthesis

While the results of this investigation made progress in isolating the transcriptional regulator of flavonol biosynthesis in grapevine, attempts to isolate grapevine regulators and functionally test them with the *VvFLS1* promoter were unsuccessful (**Chapter 5**). However, there are immediate experiments that can be performed to continue this work in unravelling the molecular mechanisms of flavonol biosynthesis. These include, isolation of additional *VvFLS1* promoter sequence and isolation of transcription factor candidate genes. Experiments isolating more *VvFLS1*promoter sequence should be made easier by the fact that more of the *VvFLS1* promoter sequence is now available (**Chapter 5**). It would also be of interest to functionally test the two candidate transcription factors identified in **Chapter 5** using the transient cell assay developed.

With limited knowledge of the number of transcription factors present in grapevine, combined with the fact that these genes are highly homologous and typically expressed at lower levels and at

different times, elucidation of the gene(s) responsible for the light induction of *VvFLS1* in grapevines is likely to be a challenge. However, questions that still remain include:

-Is there a specific regulator of VvFLS1?

-Is this regulator(s) controlled differently during development and in response to light exposure?

Mutant plants have been effective tools in isolating different regulators of the flavonoid biosynthetic pathway. There are a number of mutant lines defective in flavonoid biosynthesis identified in *Arabidopsis* on the basis of altered seed coat colour and are known as the *transparent testa* or *tt* mutants (Koornneef et al. 1991; Lepiniec et al. 2006; Shirley et al. 1995). Some of these mutants are deficient in anthocyanins and tannins, which has allowed the genes involved in regulation to be determined (Abrahams et al. 2002; Marinova et al. 2007; Ramsay et al. 2003; Shirley 1996; Shirley et al. 1995). It could be that in the future, the use of these types of mutant plants may play a role in helping to determining the regulation of the flavonoid pathway in grapevines and moreover could also be used to look at the processes involved in post-transcriptional modification. It is anticipated that with a greater understanding of the regulation of the flavonoid biosynthetic pathway that genetic manipulation technologies may be able to improve the types and amounts of flavonoids accumulated in plants.

6.7 Summary

This research contributes to our knowledge of flavonoid synthesis in grapes; how it is coordinated, the relationship with wine quality, and the influence of light particularly on synthesis of flavonols. It also explores the molecular mechanisms of *VvFLS1* control, through isolation of the *VvFLS1* promoter and identification of potential transcription factors, which may regulate it. However, many aspects of flavonoid biogenesis remain to be elucidated. While this research has closely examined the cultivars Shiraz and Chardonnay, the patterns observed here may not be representative of the whole *Vitis vinifera* species; therefore it may be necessary to expand these investigations to other cultivars. An understanding of the synthesis of flavonoids and how they may be coordinated, particularly in response to light, will allow viticulturalists and winemakers to optimise the levels of these quality enhancing factors in grapes and wine.

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