An Investigation of the Role of the Regulatory Gene *VvMYBA1* in Colour, Flavour and Aroma Metabolism Using Transgenic Grapevines

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ABSTRACT

Anthocyanins are flavonoid compounds responsible for most of the red, purple and blue colours of leaves, fruit and flowers of many plant species. They are produced through the anthocyanin biosynthesis pathway and in grapevine the VvMYBA1 and VvMYBA2 transcription factors are responsible for the transcriptional activation of genes encoding enzymes required for their synthesis. White grapevine cultivars contain inactive versions of the *VvMYBA1* and *VvMYBA2* genes and hence cannot produce anthocyanins in berries. While much is now known about anthocyanin biosynthesis in grapevine, there are still some genes involved in anthocyanin modification and transport which have not yet been identified. In several other plant species recent research has established a link between anthocyanin biosynthesis and the synthesis of volatile aroma compounds.

In this research project, the aim was to further characterise VvMYBA and its role in anthocyanin and flavour metabolism. To do this, transgenic and natural mutant grapevines in which berry colour has been altered due to differential expression of *VvMYBA* genes were used. Two different approaches were taken to investigate the effect of *VvMYBA* gene expression on the transcriptome and flavour metabolism in berries, with the aim of linking transcriptomic changes to metabolomic changes. Microarray analysis was performed to identify differences in global transcription levels in berries differing in their *VvMYBA* gene expression. Microscale wines were also made from both whole berries and free run juice and volatile wine flavour/aroma compounds were analysed using HS-SPME-GC/MS.

This research has shown that the presence of VvMYBA in berries does have an effect on the abundance of volatile flavour/aroma compounds in wines; however this was often in a cultivar specific manner. One conserved difference was that red wines, made from berries expressing *VvMYBA*, contained less linalool compared to white wines, made from berries not expressing *VvMYBA*. Light exclusion studies and transcript analysis of genes associated with linalool metabolism have suggested that the accumulation of anthocyanins in red grapes may cause a shading effect which down-regulates linalool synthesis.

From microarray studies, two putative acyltransferase genes were identified, one belonging to the BAHD protein family and the other to the serine carboxypeptidase-like (SCPL) family. At the commencement of this study, no anthocyanin acyltransferases had been identified in grapevine and it was hypothesised that one or both of these genes could have this function.

Acylation of anthocyanins has been shown to change the hue of the pigment in the fruit and flowers of various plant species, and to increase their stability in products such as wine. Gene expression studies, bioinformatics analyses and *in vitro* and *in planta* functional assays were used to characterised these two genes. Through these studies the first *Vitis vinifera* anthocyanin acyltransferase gene (*VvAnAT*) was identified. VvAnAT belongs to the BAHD acyltransferase protein family and recombinant enzyme kinetic studies show that it can utilise a range of CoA thioester acyl donors and shows a preference towards monoglucoside anthocyanins as the acyl acceptor substrate. Using promoter activation assays the ability of the VvMYBA1 transcription factor to activate the transcription of the *VvAnAT* gene was shown. The putative SCPL gene did not function as an anthocyanin acyltransferase in *in planta* experiments; further studies are required to understand the function of this gene.

The outcomes of this PhD project have added to the current understanding of anthocyanin synthesis and its regulation in grapevine. Knowledge and identification of a grapevine anthocyanin acyltransferase gene can be used in breeding programs aiming to improve grapevine cultivars that cannot currently produce acylated anthocyanins, and hence increase their potential wine colour stability properties.

DECLARATION

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

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SIGNED DATE 12/6/14

Amy Rinaldo

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ABBREVIATIONS

Units

°C	degrees Celcius
aa	amino acid
bp/kb/Mb	base pairs/kilobase pairs/megabase pairs
g	relative centrifugal force
g; mg; µg	gram; milligram; microgram
h	hour
kPa	kilopascal
L; ml; µl	litre; millilitre; microlitre
M; mM; µM; nM	molar (moles per L); millimolar; micromolar; nanomolar
min	minute
s	second
TTS	total soluble solids
Vol	volume
w/v	weight per volume
wpf	weeks post flowering
wpv	weeks post veraison

Flavonoid pathway

anthoMATE	anthocyanin multidrug and toxic efflux transporter
4CL	4-coumaroyl CoA ligase
ANR	anthocyanidin reductase
bHLH	basic helix-loop-helix
CHI	chalcone isomerase
CHS	chalcone synthase
DFR	dihydroflavonol 4-reductase
F3'5'H	flavonoid 3',5'-hydroxylase
F3H	flavanone-3-hydroxylase
F3'H	flavonoid 3'-hydroxylase
FAOMT	flavanol and anthocyanidin-glucoside 3',5'-*O*-methyltransferase
FGT	flavonol glucosyltransferase
FLS	flavonol synthase
GST	glutathione-S-transferase
LAR	leucoanthocyanidin reductase
LDOX	leucoanthocyanidin dioxygenase
MYB	transcription factor family named after the first gene identified in the family *Myeloblast*
MYC	transcription factor family named after the first gene identified in the family *myelocytomatosis viral oncogene*
PA	Proanthocyanidin (condensed tannins)

Flavonoid pathway continued....

PAL	phenylalanine ammonia lyase
R2R3-MYB	class of MYB TFs containing a two-repeat R2R3 DNA binding domain
UFGT	UDP-glucose flavonoid 3-*O*-glucosyltransferase
WD40 TF	A class of transcription factors containing tandem repeats of a structural motif terminating in a tryptophan-aspartic acid (W-D) dipeptide
WDR	tryptophan-aspartic acid repeat protein
WRKY TF	A class of DNA binding transcription factors that contain a conserved WRKYGOK amino acid sequence

Methylerythritol (MEP) pathway and linalool synthesis

bOci	E-β-ocimene synthase
CDP-ME	4-(cytidine 5'-diphospho)-2-C-methyl-D-erythritol
CDP-MEP	2-phospho-4-(cytidine 5'-di-phospho)-2-C-methyl-D-erythritol
CMK	CDP-ME kinase
CMS	CDP-ME synthase
DMAPP	dimethylallyl diphosphate
DXP	1-deoxy-D-xylulose 5-phosphate
DXR	DXP reductoisomerase
DXS	DXP synthase
G3P	glyceraldehyde 3-phosphate
GPP	geranyl diphosphate
GPS	GPP synthase
HDS	HMBPP synthase
HMBPP	4-hydroxy-3-methylbut-2-enyl diphosphate
IDI	IPP isomerase
IDS	IPP/DMAPP synthase
IPP	isopentenyl prenyldiphosphate
MCS	ME-cPP synthase
ME-cPP	2-C-methyl-D-erythritol 2,4-cyclodiphosphate
MEP	methylerythritol

General

35S	35S constitutive promoter from the *Cauliflower Mosaic Virus*
A, C, G, T	adenine, cytosine, guanine, thymine
ABC transporter	ATP-binding cassette transporter
AMP	adenosine monophosphate
ANOVA	analysis of varience
ATP	adenosine triphosphate
BAHD	A gene and protein family named after the first letter of the first 4 characterised proteins BEAT, AHCT HCBT and DAT

General continued...

BLAST	basic local alignment search tool
cDNA	complementary DNA
cp	cycle threshold
CSIRO	Commonwealth Scientific and Industrial Research Organisation
DNA	deoxyribonucleic acid
ELIP	early light-inducible protein
EST	expressed tag sequence
ER	endoplasmic reticulum
FC	fold change
GC	gas chromatography
gDNA	genomic DNA
HPLC	high performance liquid chromatography
HS	headspace
LC	liquid chromatography
MS	mass spectrometry
NADH	nicotinamide adenine dinucleotide
NCBI	National Centre for Biotechnology Information
N-terminal	amino-terminal
PLACE	plant cis-acting regulatory DNA elements
qPCR	quantitative polymerase chain reaction
QTL	quantitative trait locus
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
SAM	significance analysis of microarray
SCP	serine carboxypeptidase
SCPL	serine carboxypeptidase-like
SMT	1-*O*-β-sinapoylglucose:L-malate sinapoyltransferase
SPME	solid phase microextraction
TF	transcription factor
UTR	untranslated region
UV	ultra violetlight

Chapter 1: Introduction

1.1 Introduction

Wine grapes have been used to produce wine for many millennia. There are thousands of different grapevine cultivars, which are used to produce a vast array of different wine styles. Red and white wines, made from red and white grapes respectively, have very distinctive flavour and aroma profiles. While this could be partially attributed to the different methods used to produce these wines, it may also be a result of distinguishing metabolites in the red and white grapes, including flavour and aroma precursors or compounds such as flavonoids. Flavonoids are a group of compounds which have been linked to important grape and wine attributes including colour, mouthfeel and overall quality. The red pigment found in the skins of red grapes is due to the presence of a class of flavonoids known as anthocyanins. The biosynthesis of these, and other flavonoid compounds, has been extensively studied and the regulation of anthocyanin synthesis is well understood. There are, however, still gaps in our knowledge of anthocyanin synthesis in grapevine, as some genes involved in modification and transport of anthocyanins have not yet been identified. In other flowering plant species, including petunia (*Petunia x hybrida*) and carnations (*Dianthus caryophyllus* L.), a link between anthocyanin synthesis and other volatile metabolite pathways has been inferred. If such a link existed in grape berries then these altered volatiles may have an effect on the final flavour and aroma of wine. Having a greater understanding of the metabolism of colour and flavour in grapes, and how these two important quality traits may be linked, could provide valuable information to the grape and wine industry.

1.2 The Grapevine

Grapevines are classified as members of the genus *Vitis*, within the family Vitaceae. The fruit of grapevine (grapes) are non-climatic, meaning that their ethylene production is low and fruit ripening does not seem to be controlled through ethylene signalling (Tira-Umphon et al., 2007). There are over 7000 grapevines cultivars and almost all of those used to produce wine belong to the species *Vitis vinifera* which are composed of tall woody vines with flaky bark that can grow up to 35 m tall. These domesticated grapevines are grown in a clonal manner as rooted cuttings or grown on non-vinifera rootstocks (Jackson, 2000). Grapevines are diploid organisms and the genome from the heterozygous red berried variety Pinot Noir as well as

and a near homozygous Pinot Noir derived inbred line (PN40024) has been sequenced (Jaillon et al., 2007; Adam-Blondon et al., 2011).

1.3 Grape and wine flavour

The fermentation of grape juice by yeast, to produce wine, is a natural process which has been harnessed by humans for thousands of years. During this process, yeast converts sugar into energy in the absence of oxygen, and alcohols, carbon dioxide, esters and acids are formed as by-products (Nykanen, 1986). The result is a beverage (wine) which has flavour and aroma complexity and an alcohol content that exerts physiological and psychological effects perceived to be pleasant by humans. For this reason, wine has been much enjoyed by humans for many millennia. Archaeological records show evidence of wine residues from over 7500 years ago (McGovern et al., 1996). The first evidence of intentional wine making came in the form of wine presses found in Egypt dating from some 5000 years ago (Petrie, 1923).

Continual refinement of the wine making process is ongoing and research on all aspects involved is necessary to continue to deliver an exceptional product in a very competitive market. The Australian Bureau of Statistics (ABS) calculated that in the 2011-12 financial year the Australian wine and grape industry contributed approximately $450 million in domestic sales and $720 million in export sales to the Australian economy (ABS, 2013). In 2009 Australia was ranked as possessing the 4th highest value share (7.1%) of world wine exports that year, outperformed by only France, Italy, and Spain (Anderson and Nelgen, 2011). While these statistics show it is clear that Australian wines are enjoyed by international markets today, it is important that our wine continues to compete on the global stage so that the industry can maintain its large input into the Australian economy. New and innovative contributions to both the technology used in wine making and our understanding of the components of wine will surely aid in maintaining this international reputation.

1.3.1 Grape contribution to wine flavour

The flavour and aroma components of wine can originate from a number of sources including the grapes, the yeast strain, and post-fermentation treatments. The distinctive varietal flavour and aroma differences in wine generally arise from compounds found in the berries. Varietal wine flavours are a result of genetic differences between different grapevine cultivars

(reviewed in Roubelakis-Angelakis et al., 2009). Both 'neutral' (common to all varieties) and 'impact' varietal compounds can be present in the berry in their free form, or as flavourless non-volatile compounds bound to sugars or other molecules. During the process of fermentation, yeast enzymes cleave the sugar molecule from the glyco-conjugate releasing the volatile form which can then affect the flavour/aroma profile of the finished wine product (reviewed in Francis and Newton, 2005). Many grape-derived impact volatiles have now been identified, for example Muscat wines can be distinguished by their high levels of linalool and *cis*-rose oxides which give rise to their 'floral' characters (Berger, 2007). In contrast, both Sauvignon Blanc and Cabernet Sauvignon cultivars are often described to possess fresh 'green' aromas which are present in the form of methoxypyrazine derivatives (Berger, 2007). Sauvignon Blanc is a parent of Cabernet Sauvignon cultivar (which is heterozygous for colour), demonstrating how a genetic link between these grapes has resulted in a similarity in the flavour of their wine (Bowers and Meredith, 1997).

1.3.2 Red and white wine flavour: what is the difference?

Table wine can be categorised into three wine styles: red, white and rosé. An obvious difference between these three wine categories is their colour. Red and rosé wines contain anthocyanins, which are red pigments originating from the skin of red berries used to make the wine. A second difference between red, rosé and white wines is their level of astringency. Higher astringency in red wines and to a lesser extent in rosés, results in a more textured mouth-feel compared to whites and this is due to their increased levels of proanthocyanidins (PAs, otherwise known as condensed tannins). Both the anthocyanins and PAs in these two wine styles arise from the winemaking processes used to produce them, which is different from the 'white' winemaking style. In all cases the berries are first crushed. For white wine the crushed matter is then gently pressed and the juice separated from the pomace (skin, flesh, seeds etc.) before fermentation. During red wine making, fermentation is carried out directly on the crushed grape matter i.e. in the presence of the skin and seeds, resulting in an array of compounds found within these tissues being extracted into the wine, including anthocyanins and PAs. Rosé wines are made from red berries, but fermentation in the presence of the skin and seeds is only carried out for a short time. As a result fewer anthocyanins and PAs are extracted and are therefore present at lower levels in the finished wine (reviewed in Jackson, 2000). The result is a style of wine which possesses attributes of both red and white wine. For

all wine styles PAs can also be present due to post-fermentation treatments such as the addition of oak (Waterhouse, 2002).

Colour and astringency are not the only distinguishing factors differentiating the three table wine categories; there is an abundance of sensory data which separates these wines through their aroma. A study was conducted where participants were presented with 18 wines in dark glasses to conceal their colour, to assess if they could correctly categorise the wines by their odour. The sample set was composed of 6 white, 6 rosé and 6 red wines, and the results clearly showed that the participants were able to easily categorise the white and red wines; however it was more difficult to accurately place the rosé samples (Ballester et al., 2009). When participants were then asked to give descriptors for each of the wine odours, it was found that the white wines were commonly categorised by yellow and orange fruity notes such as pineapple, citrus, and apricot/peach. The red wines were described as containing red berry notes in their aroma such as strawberry and blackberry. Another study by Escudero et al. (2007) aimed to characterise the aroma of different premium red wines using both sensory and analytical data. They were able to identify 9 volatile ester compounds that were responsible for the red berry notes in these wines using gas chromatography – olfactometry (GC-O). They also identified several norisoprenoids (β-damascenone and β-ionone) and dimethyl sulphide compounds which did not directly give fruity odours when analysed separately, but when present in the wine increased the intensity of these odours from other volatile compounds present. These studies bring up the question of where do these impact volatiles separating red and white wines arise from? Are they simply a result of skin contact in red wine making or are there other contributing factors?

1.3.3 Factors contributing to red and white wine flavour differences

There is currently much evidence to suggest that the presence of anthocyanins and PAs in red wine have a significant impact on wine aroma. In the past there have been studies where white wines has been made in the style of reds, using fermentation on skins and seeds, and the finished product has had no resemblance to a red wine (Singleton et al., 1975). It is now understood that the textured mouth-feel associated with red wines is due to the presence of both PAs and anthocyanins, which form pigmented polymers required to retain the PAs in solution and prevent anthocyanins from oxidising to a brown colour (Singleton and Trousdale, 1992).

More recently, it has come to light that differences in the non-volatile matrices (e.g. carbohydrates, proteins and polyphenols) of red and white wine also have an effect on the release and retention of certain volatiles affecting the overall wine flavour and aroma. A significant example of this was shown by Saenz-Navajas and colleagues (2010) who extracted and separated the non-volatile matrix from the volatile mixtures of red and white wine. They exposed each volatile mixture to the two different non-volatile matrices and found that this resulted in different volatile interactions, giving rise to altered sensory attributes. Whether this phenomenon can account for all of the distinguishing factors of red and white wine is unknown. Furthermore it is widely acknowledged that varietal differences in wine are a result of the genetic variation of those cultivars as discussed previously in section 1.3.1. Much is now known about the genetic difference between red and white-berried grapevines but the question of how these differences may affect wine flavour has not yet been investigated.

1.3.4 Is there a link between berry colour and wine flavour/aroma?

One question that has not yet been investigated is whether some of the flavour and aroma differences between red and white wine might be due to the flavour compounds present in their berries. It is commonly acknowledged that varietal differences of wine are due to the genetic variations of their grape cultivars. It therefore seems logical that the distinctive flavours carried through all red wine varieties, such as the red berry flavours not common in white wine, may originate from precursors found only in red grapes. Recent studies in other plant species have been aimed at investigating a possible link in the regulation of colour and scent production in flowering plants. It has been hypothesized that it would be advantageous for a plant to coordinate the synthesis of colour and scent in parallel, as these traits are both known to attract pollinators and seed dispersers (reviewed in Majetic et al., 2010). Salzmann and Schiestl (2007) analysed the volatile release from red and yellow colour morphs of the orchid species *Dactylorhiza romana* and found that higher amounts of benzaldehyde were emitted from the yellow morphs, while red morphs released greater amounts of linalool. Several research groups have altered the colour of flowers through genetic modification of the anthocyanin biosynthesis pathway and as a consequence also altered the release of volatile aroma compounds (Zuker et al., 2002; Zvi et al., 2008; Colquhoun et al., 2011; Zvi et al., 2012). These studies have provided evidence that the regulation of pigment pathways, and in particular anthocyanin biosynthesis, may, in some plant species, also be linked to the regulation of volatile and other flavour compound pathways. If such a phenomenon occurred

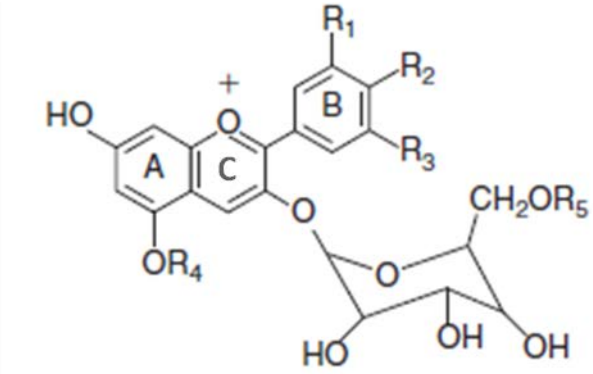
in the berries of grapevine, this could contribute to the differences in flavour and aroma of red and white wine, a concept which has formed the primary hypothesis of this PhD project.

1.4 Anthocyanins: their synthesis and regulation

1.4.1 Anthocyanins

Anthocyanins, a group of water-soluble flavonoid compounds, are produced by almost all vascular plants and have been shown to have a diverse range of biological functions. They are major contributors to the orange, red, purple and blue colours seen in the leaves, fruit and flowers of many plant species and hence have important roles in attracting pollinators and seed dispersers (Schaefer et al., 2004). It has been suggested that they also act as protection agents against UV (Markham, 1988) and are involved in plant stress responses (Dixon and Paiva, 1995; Treutter, 2006). Anthocyanins have potent antioxidant capacity, which can explain their numerous health-promoting properties including cardiovascular disease prevention, anti-inflammatory, antimicrobial and anti-carcinogenic activities (He and Giusti, 2010).

Anthocyanins are glycosylated anthocyanidins which contain a flavylium three ring C6-C3-C6 structure (Figure 1.1). They have a heterocyclic benzopyran ring (as the C ring), one fused aromatic ring (as the A ring) and one phenyl constituent (as the B ring) (Mazza and Francis, 1995). The hydroxylation and methylation patterns of the B ring of anthocyanins greatly affect the hue and colour stability of these pigments, as does the number and types of sugar and acyl moieties that are attached (He et al., 2010). Red wine grapes (sp. *Vitis vinifera*) contain both 3-*O*-monoglucoside and 3-*O*-acyl monoglucoside anthocyanins which are derived from 5 main anthocyanidin aglycones: delphinidin, cyanidin, peonidin, petunidin and malvidin. The proportions and amounts of these different types of anthocyanins give rise to the huge range of different rose, red, purple and black skinned berry phenotypes. Over the years a large amount of research has been undertaken to understand how anthocyanins are synthesised in plants, including grapevine, and this is now well understood (reviewed in Tanaka et al., 2008).



NAME	R1	R2	R3
Cyanin	OH	OH	H
Peonin	OCH_3	OH	H
Delphinin	OH	OH	OH
Petunin	OCH_3	OH	OH
Malvin	OCH_3	OH	OCH_3

DERIVATIVES
R4 – H: monoglucoside; glucose: diglucoside
R5 – acetyl, *p*-coumaroyl, caffeoyl

Figure 1.1 - General anthocyanin structure found in grapevine.

Shows the three ring flavylium anthocyanidin conjugated to a sugar molecule. They contain a heterocyclic benzopyran ring (C), a fused aromatic ring (A) and a phenyl constituent (B). Hydroxylation and methylation patterns are different at the R1, R2 and R3 position of the B ring for different anthocyanins, which can also be mono- or di-glycosylated. The R5 position on the glucose molecule can be acylated by an acetyl, p-coumaroyl or caffeoyl group. (after Jackson, 2000)

1.4.2 The anthocyanin biosynthesis pathway

The anthocyanin biosynthesis pathway has been well characterised in many plant species; maize, petunia and snapdragon being some of the earliest (Dooner et al., 1991; Gerats and Martin, 1992; Martin and Gerats, 1993; Holton and Cornish, 1995). Anthocyanins are produced through the flavonoid biosynthetic pathway (Figure 1.2) which is also responsible for the co-ordinated production of flavonols and PAs (Stafford, 1990). Phenylalanine is the precursor of the flavonoid pathway and is converted to its derivative 4-coumaroyl-CoA in a three step process catalysed by the enzymes phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumaroyl CoA ligase (4CL). Chalcone synthase (CHS) is the first committed enzyme within the flavonoid pathway, catalysing the conversion of 4-coumaroyl-CoA to naringenin chalcone. To produce anthocyanins this conversion is then followed by a series of enzymatic steps, each catalysed by a separate enzyme (see Figure 1.2, Anderson and Jordheim, 2006). These enzymes are chalcone isomerase (CHI), flavanone-3-hydroxylase (F3H), flavonoid 3'-hydroxylase (F3'H), flavonoid 3',5'-hydroxylase (F3'5'H), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) [sometimes referred to as anthocyanin synthase (ANS)], and UDP-glucose flavonoid 3-*O*-glucosyltransferase (UFGT). The final step in anthocyanin biosynthesis involves the transfer of a glucosyl moiety to the anthocyanidin molecule by UFGT, producing stable anthocyanins (Larson and Coe, 1977). Metabolites at certain steps within this pathway can also be utilised in other branches of the flavonoid pathway. Flavonols are synthesized from dihydroflavonols, and catechin and epicatechin PA precursors are derived from leucocyanidin (catalyzed by leucoanthocyanidin reductase (LAR)) and cyanidin (catalyzed by anthocyanidin reductase (ANR)) respectively (see Figure 1.2) (Abrahams et al., 2003). The core anthocyanin biosynthesis genes were first identified in grapevine by Sparvoli et al. (1994) except for *F3'H* and *F3'5'H* which were characterised by Bogs et al. (2006).

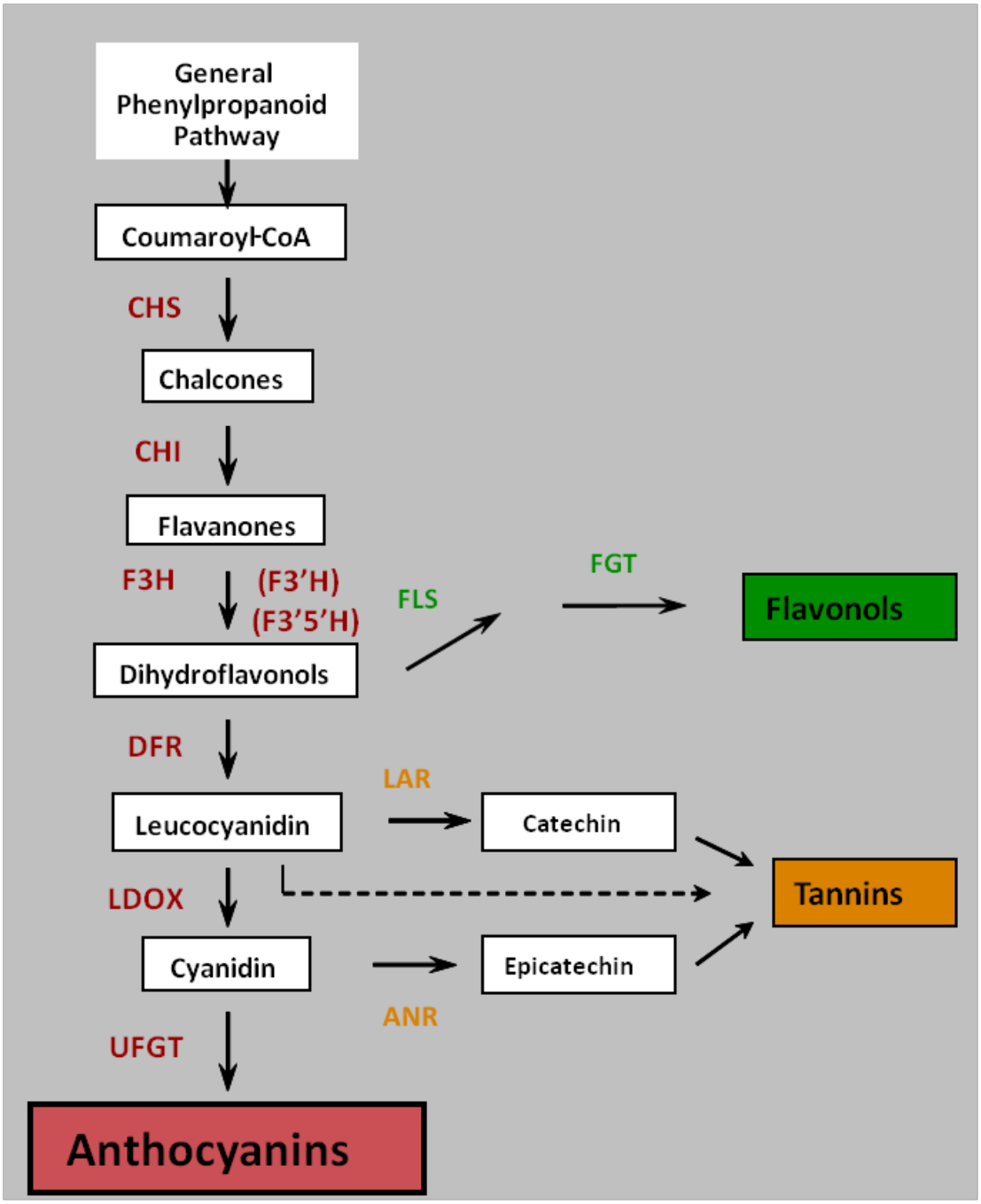


Figure 1.2 - A schematic of the general flavonoid biosynthetic pathway.

Metabolites are boxed with enzymes catalysing each biochemical reaction annotated in red. CHS = chalcone synthase, CHI = chalcone isomerase, F3H = flavanone-3-hydroxylase, F3'H = flavonoid 3'-hydroxylase, F3'5'H = flavonoid 3',5'-hydroxylase, DFR = dihydroflavonol 4-reductase, LDOX= leucoanthocyanidin dioxygenase, UFGT = UDP glucose-flavonoid 3-*O*-glucosyltransferase, FLS = flavonol synthase, FGT = flavonol glucosyltransferase, LAR = leucoanthocyanidin reductase, ANR = anthocyanidin reductase. F3'H and F3'5'H convert a number of substrates to their hydroxylated forms. (reviewed in Anderson and Jordheim, 2006)

Post glucosylation, anthocyanins can be further modified by anthocyanin *O*-methyltransferases (AOMTs), which add methyl groups to the 3' and 5' positions of the B-ring (Yonekura-Sakakibara et al., 2008; Yonekura-Sakakibara et al., 2009). Several of these genes have been identified from *V. vinifera* including *S-adenosyl-l-methionine: cyanidin 3-glucoside 3'-O-methyltransferase* (Bailly et al., 1997), *anthocyanin O-methyltransferase* (Hugueney et al., 2009), *flavonol and anthocyanin 3'5'-O-methyltransferase* (*VvFAOMT*) (Lücker et al., 2010) and *anthocyanin O-methyltransferase* 2 (Fournier-Level et al. 2011). Anthocyanins can also be acylated through the addition of aromatic and/or aliphatic substituents generally attached to the glycosyl moiety. These reactions are catalysed by anthocyanin acyltransferases belonging to 2 different enzyme families, the BAHD superfamily and/or the serine carboxypeptidase-like (SCPL) family (Yonekura-Sakakibara et al., 2008). In *V. vinifera* three acyl groups have been found attached to the C6'' position of the glucosyl producing 3-*O*-acetyl, 3-*O*-coumaroyl, and 3-*O*-caffeoyl-monoglucosides (Mazza and Francis, 1995). There have been no grapevine anthocyanin acyltransferases identified to date.

There is mounting evidence within many plant species that anthocyanin synthesis occurs on the cytoplasmic face of the surface of the endoplasmic reticulum (ER) (Grotewold et al., 1998; Zhang et al., 2006; Hsieh and Huang, 2007; Poustka et al., 2007). It is also possible that at least the early steps in the pathway are carried out by one or several multi-enzyme complexes containing a number of the flavonoid pathway enzymes (Winkel, 2004). Once synthesised, the anthocyanins are transported to, and then across, the vacuolar membrane where they are stored. Exactly how this transportation occurs is still under debate with proposed models including vesicular transport and ligandin transport (Grotewold and Davies, 2008). In grapevine a number of transporters have been identified that are involved in the sequestration of anthocyanins including a number of glutathione-S-transferases (GSTs) (Ageorges et al., 2006; Conn et al., 2008) and two anthocyanin multidrug and toxic efflux transporters (anthoMATEs), VvanthoMATE1 and VvanthoMATE3 (Gomez et al., 2009). A recent study by Gomez et al. (2011) showed evidence suggesting that both vesicular and ligandin transport models of anthocyanin transport from the ER to the vacuole are probably occurring concurrently and that VvGST is likely to be involved in the ligandin transport mechanism and VvanthoMATE1 and VvanthoMATE3 with the vesicular transport

mechanism. It is also possible that other transporters yet to be identified may be involved in this process.

1.4.3 Transcriptional regulation of the anthocyanin biosynthesis pathway

The accumulation of anthocyanins in grapevines is tightly regulated. In the berries they are usually only found in the skins and begin to accumulate after veraison. Veraison is defined as the onset of ripening and is the point where the berries begin to soften and accumulate sugar and anthocyanins. Boss et al. (1996c) analysed the expression of the flavonoid structural genes involved in anthocyanin biosynthesis within different grapevine tissues and at different developmental stages of berry ripening. The results showed that all of these genes except *VvUFGT* were expressed in berry skins pre-veraison, but that most of the genes were also expressed in other tissues. Boss et al. (1996a) predicted that the expression of these earlier structural genes was due to their role in producing other flavonoids besides anthocyanins. Two studies conducted by Downey and colleagues (2003a and 2003b) analysed the flavonoid accumulation in grapes and other tissues during development, and showed that PAs, among other flavonoids, were predominately synthesized pre-veraison followed by a steady accumulation of anthocyanins post-veraison.

Due to the convenient visual signs of active anthocyanin synthesis, mutant phenotypes from altered genes associated with this pathway can be readily identified, and these have been used to study its regulation. There are many examples of bud sports of black and red skinned grapevine cultivars where the berry colour has been either reduced (to bronze or rose) or is completely absent, due to spontaneous mutagenesis. Boss et al. (1996b) analysed a combination of sports where anthocyanin synthesis had been both lost and gained through this mechanism. This research showed that the presence or absence of anthocyanins in the berry skin could be directly related to the presence or absence of *VvUFGT* gene expression. Using a probe designed to *VvUFGT*, southern blot hybridisation analysis of genomic DNA (gDNA) from white-skinned berry cultivars showed that all of these cultivars still contained this gene in their genomes. This suggested that the absence of *VvUFGT* gene expression in these cultivars was rather due to its transcriptional control. Kobayashi and colleagues (2002) were the first to clone several genes, from the Kyoho grape cultivar, relating to the R2R3-*MYB* gene family known to code for transcription factors (TFs). The activity of these genes, named *VvMYBA1* and *VvMYBA2*, was tested in transient expression assays using somatic embryos

which showed that their proteins induced both pigmentation and *VvUFGT* gene expression. These transcripts and their upstream elements were sequenced and a retrotransposon was found within the *VvMYBA1* gene promoter of white-skinned varieties rendering it inactive (Kobayashi et al., 2004). A later study showed that these two genes are adjacent on a single colour locus, as were two closely related genes, *VvMYBA3* and *VvMYBA4*, which are potentially pseudogenes and were shown not to be involved in anthocyanin synthesis (Walker et al., 2007). This study also showed that white cultivars possessed a mutated version of the *VvMYBA2* gene resulting in a frameshift and a mutated protein that is unable to activate *VvUFGT* gene transcription. This phenomenon was used to explain the evolution of red and white grape cultivars, being that all white-skinned berries tested have arisen from the same mutations within a red cultivar genome.

Other R2R3-MYB TFs have been identified in grapevine that also play a role in regulating the flavonoid pathway. VvMYBPA and VvMYBF1 up-regulate the PAs and flavonol branches of the pathway respectively (Bogs et al., 2007; Czemmel et al., 2009) while VvMYB5a and VvMYB5b up-regulate the expression of the earlier structural genes affecting both PAs and anthocyanin biosynthesis (Deluc et al., 2006; Deluc et al., 2008).

Regulation of the flavonoid pathway in other plant species has guided the identification of a putative regulatory complex that includes the R2R3-MYB TFs along with a basic helix-loop-helix (bHLH or MYC) and a tryptophan-aspartic acid repeat (WD40) protein. Through analysis of this complex in *Arabidopsis thaliana* (herein *Arabidopsis*) it was hypothesized that these three proteins alter the expression of flavonoid structural genes by binding to responsive elements found in their promoters and activating transcription (Figure 1.3, Baudry et al., 2006). These particular responsive elements have been found in various grape flavonoid structural gene promoters including *VvDFR* (Gollop et al., 2002) and *VvANR* (Fujita et al., 2005), indicating that this tri-protein complex is also likely to regulate this pathway within the *Vitis* species. The first MYC and WD40 genes from *Vitis* were recently cloned by Matus et al. (2010). The expression patterns of these genes, named *VvWDR-1* and *VvMYCA1*, were found to correlate with *VvMYBA1-2* and *VvUFGT* gene expression as well as anthocyanin accumulation, strongly supporting the hypothesis of this tri-protein regulatory complex in grapevine.

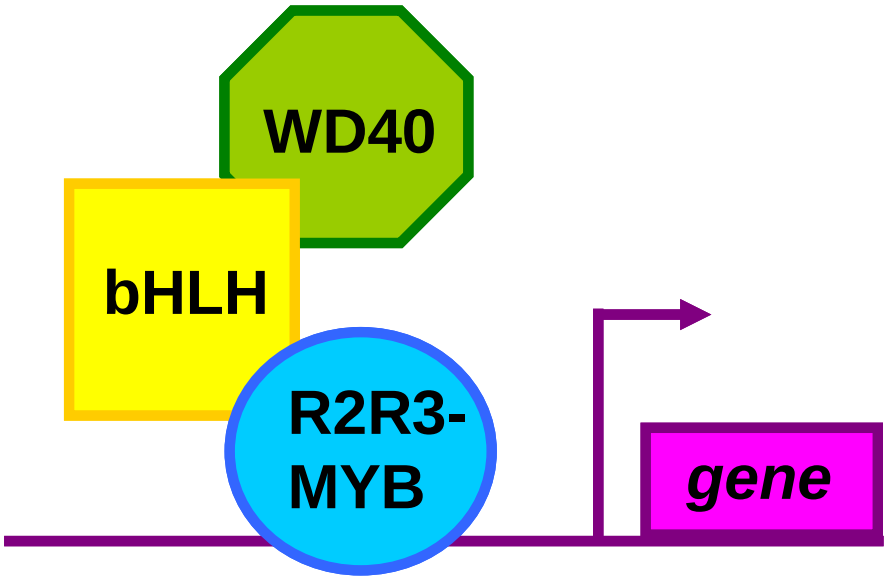


Figure 1.3 - Proposed model of how the MYB/bHLH/WD40 complex activates flavonoid structural genes by binding to responsive elements in their promoters.

(Koes et al., 2005)

While the majority of studies on the regulation of flavonoid synthesis have focused on TFs that up-regulate this pathway, there have been a few studies which have characterised MYBs that act as negative regulators of the pathway. AtMYB4 has been shown to repress the expression of the early phenylpropanoid pathway gene *AtC4H* and *Arabidopsis* lines mutant for this TF contained increased levels of sinapate esters as a consequence (Jin et al., 2000). Expression of the apple protein MdMYB6 in transgenic *Arabidopsis* resulted in the accumulation of less anthocyanins under high osmotic stress conditions and lower expression of early and late anthocyanin biosynthesis genes, indicating that it may act as a repressor of this pathway (Gao et al., 2011). Similarly, the strawberry MYB TF FaMYB1 is capable of lowering the expression of anthocyanin and flavonol biosynthetic genes in transgenic tobacco (Aharoni et al., 2001). A number of *Arabidopsis* R3-type, single domain MYB factors, including AtMYBL2, AtMYB60 and AtCAPRICE, can also inhibit anthocyanin synthesis in transgenic plants (Matsui et al., 2008; Park et al., 2008; Zhu et al., 2009). Matus et al. (2008) identified an AtMYB4 orthologue, VvMYB4, from grapevine, and Huang et al. (2013) identified VvMYBC4-L2, both of which are likely to function as a negative regulators of anthocyanin biosynthesis.

1.5 Evidence linking MYB transcription factors to the regulation of anthocyanin and flavour/aroma biosynthesis

Recent studies have provided evidence that some MYB TFs responsible for the regulation of anthocyanin or flavonoid biosynthesis may also have roles in the regulation of volatile flavour/aroma compound production. In one study, a flavonoid regulatory gene from *Arabidopsis*, called *production of anthocyanin pigment 1* (*AtPAP1)*, known to up-regulate anthocyanin synthesis, was inserted into petunia. The transgenic AtPAP1 expressing plants had increased levels of pigment within their floral tissue as well as an increased release of phenylpropanoid and benzenoid volatiles (Zvi et al., 2008). Bendon et al. (2010) expressed the *Pinus taeda MYB14* gene in *Picea glauca* and found that this resulted in the accumulation of both anthocyanins and sesquiterpenes in the tissues expressing this gene. In another study, the grapevine TF gene *VvMYB5A* was expressed in tomato plants which were subsequently shown to have both increased anthocyanin and terpenoid levels in their transgenic fruit (Mahjoub et al., 2009).

A critical analysis of these studies has led to the formation of this PhD project's primary hypothesis; VvMYBA may have a role in regulating the synthesis of flavour and aroma compounds in red grapes which may contribute to the flavour differences of red and white wine. Recently a transcriptomic approach to analysing the role of VvMYBA in grapevine was undertaken by Cutanda-Perez *et al.* (2009). They used microarray to compare the transcriptome between transgenic *V. vinifera* hairy root tissue expressing the *MYBA1* gene from *V. labruscana* (*VlMYBA1*) and controls. They found that 70 genes had altered expression (1.5 fold cut off) in the transgenic tissue, many of which were related to flavonoid biosynthesis and transport. Their conclusion was that VvMYBA was only involved in the regulation of the anthocyanin biosynthesis pathway and genes associated with anthocyanin transport, and no links to other volatile compound synthesis were made. Yet as roots are not the natural tissue in which *VvMYBA* genes are expressed in grapevine, all direct targets of this TF may not be regulated in this tissue due to a number of reasons. For example a lack of co-factors not present in roots could prevent VvMYBA from regulating some targets, or other TFs which may complex with VvMYBA and could be required for another regulatory function may not be present. There could also be differential processing of transcripts between these two tissues. Hence, this model system is not optimal for studying the regulatory function of VvMYBA. Similar microarray studies where *VvMYBA* gene expression has been altered in the fruit of grapevines would provide a superior genetic background for such an investigation.

1.6 Natural and transgenic grape varieties with altered *VvMYBA* gene expression used in this study

To further study the role of *VvMYBA* in anthocyanin biosynthesis and to elucidate whether it may also regulate flavour and aroma metabolism, grapevines with the same genetic background but differing in their expression of the VvMYBA regulator are required. Chardonnay, Shiraz and Cabernet Sauvignon are the most commonly grown cultivars in Australia (Pink, 2009), and would therefore be the most logical candidates for use in such studies. Fortunately, nature itself has provided a set of colour mutations in vines of the cultivar Cabernet Sauvignon growing at Langhorne Creek in South Australia. Cabernet Sauvignon is heterozygous for colour and is thought to originated from a cross between Cabernet Franc and Sauvignon Blanc, obtaining its red and white allele of the berry colour

locus from each parent respectively (Bowers and Meredith, 1997; Walker et al., 2007). A bud sport from Cabernet Sauvignon first emerged in 1977, when a single cane exhibiting bronze coloured berries was observed. Cuttings from this cane were propagated and the bronze Cabernet Sauvignon mutant is named Malian (Cleggett, 2002). In 1991 a white-skinned bud sport arose from the Malian variety, which is now called Shalistin (Cleggett, 2003). From a genetic analysis of these vines it was proposed that the bronze variety arose due to a deletion in its genome, which included the 'red' colour allele, in the L2 cell layer of a developing meristem. This deletion included the *VvMYBA* genes which resulted in the loss of anthocyanin synthesis in these cells. This has resulted in the loss of anthocyanin accumulation in all skin cell layers except the outermost epidermal layer, giving the berries a bronze/rose coloured phenotype. It has been suggested that the Shalistin bud sport emerged due to an incorporation of some L2 cells into the L1 cell layer of a meristem from which the berry epidermal cell layer was formed (see Figure 1.4, Walker et al., 2006). These three varieties are grown within the same vineyard in close proximity to one another.

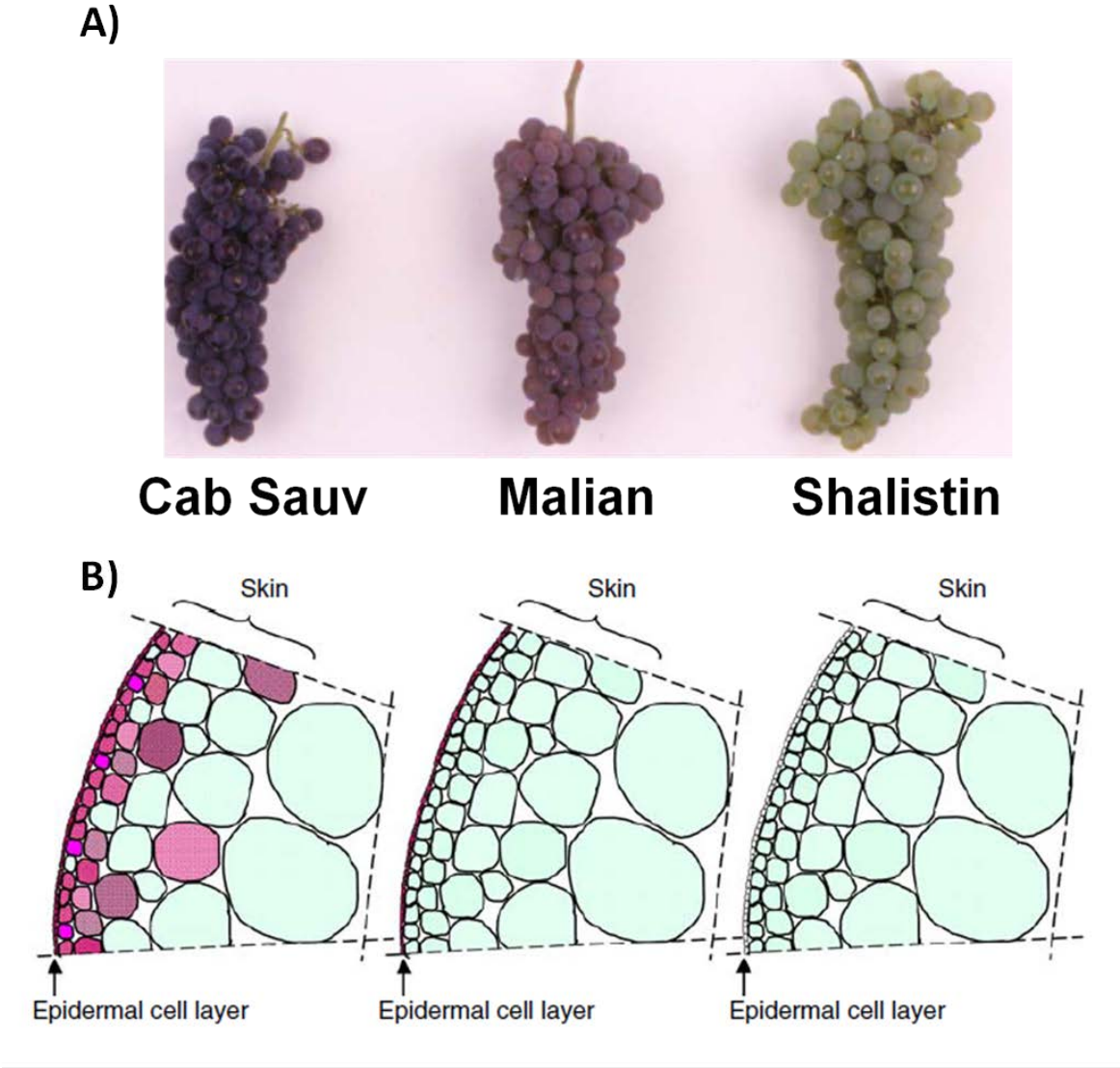


Figure 1.4 - Cabernet Sauvignon bud sports, Malian and Shalistin

Photograph of Cabernet Sauvignon bud sports and new colour varieties Malian and Shalistin located at Langhorne Creek, South Australia. Malian arose due to a mutation in the Cabernet Sauvignon colour locus in the L2 cell layer of berry skins knocking out anthocyanin synthesis. Shalistin arose due to L2 cell invasion into epidermal cell layer. **B)** Schematic of Cabernet Sauvignon, Malian and Shalistin berry skin cells to illustrate this. (from Walker et al., 2006)

Walker and colleagues (unpublished) used an A*grobacterium*-mediated transformation system to generate stably transformed Shiraz and Chardonnay plants with altered *VvMYBA* gene expression using methods described by Iocco et al. (2001). Two gene constructs have been inserted into the Chardonnay variety, both containing the *VvMYBA1* gene under the control of either its native promoter (*pVvMYBA1*), or the 35S constitutive promoter from the *Cauliflower Mosaic Virus* (pCaMv*35S*). The result has been the production of two groups of plants, red Chardonnay lines where *VvMYBA* gene expression and anthocyanin synthesis is detected in all plant tissues giving the whole plant a purple coloured phenotype, and another where anthocyanin synthesis is visible only in the skin of post-veraison berries (see Figure 1.5).

Only one construct was inserted into the Shiraz genome (*VvMYBA1*si) and this was designed to silence *VvMYBA1* and *VvMYBA2* gene expression through anti-sense gene technology. From this transformation three different phenotypes were observed: i.e. vines containing black, rose and white berries (see Figure 1.5). Quantitative PCR (Q-PCR) was used to show that these phenotypes correlated with the amount of *VvMYBA1* gene silencing achieved in each line. Apart from their pigmentation differences, transgenic grapevines displayed no other obvious differences in their appearance or fitness compared to their non-transgenic controls (Walker, personal communication).

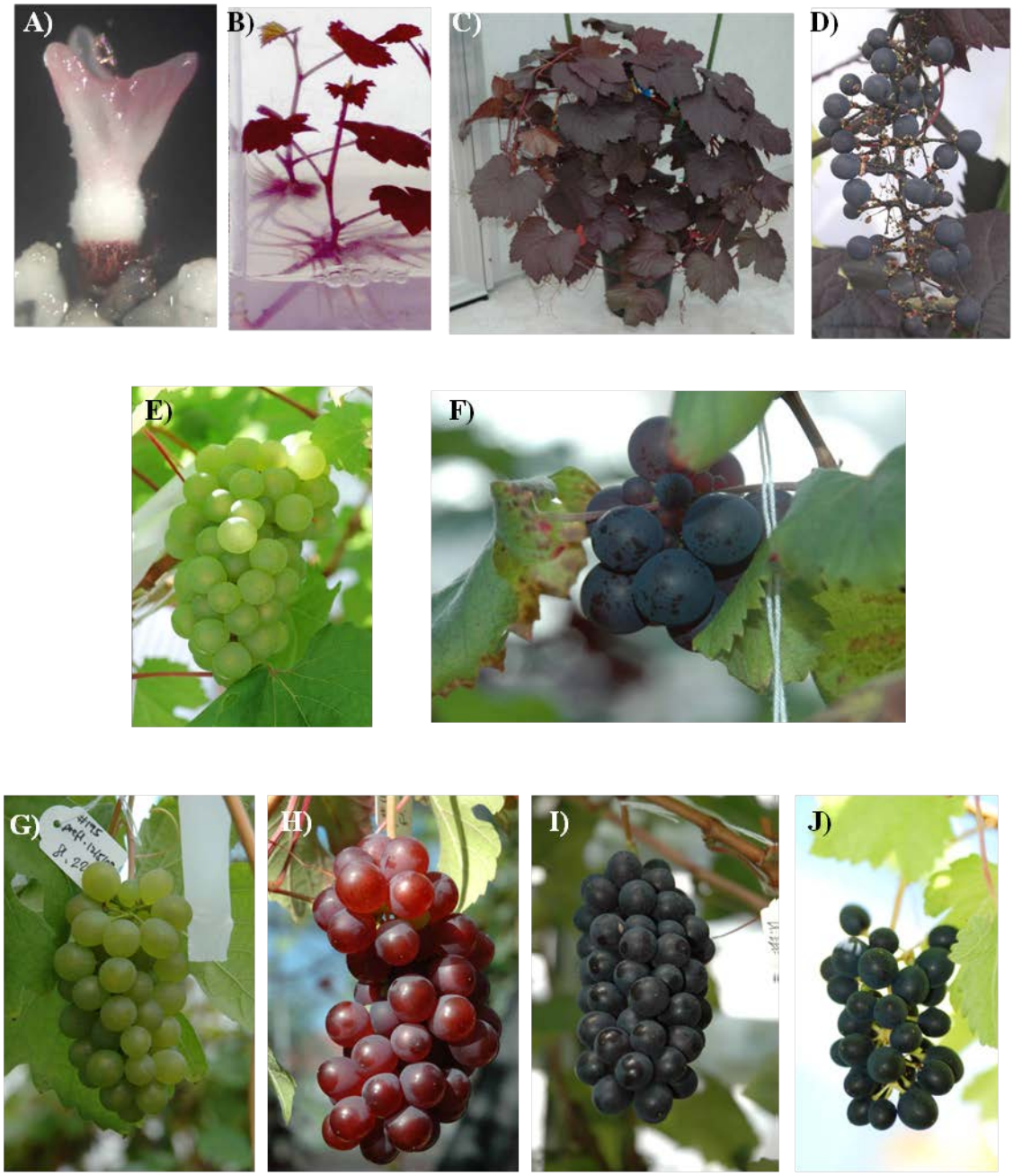


Figure 1.5 - Transgenic grapevines with altered pigmentation and Vv*MYBA1* gene expression.

A-D) Chardonnay containing *pCaMv35S:VvMYBA* constructs. The purple phenotype in all tissues is due to anthocyanin accumulation. Shown above embryos (**A**), plantlets in tissue culture with purple leaves, stems and roots (**B**), purple leaves of mature grapevine (**C**), purple pre-veraison berries (**D**). **E**) Chardonnay control. **F**) Transgenic chardonnay containing *pVvMYBA1:VvMYBA1* gene construct has red pigmentation in skins of berries only post-veraison. **G-I**) Transgenic shiraz containing *VvMYBAsi* silencing construct resulted in three different phenotypes: white (**G**), rose (**H**) and black (**I**) depending on level of gene silencing. **J**) Shiraz control. (Photos by A. Walker)

1.7 Summary and project aims

Much research has been undertaken to fully characterise the anthocyanin biosynthetic pathway and its regulation in grapevine, and this has clearly identified the genetic determinant of grape colour (red/black or white) i.e. the presence or absence of VvMYBA. There has been limited research aimed at investigating further regulatory roles that VvMYBA may have in grapevine but these studies have been conducted using the model hairy root system. Research in species other than grapevine has suggested that transcription factors involved in anthocyanin synthesis may also regulate volatile composition in plants, and a grapevine flavonoid transcription factor VvMYB5b has been shown to increase terpenoid production in genetically modified tomatoes. To our knowledge there have been no studies reported on any grapevine flavonoid TFs and their effect on volatile production within grapes.

This laboratory has optimised and now utilises a successful grapevine transformation method which has enabled the production of transgenic grapevine with altered *VvMYBA* gene expression. This has resulted in the production of 'white' Shiraz and 'red' Chardonnay grapes. These will be utilised to further analyse the role of VvMYBA in anthocyanin biosynthesis as well as flavour and aroma metabolism as the genetic background of these plants are identical and also optimal for the expression of this gene. This research may further our understanding of the flavour components of wine and their origins and possibly add to the growing body of information on anthocyanin synthesis in grapevine.

1.7.1 Aims/objectives

This project aims to further investigate the role of VvMYBA1 in anthocyanin biosynthesis, and analyse its influence on flavour and aroma compounds found in wine. It is expected that the presence of VvMYBA can alter the profile of berry flavour precursors ultimately contributing to the differences that distinguish between red and white wine. In order to explore this hypothesis, transgenic grapevines with altered *VvMYBA* gene expression, along with natural colour mutants originating from the Cabernet Sauvignon grape variety, will be utilised. This project aims to meet the following objectives:

- To analyse the differences in transcriptomes of transgenic Chardonnay and Shiraz berries with altered *VvMYBA1* gene expression and identify potential uncharacterised

targets of this transcription factor that may be involved in anthocyanin or flavour/aroma metabolism.

- To characterise the function of any uncharacterised targets of interest of VvMYBA through gene expression studies and other biochemical analyses
- To identify differentiating compounds in wine made from grapes differing only in the expression of the *VvMYBA* colour regulator genes.
- To determine the origins of these differences, whether they are through interactions of the wine matrix or through an altered regulation of flavour precursor pathways.

Chapter 2: Transcriptomic analysis of berries with altered *VvMYBA* gene expression in transgenic grapevines

Note about the experimental work in this Chapter

Most of the exerimental work presented in the following chapter was carried out by Amy Rinaldo (the author of this thesis). However the cDNA synthesis and microarray hybridizations were performed, and raw data from these were processed, in a collaborating laboratory in the Department of Biotechnology, University of Verona, Verona, Italy, by Dr. Marianna Fasoli (sections 2.2.4 and 2.2.5 of the methods). These raw data have been depositied in the Microarray Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cnmxwmmsfjctvgt&acc=GSE56915). The preparation of this Chapter was done entirely by Amy Rinaldo as was the interpretation of all the results.

2.1 Introduction

One of the aims of this PhD project (as outlined in section 1.7.1) was:

To analyse the differences in transcriptomes of transgenic Chardonnay and Shiraz berries with altered VvMYBA1 gene expression and identify potential uncharacterised targets of this transcription factor that may be involved in anthocyanin or flavour/aroma metabolism.

To meet this aim, transgenic Chardonnay and Shiraz grapevines in which *VvMYBA* gene expression and hence berry colour had been altered were utilized. These vines were discussed in section 1.6.

A *Vitis* NimbleGen microarray chip representing 29,549 predicted genes based on the 12X grapevine genome sequence (V1 gene prediction version, http://genomes.cribi.unipd.it/grape/) was available in a collaborating laboratory in the Department of Biotechnology at the University of Verona in Italy. Microarray experiments were therefore the method chosen to analyse the transcriptomes of berries from the transgenic grapevines and their controls.

2.1.1 Specific aims of this research

When designing the microarray experiments there were several research questions which were of interest. The first of these was:

- What effect does altering *VvMYBA* gene expression have on the transcriptomes of Chardonnay and Shiraz berries?

To answer this question, a comparison of the transcriptomes of transgenic berries, with altered *VvMYBA* gene expression, and that of non-transgenic controls, within each cultivar (Chardonnay or Shiraz) was required.

One benefit of having transgenic plants from two grapevine cultivars is that transcriptomic changes within the cultivars could be compared to one another. It was expected that this comparison would highlight conserved roles of gene regulation by VvMYBA which occur in both varieties. So the second research question was:

- What genes had expression levels which were changed in a consistent manner in relation to *VvMYBA* gene expression in both varieties?

Having the transgenic Chardonnay vines which constitutively expressed *VvMYBA1* (since they contained the *35S:VvMYBA1* construct) meant that berries not only expressed this gene in their skins, but also in the other tissues within the berry. Therefore differences in transcriptomic changes between different berry tissues could also be analysed. Of particular interest was the answer to the third research question:

- Are there differences in gene expression changes, due to altered *VvMYBA1* gene expression, when comparing skin and whole berry samples from transgenic Chardonnay and controls?

2.2 Materials and methods

2.2.1 Plant Material

Transgenic Chardonnay/Shiraz and non-transformed WT controls were all grown in the same glasshouse in ambient light, with a night break. Day and night temperatures were about 27^{o}C and 22^{o}C respectively. Whole berries were sampled from independent transgenic lines: three from transgenic Chardonnay and four from transgenic Shiraz, resulting in three and four biological replicates respectively. Bunches were harvested close to ripeness based on average total soluble solids (TSS, measured as ^{o}Brix). This was aimed to be between 20 – 24 ^{o}Brix (Appendix A) determined from TTS of a subsamples from each bunch. A sample consisted of all remaining berries from a single bunch except when there were <100 berries in which case more than one bunch was used in the one replicate.Whole berries were immediately frozen in liquid nitrogen. For skin samples, the skins were first removed from fresh berries then immediately frozen in liquid nitrogen. All samples were stored at -80^{o}C. Due to unsynchronized flowering of the glasshouse grown vines, sampling occurred throughout the year and during the light period of the day.

2.2.2 RNA extractions

Frozen whole berry or skin samples were ground to a fine powder under liquid nitrogen using a chilled grinding mill (IKA®, Germany) and a mortar and pestle. Total RNA was extracted using a modified perchlorate method previously described in Boss et al. (2001). Genomic DNA was removed using RNAse-free DNAse (Qiagen, Nimburg, Netherlands) in conjunction with the RNeasy Mini kit (Qiagen) according to their protocol. A NanoDrop® 1000

spectrophotometer (V3.7.1, Thermo Fisher Scientific, Massachusetts, USA) was used to determine RNA quantity and purity by ensuring that absorbance ratios A260/280 and A260/230 were both between 1.8 and 2.0. RNA samples were sent on dry ice to the Australian Genome Research Facility (Sydney, Australia) where RNA integrity was analysed using a Bioanalyser Chip RNA 7500 series II (Agilent, CA, USA). Only samples with an RNA integrity number (RIN) greater than 1.7 were used in microarray experiments.

2.2.3 Experimental plan

Two microarray experiments were conducted each containing cDNA from 12 samples:

1. The Chardonnay microarray compared the transcriptomes of 3 replicates each of skin only and whole berries from transgenic 'red' Chardonnay (containing the *35S:VvMYBA1* gene construct) and controls.
2. The Shiraz microarray compared the transcriptomes of 4 replicates each of control shiraz whole berries and transgenic 'rose' and 'white' shiraz whole berries expressing the *VvMYBAsi* silencing construct.

Each replicate was composed of berries pooled from 1-3 bunches from a single vine. The plant IDs and transformant lines used are summarised in Appendix B.

2.2.4 cDNA synthesis, labelling and microarray experiments

The cDNA synthesis and labelling, chip hybridization and washing reactions were all carried out according to the NimbleGen Arrays User's Guide: *Gene Expression Analysis* v3.2 protocols (Roche, Penzburg, Germany) in Verona, Italy (see note on page 24). The NimbleGen microarray 090818 Vitis exp HX12 (Roche) was used for all hybridizations. Each gene is represented by four individual DNA spots (probes) on the chip. The design of this chip can be found at http://ddlab.sci.univr.it/FunctionalGenomics/.

The microarray was scanned using a ScanArray 4000XL (Perkin-Elmer, Waltham, USA) at 532 nm (Cy-3 absorption peak) in conjunction with GenePix Pro7 software (Molecular Devices, CA, USA) to produce high resolution images. Images were then analysed using NimbleScan v2.5 software (Roche) which used a Robust Multichip Average (RMA) procedure to produce normalised expression data for each gene derived from the average of the signal intensities of the four probes for that gene.

2.2.5 Analysis of microarray data

2.2.5.1 Identification of genes with significantly altered expression levels in transgenic berries

Normalised expression values were converted to log2 values and a Pearson Correlation analysis was carried out to evaluate the robustness of the biological replicates in each sample. A gene was considered to be expressed if the normalised expression value for at least two of the three biological replicates was higher than the value obtained by averaging the fluorescence of negative controls present on the chip. A Multi-class Significance Analysis of Microarray (SAM) was utilised using TMeV software (http://www.tm4.org/mev) to remove genes which were not significantly modulated compared to the controls. The false discovery rate was set to 1% for the Chardonnay dataset but was increased to 2.5% in the Shiraz dataset due to a much smaller number of genes being detected as having significant differences in transcript levels between different coloured berry samples. A two-class unpaired SAM analysis was then used to compare the expression values both between controls and also between transgenic lines.

2.2.5.2 Identification of genes that were modulated in relation to *VvMYBA1* gene expression consistently in Chardonnay and Shiraz

Genes whose expression was significantly altered in the transcriptomes of transgenic 'red' Chardonnay and 'white' Shiraz whole berries compared to controls were analysed. To find genes whose regulation was altered by the presence/absence of the VvMYBA TFs in a consistent manner, genes that were present in both data sets were analysed individually. Genes that were up-regulated in the 'red' Chardonnay (positive red/white ratio) and conversely down-regulated in 'white' Shiraz (negative white/red ratio), or that were down-regulated in the 'red' Chardonnay and up-regulated in 'white' Shiraz were of interest. No fold change (FC) cut off was used for this analysis. Only genes where the SAM found the FCs as being significantly different in both the Chardonnay and Shiraz datasets were included. When genes were represented in the dataset more than once due to multiple copies of the genes, the copy that had the largest FC ratio was chosen to be presented in Table 2.1.

2.2.5.3 Identification of genes with differential expression changes when comparing skin and whole berry Chardonnay datasets

FC ratios comparing gene expression between 'red' Chardonnay and non-pigmented controls in whole berry samples were compared to those from skin only samples. Where the two ratios differed by a multiplication value of ≥ 2 then they were considered significantly different. Only genes that had FC ratios of ≥ ±2 in at least one of the tissues (whole berry or skin) were considered in this analysis, due to the large number of genes present in the Chardonnay microarray dataset and the fact that this experiment was comparing controls with over-expression lines and hence gene expression changes were expected to be exaggerated.

2.2.5.4 Analysis of gene expression trends in relation to berry colour in Shiraz

All genes which had significant FC ratios when comparing transgenic 'white' or 'rose' Shiraz whole berry transcriptomes to non-transgenic controls (red berries) were considered in this analysis and no FC cut off was used. For each gene the FC ratios between 'white' Shiraz and controls (white/red) and 'rose' Shiraz and controls (rose/red) were compared. A FC ratio was considered significant if determined so by the SAM test, regardless of the ratio value. Where the multiplication value between these two ratios was ≥ 2 then the ratios were considered significantly different. The genes were sorted into specific gene expression trends relating to berry colour, which are outlined in Figure 2.3.

2.2.6 Bioinformatics

The microarray gene IDs, relating to the individual probes on the microarray chip, were annotated with known, putative or unknown functions within the microarray array data file. Where possible, they were also annotated with any gene networks that these genes have been previously shown to be associated with (data not shown, can be found at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cnmxwmmsfjctvgt&acc=GSE56915).
For some genes of interest further investigation into their function was required. This was done by investigating homology to other genes and proteins using nucleotide or translated nucleotide Basic Local Alignment Search Tool (BLAST) searches. These were performed in the National Centre for Biotechnology Information (NCBI) server (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.3 Results

2.3.1 Altering *VvMYBA* gene expression in grapevine changes transcription in many cellular pathways

To analyse the effect of altering *VvMYBA* gene expression on global transcription the transcriptomes from whole berries of transgenic 'red' Chardonnay (containing the *35S:VvMYBA1* construct) and 'white' and 'rose' Shiraz (silencing lines containing the *VvMYBAsi* construct) were compared to that of non-transgenic controls using microarray technology. 636 and 488 genes were significantly up- and down-regulated respectively (with a FC of ≥ 1.5 or ≤ -1.5) in the *VvMYBA1* over-expressing 'red' Chardonnay berries compared to non-pigmented controls. 115 and 93 genes were significantly up- and down-regulated respectively in transgenic 'white' Shiraz berries compared to non-transgenic red-berried controls. Of these, 75 and 71 had FCs of ≥ 1.5 or ≤ -1.5. When comparing the 'rose' Shiraz transcriptome to controls 134 and 128 genes were significantly up- and down-regulated respectively, of which 102 and 103 had FCs of ≥ 1.5 or ≤ -1.5. These raw data have been depositied in the Microarray Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cnmxwmmsfjctvgt&acc=GSE56915).

2.3.1.1 The expression of some flavonoid pathway genes was altered in transgenic berries

Most genes whose expression FCs were greatest in transgenic Chardonnay and Shiraz berries (compared to controls) are known to be involved in the anthocyanin, flavonoid and general phenylpropanoid biosynthetic pathways or anthocyanin transport. In the 'red' Chardonnay berries, *VvGST* was most highly upregulated, with a FC of 806. This was followed by *VvF3'5'H* (FC of 388), *flavonol and anthocyanidin-glucoside 3',5'-O-methyltransferase* (*VvFAOMT*) (FC of 115), *VvUFGT* (FC of 62), and *VvCHS* (FC of 35). *VvanthoMATE1* (FC of 6) was also up-regulated as were some general phenylpropanoid pathway genes on a smaller scale including a *flavonoid-3-monooxygenase* (FC of 5), *VvPAL2* (FC of 2.6) *cinnamoyl alcohol dehydrogenase* (FC 1.5), *cinnamoyl-CoA reductase* (FC 1.5), a putative *LDOX-like* gene (FC 1.7) and *stilbene synthase* (FC 2.8). Flavonoid genes that were down-regulated in these berries included: *VvANR* (FC of -3.7), *VvLAR* (FC of -2.8), *VvF3H* (FC of -1.6), *Vv4CL* (FC of -3) and a putative *UDP-rhamnose/rhamnosyltransferase* (FC of -1.6). The same flavonoid genes up-regulated in the 'red' Chardonnay berries were down-regulated in the transgenic 'white' Shiraz berries (compared to controls). *VvF3'5'H* (FC of -66), *VvCHS*

(FC of -44), *VvFAOMT* (FC of -33.8), *VvGST* (FC of -12.3), *VvanthoMATE1* (FC of -3.3), *VvPAL2* (FC of -2.1), *cinnamoyl-CoA reductase* (FC of -2.3), *VvLDOX* (FC of -2) and stilbene synthases (FC of -1.8) were all down-regulated when the expression of *VvMYBA* genes were silenced. Conversely, in 'white' Shiraz there was not a comparative up-regulation of those flavonoid genes which were down-regulated in the 'red' Chardonnay. There were a small number of flavonoid pathway genes in Chardonnay and Shiraz which had dissimilar transcriptional responses to the presence or absence of *VvMYBA* transcripts. Unlike *VvPAL2*, which was up-regulated in 'red' Chardonnay and down-regulated in 'white' Shiraz, *VvPAL* was down-regulated in both cultivars ('red' Chardonnay FC of -3.31, 'white' Shiraz FC of -1.63). *VvCHI* gene expression was also significantly down-regulated in 'white' Shiraz berries (FC of -1.8) but was not significantly altered in 'red' Chardonnay berries.

2.3.1.2 Transcription in other cellular pathways was also altered in transgenic berries expressing *VvMYBA1*

Apart from flavonoid and general phenylpropanoid pathway related genes, many other genes, with a broad range of functions, were also shown to have altered gene expression in the transgenic Chardonnay and Shiraz berries. These included genes involved in many primary and secondary metabolism pathways including photosynthesis and amino acid, sugar, fatty acid, terpenoid, and cell wall metabolism. A large number of TF genes and genes involved in hormone signalling including abscisic acid (ABA), auxin and ethylene signalling also had altered gene expression as well as genes involved in stress and defence responses. Figure 2.1 shows a pie graph representing the number of genes, categorised by their functions, which had altered expression in transgenic 'red' Chardonnay berries compared to non-pigmented controls. There was not a large difference between the percentages of genes within each functional category when comparing those which were up- and down-regulated. A slightly larger percentage of genes were down-regulated that are annotated to have roles in primary metabolism, compared to those that were up-regulated.

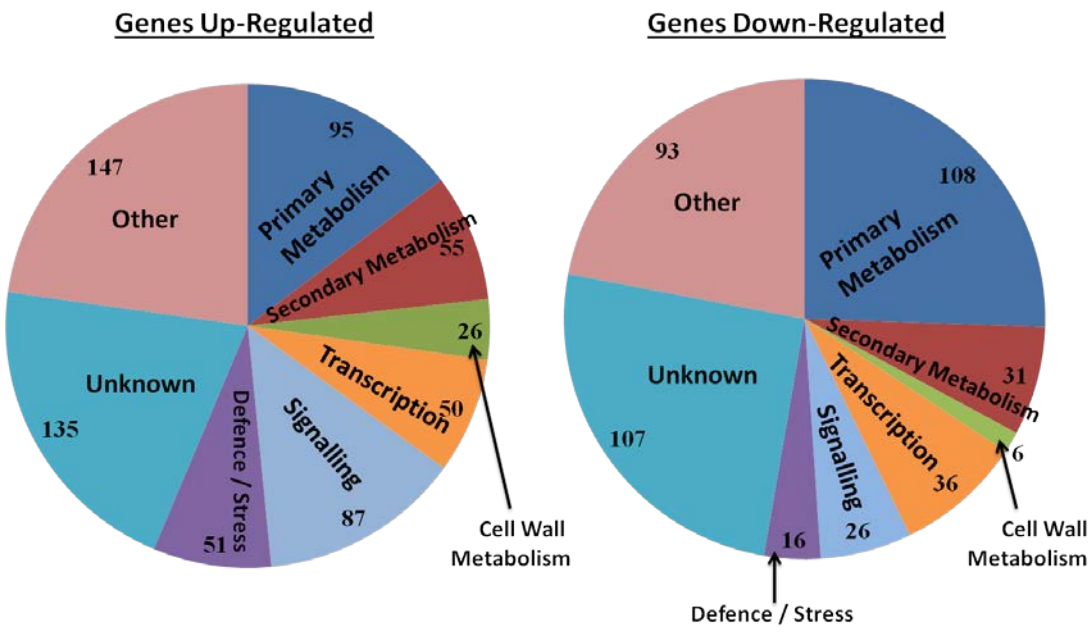


Figure 2.1: Pie chart representing number of genes with altered expression in transgenic 'red' Chardonnay categorised by function

The pie charts represent the number of genes, sorted into functional categories, with significantly altered expression in transgenic 'red' Chardonnay (containing the *35S:VvMYBA1* construct) compared to non-transgenic controls. Microarrays were performed using the NimbleGen microarray 090818 Vitis exp HX12 chip and all data are representative of the mean of 3 biological replicates.

2.3.2 Comparison of Chardonnay and Shiraz microarray datasets

In this study there was a particular interest in identifying genes whose transcription was consistently altered in response to *VvMYBA* gene expression, in the same manner in both Chardonnay and Shiraz cultivars. This could reveal any conserved functions in gene regulation by VvMYBA that were not already known. For this reason the microarray data was further analysed for genes whose expression was consistently up- or down-regulated in response to the presence/absence of *VvMYBA* gene expression in both Chardonnay and Shiraz. Genes that were up-regulated in 'red' Chardonnay berries and conversely down-regulated in 'white' Shiraz were considered to be up-regulated by the presence of VvMYBA. Genes that were down-regulated in 'red' Chardonnay and up-regulated in 'white' Shiraz were considered to be down-regulated by the presence of VvMYBA. This analysis revealed that only 15 and 11 genes were up- and down-regulated respectively, by VvMYBA in a consistent manner between the two cultivars (Figure 2.2 and Table 2.1).

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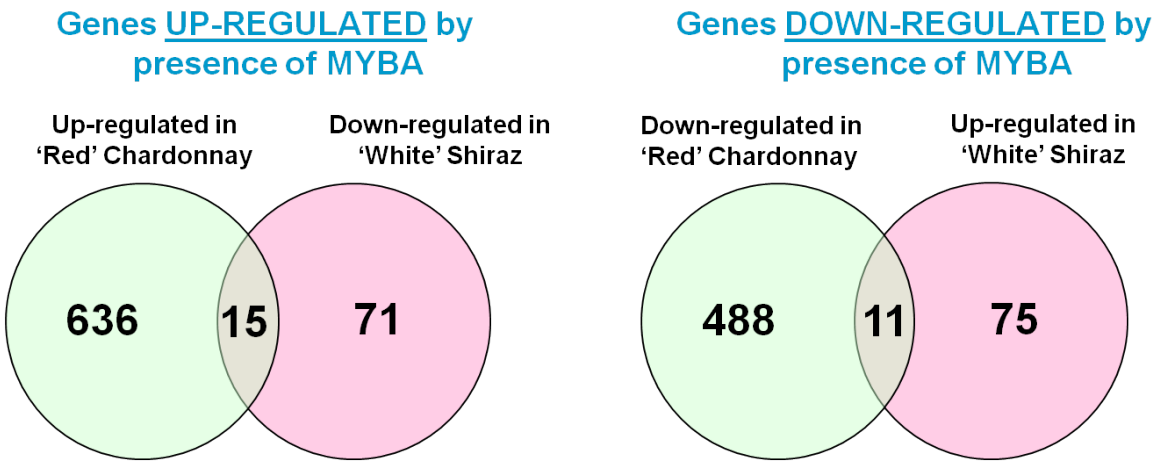


Figure 2.2: Comparison of numbers of genes with altered transcription in response to *VvMYBA* gene expression in transgenic Chardonnay and Shiraz berries

Venn diagrams showing number of genes with altered expression (with FC ≥ 1.5 and ≤ -1.5) in whole berry samples of transgenic 'red' Chardonnay containing the *35S:VvMYBA1* construct and transgenic 'white' Shiraz containing the *VvMYBAsi* silencing construct, compared to non-transgenic controls. Gene expression was determined by microarray analysis using the NimbleGen microarray 090818 Vitis exp HX12. The number of genes with expression changes consistent in relation to the presence or absence of VvMYBA in both varieties is shown in the overlap between the circles.

Table 2.1: Genes with altered transcription levels in response to *VvMYBA* gene expression in a consistent manner in both transgenic Chardonnay and Shiraz berries

		'red' Chardonnay* / control		'white' Shiraz# / control	
Microarray gene ID	**Annotation/Blast Hits**	**red / white FC**	**significant (SAM)^**	**white/red FC**	**significant (SAM)^**
VIT_04s0079g00690	glutathione S-transferase (VvGST)	805.99	Y	-12.29	Y
VIT_06s0009g02840	flavonoid 3',5'-hydroxylase (VvF3'5'H)	388.73	Y	-65.96	Y
VIT_01s0010g03490	*O*-methyltransferase (VvFAOMT)	106.63	Y	-33.84	Y
VIT_03s0017g00870	BAHD family acyl transferase	64.90	Y	-23.81	Y
VIT_16s0039g02230	UDP-glucose:flavonoid 3-*O*-glucosyltransferase (VvUFGT)	62.75	Y	-21.18	Y
VIT_16s0022g01020	chalcone synthase (VvCHS)	35.72	Y	-30.73	Y
VIT_03s0091g01240	serine carboxypeptidase-like gene	22.55	Y	-10.43	Y
VIT_16s0050g00910	anthoMATE1 transport protein (VvanthoMATE1)	5.98	Y	-3.29	Y
VIT_02s0087g00330	glycosyl transferase family 1 protein	4.49	Y	-1.69	Y
VIT_13s0074g00400	petal loss-like protein	4.17	Y	-1.56	Y
VIT_11s0016g04920	early nodulin 93	3.77	Y	-1.32	Y
VIT_14s0128g00160	protein kinase CDG1	3.42	Y	-2.92	Y
VIT_10s0116g00820	adenosine/AMP deaminase	2.89	Y	-1.53	Y
VIT_13s0019g04460	phenylalanine ammonia-lyase 2 (VvPAL2)	2.56	Y	-2.07	Y
VIT_08s0007g05430	pyruvate kinase	2.51	Y	-2.43	Y
VIT_16s0050g02480	ABC transporter C member 15	-1.23	Y	1.13	Y
VIT_18s0001g02460	Unknown protein – no hits	-1.28	Y	1.44	Y
VIT_18s0001g13790	p450	-1.37	Y	1.13	Y
VIT_00s1206g00010	aspartic proteinase nepenthesin-1 precursor	-1.40	Y	1.77	Y
VIT_05s0020g04110	early light-inducible protein (ELIP1)	-1.43	Y	1.47	Y

Table 2.1 continued

		'red' Chardonnay* / control		'white' Shiraz# / control	
Microarray gene ID	**Annotation/Blast Hits**	**red / white ratio**	**significant (SAM)^**	**white/red ratio**	**significant (SAM)^**
VIT_05s0049g00220	2-oxoglutarate-dependent dioxygenase	-1.46	Y	1.56	Y
VIT_19s0140g00210	SOUL heme-binding	-1.50	Y	1.82	Y
VIT_12s0134g00030	E-beta-ocimene synthase	-1.70	Y	2.73	Y
VIT_11s0016g03830	protein kinase	-1.81	Y	1.77	Y
VIT_06s0004g00610	accelerated cell death 1(ACD1)	-1.87	Y	1.84	Y
VIT_01s0011g02260	metal-nicotianamine transporter YSL7	-2.61	Y	1.39	Y

Transcript levels determined using microarrays

*'red' Chardonnay contained a *35S:VvMYBA1* construct and expressed the *VvMYBA1* gene. Non-transgenic un-pigmented Chardonnay berries were the control for these experiments

#'white' Shiraz contained a *VvMYBAsi* construct which completely silenced the expression of *VvMYBA1/2* genes. Non-transgenic red/black Shiraz berries were used as the control for these experiments.

^SAM = Significance Analysis of Microarray. Y indicates that the fold change ratio was significant as determined by a SAM

FC = Fold change

2.3.2.1 The transcription of some flavonoid pathway genes was consistently altered by *VvMYBA* gene expression

Of the 26 genes whose expressions were altered in a consistent manner in relation to *VvMYBA* gene expression in Chardonnay and Shiraz berries, 7 of these were previously characterised flavonoid related genes. All were up-regulated in red berries (i.e. when *VvMYBA* was expressed). Two genes encoded anthocyanin transporters, VvGST and VvanthoMATE1, three were anthocyanin biosynthetic genes *VvCHS*, *VvF3'5'H*, and *VvUFGT*, and the other two were phenylpropanoid biosynthetic genes *VvPAL2* and *VvFAOMT*. There were no flavonoid biosynthetic genes that were consistently down-regulated in red berries in both grapevine cultivars.

2.3.2.2 *VvMYBA* gene expression consistently altered the transcription levels of potential flavour/aroma biosynthetic genes

Two genes were found to be down-regulated in both Chardonnay and Shiraz berries expressing *VvMYBA* (i.e. red berries), which could have roles in the production of flavour compounds. One of these was *E-beta-ocimene synthase* (microarray ID VIT_12s0134g00030, Chardonnay white/red ratio 2.73 FC, Shiraz red/white -1.7 FC) which has been functionally characterised *in vitro* and is known to synthesise the *E*- and *Z*- isomers of the monoterpene *beta*-ocimene (Martin et al., 2010). The other gene, which had smaller FCs (Chardonnay white/red ratio 1.13 FC, Shiraz red/white -1.37 FC) is uncharacterised but has homology to the p450 family (microarray ID VIT_18s0001g13790) and was annotated as belonging to a monoterpene synthesis gene network (vv10902Monoterpenoid_biosynthesis).

2.3.2.3 Two potential acyl-transferase genes were upregulated in red berries

There were two uncharacterised genes which were up-regulated to similar levels to the flavonoid genes discussed in section 2.3.2.1. One, with a FC of 65, had homology to the BAHD protein superfamily and was up-regulated to very similar levels to that of *VvUFGT* (FC of 63). The other, with a FC of 23, had homology to the serine carboxypeptidase-like (SCPL) protein family. Members of both the BAHD and SCPL families are known to act as acyl-transferases in plants (Milkowski and Strack, 2004; D'Auria, 2006).

2.3.2.4 Other genes were affected by *VvMYBA* gene expression consistently in both cultivars

There were a number of other genes which were up- or down-regulated in a consistent manner in relation to *VvMYBA* gene expression in both Chardonnay and Shiraz berries. These genes are annotated to have diverse functions that are not necessarily related. Those that were up-regulated when *VvMYBA* was expressed were annotated to code for, or show homology to the following proteins or protein families: a glycosyl transferase family 1, PTL (PETAL LOSS), early nodulin 93, protein kinase CDG1, adenosine/AMP deaminase, and pyruvate kinase. Genes that were down-regulated by the presence of VvMYBA coded for, or had homology to the following proteins or protein families: an ATP-binding cassette (ABC) transporter C member 15, aspartic proteinase nepenthesin-1 precursor, early light-inducible protein 1 (ELIP1), 2-oxoglutarate-dependent dioxygenase, SOUL heme-binding protein, protein kinase, accelerated cell death 1 (ACD1), metal-nicotianamine transporter YSL7, and one unknown protein with no BLAST hits.

2.3.3 Over-expression of VvMYBA1 in Chardonnay results in differential transcriptomic changes in skins compared to whole berries

As the transgenic 'red' Chardonnay berries expressed *VvMYBA1* in all berry tissues, transcriptomic changes occurring in the skins only were able to be compared to that of whole berries. Genes with different FC ratios when comparing 'red' chardonnay skins to control skins and 'red' Chardonnay whole berries to control whole berries were of interest. If these FC ratios differed by ≥ 2 or ≤ -2 then they were considered to be significantly different and to have differential expression between the tissue types.

Table 2.2 shows the 167 genes which were differentially expressed in skins compared to whole berries: 39 had increased up-regulation in whole berries, while 74 had increased up-regulation in skins. Another 21 genes had increased down-regulation in whole berries, and 34 had increased down-regulation in skins.

2.3.3.1 Genes with increased up-regulation in whole berries compared to skins

Thirty-nine genes were up-regulated to higher levels in transgenic 'red' Chardonnay whole berries compared to in skins. The genes with the greatest difference (berry/skin ratio) were the flavonoid related genes *VvGST*, *VvF3'5'H*, *VvFAOMT* and *VvUFGT*. Both of the

uncharacterised putative BAHD and SCPL acyltransferase genes also fell into this category as did the general phenylpropanoid pathway gene *VvPAL2*. The functions of the other genes in this category were varied. They included several genes associated with, or involved in, the cell wall and its metabolism such as putative *extensin*, *annexin* and *fasciclin arabinogalactan* (*FLA7*) genes (Kieliszewski and Lamport, 1994; Clark et al., 2001; Johnson et al., 2003)

2.3.3.2 Genes with increased up-regulation in skins compared to whole berries

Seventy-four genes had increased up-regulation in 'red' Chardonnay skins compared to whole berries. 10 of these are annotated to have homology to disease resistance or defense related proteins. Genes with homology to cellulose synthase proteins are also included in this category along with a number of putative transporter proteins including a sulphate transporter, a gene belonging to the proton-dependent oligopeptide transport (POT) family, and an amino acid permease. A number of genes relating to hormone signalling were also more highly up-regulated in transgenic skins compared to whole berries including an auxin efflux carrier, an auxin-binding protein, and a putative gibberellin-regulated protein 1 (GASA1).

2.3.3.3 Genes with increased down-regulation in whole berries compared to skins

Twenty-one genes had an increased down-regulation in transgenic 'red' Chardonnay whole berries compared to skins. The majority of these genes and their products were annotated to have roles in photosynthesis (e.g. photosystem proteins), oxidative phosphorylation (e.g. NADH dehydrogenase and NADH-plastoquinone oxidoreductase subunits) or carbon fixation (e.g. *ribulose bisphosphate carboxylase oxygenase* (RubisCo)).

2.3.3.4 Genes with increased down-regulation in skins compared to whole berries

Thirty-four genes were down-regulated to a greater extent in 'red' Chardonnay skins compared to whole berries. In particular genes involved in general phenylpropanoid biosynthesis, such as *VvPAL*, *prephenate dehydratase*, and *4-coumarate-CoA ligase*, or flavonoid biosynthesis such as *VvLAR* are represented in this group.

Table 2.2: Genes with altered expression in transgenic 'red' Chardonnay berries have differential expression when comparing skin transcriptomes to those of whole berries.

A) up-regulated more in whole berries

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)#	significant (SAM)^	berry/skin ratio
VIT_04s0079g00690	Glutathione S-transferase (VvGST)	4.47	Y	805.99	Y	180.22
VIT_06s0009g02840	flavonoid 3',5'-hydroxylase (VvF3'5'H)	6.90	Y	388.73	Y	56.32
VIT_06s0009g02880	flavonoid 3',5'-hydroxylase (VvF3'5'H)	6.63	Y	287.06	Y	43.29
VIT_06s0009g02810	flavonoid 3',5'-hydroxylase (VvF3'5'H)	7.89	Y	289.79	Y	36.71
VIT_06s0009g02970	flavonoid 3',5'-hydroxylase (VvF3'5'H)	7.14	Y	240.09	Y	33.63
VIT_06s0009g03040	flavonoid 3',5'-hydroxylase (VvF3'5'H)	7.88	Y	258.58	Y	32.79
VIT_06s0009g02830	flavonoid 3',5'-hydroxylase (VvF3'5'H)	6.39	Y	197.65	Y	30.93
VIT_06s0009g03110	flavonoid-3,5'-hydroxylase (VvF3'5'H)	7.28	Y	216.84	Y	29.80
VIT_06s0009g03050	flavonoid 3',5'-hydroxylase (VvF3'5'H)	4.97	Y	132.89	Y	26.74
VIT_06s0009g02860	flavonoid 3',5'-hydroxylase (VvF3'5'H)	7.39	Y	195.17	Y	26.39
VIT_06s0009g02920	flavonoid 3',5'-hydroxylase (VvF3'5'H)	6.58	Y	149.00	Y	22.64
VIT_01s0010g03510	Flavonoid and anthocyanin -*O*-methyltransferase (VvFAOMT)	9.75	Y	115.25	Y	11.82
VIT_16s0039g02230	UDP-glucose:flavonoid 3-*O*-glucosyltransferase (VvUFGT)	5.91	Y	62.75	Y	10.61
VIT_16s0013g00880	oleosin (OLE-4)	1.67		15.89	Y	9.49
VIT_06s0009g03010	flavonoid 3',5'-hydroxylase (VvF3'5'H)	16.56	Y	108.68	Y	6.56
VIT_03s0017g00870	BAHD acyl transferase	11.73	Y	64.90	Y	5.53
VIT_10s0003g00030	dof zinc finger protein (DOF5.3)	8.06	Y	32.35	Y	4.02

Table 2.2 **continued**

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)[#]	significant (SAM)^	berry/skin ratio
VIT_08s0056g01350	unknown protein	1.68	Y	6.53	Y	3.90
VIT_01s0010g03470	*O*-methyltransferase (VvFAOMT)	33.24	Y	97.89	Y	2.95
VIT_13s0156g00110	chaperone BCS1 mitochondrial	1.91		5.08	Y	2.66
VIT_18s0001g13210	lectin jacalin	2.72	Y	7.15	Y	2.63
VIT_05s0049g01780	caleosin	1.49	Y	3.91	Y	2.63
VIT_12s0059g00680	no hit	1.16		2.94	Y	2.54
VIT_13s0019g04460	phenylalanine ammonia-lyase 2 (VvPAL2)	1.03		2.56	Y	2.50
VIT_00s0131g00220	annexin (ANN3)	1.14		2.68	Y	2.36
VIT_06s0004g06650	1-phosphatidylinositol-4,5-bisphosphate phosphodiesterase	2.27		5.16	Y	2.28
VIT_00s0131g00130	annexin (ANN3)	1.54		3.44	Y	2.24
VIT_18s0001g04120	(-)-germacrene D synthase	2.06		4.67	Y	2.27
VIT_15s0048g00680	NSP-interacting kinase (NIK3)	1.46		3.26	Y	2.22
VIT_00s0131g00130	annexin (ANN3)	1.54		3.44	Y	2.24
VIT_13s0019g00600	annexin (ANN3)	1.17		2.52	Y	2.16
VIT_17s0000g01160	vodulin	1.80	Y	3.88	Y	2.16
VIT_03s0091g01240	serine carboxypeptidase-like gene	10.54	Y	22.55	Y	2.14
VIT_15s0048g02970	extensin	3.40	Y	7.25	Y	2.13
VIT_00s0131g00010	annexin (ANN3)	1.28		2.72	Y	2.12

Table 2.2 **continued**.

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)[#]	significant (SAM)^	berry/skin ratio
VIT_03s0038g03130	flavin containing monooxygenase 3	2.45	Y	5.16	Y	2.11
VIT_08s0007g04180	retrotransposon protein, Ty1-copia subclass	1.40		2.92	Y	2.08
VIT_12s0057g00090	wound-induced	1.05		2.15	Y	2.05
VIT_12s0059g00570	fasciclin arabinogalactan-protein (FLA7)	1.08		2.21	Y	2.05
VIT_00s0396g00020	NADH dehydrogenase subunit 1	-1.05		-5.26	Y	5.01
VIT_19s0014g03520	fiber protein	-1.02		-3.22	Y	3.16
VIT_09s0002g08340	photosystem I P700 chlorophyll a apoprotein A1	-1.09		-3.40	Y	3.13
VIT_18s0002g08340	ent-kaurene oxidase	-1.12		-3.36	Y	3.00
VIT_18s0001g06790	ripening regulated protein DDTFR18	-1.04		-2.95	Y	2.85
VIT_00s0246g00240	ribosomal protein S1 (Rps1)	-1.02		-2.75	Y	2.71
VIT_00s0246g00140	NADH-plastoquinone oxidoreductase subunit 5	-1.05		-2.78	Y	2.65
VIT_00s0505g00040	no hit	-1.02		-2.46	Y	2.41
B) Down-regulated more in whole berries						
VIT_00s0504g00010	photosystem II (PsbD)	-1.30	Y	-2.98	Y	2.30
VIT_02s0033g00980	NADH-plastoquinone oxidoreductase subunit 2	-1.05		-2.39	Y	2.28

Table 2.2 **continued**

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)[#]	significant (SAM)^	berry/skin ratio
VIT_13s0101g00210	unknown	-1.12		-2.49	Y	2.23
VIT_04s0069g01010	harpin-induced protein	-1.25	Y	-2.79	Y	2.23
VIT_00s0275g00010	photosystem II protein D2	-1.29	Y	-2.91	Y	2.25
VIT_16s0013g00330	ribulose 1,5-bisphosphate carboxylase	-1.13		-2.50	Y	2.22
VIT_07s0129g00790	ribulose bisphosphate carboxylase/oxygenase large subunit	-1.32	Y	-2.75	Y	2.09
VIT_00s0246g00190	NADH-plastoquinone oxidoreductase subunit 4	-1.01		-2.06	Y	2.04
VIT_07s0031g03000	ribulose 1,5-bisphosphate carboxylase large subunit	-1.26	Y	-2.52	Y	2.01
VIT_19s0027g00800	Ycf2	-1.16		-2.50	Y	2.16
VIT_00s2608g00010	photosystem II PsbB	-1.12	Y	-2.37	Y	2.11
GSVIVT00028664001	no hit	-1.09		-2.28	Y	2.09
C) Up-regulated more in skins						
VIT_18s0089g00200	1,4-beta-mannan endohydrolase	7.25	Y	1.35		5.38
VIT_08s0040g00940	no hit	5.53	Y	-1.05		5.27
VIT_09s0002g01320	germin-like protein	7.34	Y	1.44		5.10
VIT_18s0086g00590	auxin-binding protein (ABP19)	5.54	Y	1.20		4.61

Table 2.2 **continued**

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)[#]	significant (SAM)^	berry/skin ratio
VIT_08s0040g00960	CXE carboxylesterase	4.98	Y	1.15		4.34
VIT_02s0025g00600	Glycoside hydrolase, family 5	4.90	Y	-1.18		4.14
VIT_04s0044g01880	auxin efflux carrier	5.40	Y	1.03		5.25
VIT_19s0014g01110	curculin (mannose-binding) lectin	6.21	Y	1.20		5.18
VIT_11s0016g02800	myo-inositol oxygenase	5.10	Y	1.23		4.13
VIT_02s0025g01900	cellulose synthase (CSLG3)	5.25	Y	1.27		4.13
VIT_15s0048g02430	naringenin,2-oxoglutarate 3-dioxygenase	4.79	Y	1.19		4.01
VIT_18s0001g14260	no hit	4.29	Y	-1.10		3.90
VIT_18s0086g00410	auxin-binding protein (ABP19)	4.39	Y	1.15		3.81
VIT_06s0004g02590	carbonic anhydrase	3.46	Y	-1.03		3.37
VIT_02s0025g01940	cellulose synthase (CSLG3)	3.61	Y	-1.08		3.34
VIT_16s0098g01060	heat shock protein 26a, chloroplast	3.54	Y	-1.07		3.31
VIT_02s0025g00190	no hit	3.14	Y	1.01		3.10
VIT_07s0005g03410	globulin 11S	3.14	Y	1.02		3.08
VIT_18s0164g00100	laccase	8.96	Y	3.01	Y	2.97
VIT_05s0094g00240	chitinase, class IV [Vitis vinifera]	4.77	Y	1.61	Y	2.96
VIT_03s0063g00720	CXE carboxylesterase (CXE10)	3.41	Y	1.04		3.29
VIT_18s0001g14270	gibberellin-regulated protein 1 (GASA1)	3.91	Y	-1.20		3.24

Table 2.2 **continued**

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)#	significant (SAM)^	berry/skin ratio
VIT_05s0029g01040	aspartate aminotransferase	3.60	Y	1.15		3.14
VIT_09s0002g03340	no hit	10.13	Y	3.23	Y	3.14
VIT_12s0028g03560	Unknown protein	3.57	Y	1.22		2.92
VIT_02s0025g00610	glycoside hydrolase, family 5	3.13	Y	1.08		2.89
GSVIVT00009597001	CYP82C4	3.64	Y	-1.27		2.87
VIT_11s0016g00210	no hit	4.40	Y	1.57	Y	2.80
VIT_19s0090g00240	disease resistance protein RPS2	9.85	Y	3.56	Y	2.77
GSVIVT00023277001	disease resistance protein	4.41	Y	1.60	Y	2.76
VIT_00s2547g00010	WRKY DNA-binding protein 21	3.75	Y	1.52	Y	2.46
VIT_06s0004g06570	calcium/proton exchanger (CAX3)	2.51	Y	-1.02		2.46
VIT_11s0052g00010	no hit	2.74	Y	1.12		2.45
VIT_13s0019g04660	amino acid permease	2.69	Y	1.10		2.44
VIT_14s0060g02000	ATPP2-B14	5.13	Y	2.11	Y	2.42
VIT_12s0059g00470	unknown protein	2.67	Y	1.11		2.42
VIT_00s0316g00040	disease resistance protein	4.04	Y	1.67	Y	2.42
VIT_16s0050g00400	photoassimilate-responsive protein (PAR-1a)	2.68	Y	-1.11		2.41
VIT_01s0011g04980	sulphate transporter 91	3.05	Y	-1.12		2.71
VIT_07s0031g01260	no hit	3.44	Y	1.29		2.67

Table 2.2 **continued**

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)[#]	significant (SAM)^	berry/skin ratio
VIT_00s0316g00010	disease resistance protein	5.83	Y	2.19	Y	2.66
VIT_01s0182g00130	PHO1-like protein	2.66	Y	-1.02		2.60
VIT_04s0023g01480	dimethylaniline monooxygenase, N-oxide-forming	5.14	Y	2.04	Y	2.52
VIT_12s0035g01820	proton-dependent oligopeptide transport (POT) family protein	6.19	Y	2.46	Y	2.52
VIT_12s0028g03640	ripening induced protein	2.42	Y	1.01		2.41
VIT_02s0025g01860	cellulose synthase (CSLG3)	2.95	Y	1.24		2.38
VIT_14s0128g00570	germin	5.12	Y	-2.16		2.37
VIT_04s0008g05700	ACT domain-containing protein	2.36	Y	-1.02		2.33
VIT_15s0046g03190	myb domain protein 17	2.54	Y	-1.09		2.33
VIT_14s0128g00600	germin-like protein 3	5.26	Y	-2.28		2.31
VIT_00s0414g00060	cellulose synthase (CSLE1)	3.91	Y	1.70	Y	2.29
VIT_03s0038g00580	GATA transcription factor 25	2.79	Y	1.21	Y	2.29
VIT_01s0026g02570	kafirin cluster	3.94	Y	1.73	Y	2.28
VIT_18s0075g00350	sucrose-phosphate synthase isoform C	3.22	Y	1.41	Y	2.28
VIT_12s0034g01750	disease resistance RPP13 protein 1	2.28	Y	1.01		2.25
VIT_18s0041g01380	TIR-NBS disease resistance	2.49	Y	1.11		2.25

Table 2.2 **continued**

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)#	significant (SAM)^	berry/skin ratio
VIT_18s0001g12110	no hit	3.16	Y	1.41	Y	2.24
VIT_13s0320g00070	myosin-like protein XIF	6.04	Y	2.74	Y	2.20
VIT_18s0001g11480	CYP82C4	2.41	Y	-1.12		2.16
VIT_18s0001g04920	no hit	2.41	Y	-1.12		2.16
VIT_14s0006g01420	ser/thr receptor kinase	4.04	Y	1.87	Y	2.16
VIT_18s0001g11500	CYP81E1	2.47	Y	1.15		2.16
GSVIVT00033208001	no hit	5.69	Y	2.65	Y	2.15
VIT_18s0122g00620	cinnamoyl-CoA reductase	2.26	Y	-1.07		2.12
VIT_13s0139g00090	disease resistance protein (NBS class)	3.27	Y	1.54	Y	2.12
VIT_04s0044g01870	auxin efflux carrier	2.56	Y	-1.21		2.12
VIT_18s0001g00610	Rho GTPase activator	2.27	Y	-1.07		2.11
VIT_06s0009g02850	CYP79A2	2.95	Y	1.41	Y	2.10
VIT_02s0025g02560	O-succinylhomoserine sulphydrylase	2.57	Y	1.23		2.08
VIT_00s0153g00050	glutathione S-transferase 8 (GSTU8)	3.03	Y	1.46	Y	2.07
VIT_00s0467g00030	disease resistance protein	2.39	Y	1.17		2.05
VIT_13s0064g00830	disease resistance protein RGA2 (RGA2-blb)	2.73	Y	1.35		2.01
VIT_06s0061g01230	cellulose synthase (CSLA02)	5.08	Y	2.53	Y	2.01
VIT_04s0023g03890	atfp6	2.37	Y	1.19	Y	2.00

Table 2.2 **continued**

D) Down-regulated more in skins

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)#	significant (SAM)^	berry/skin ratio
VIT_05s0051g00680	unknown protein	-16.25	Y	-3.94	Y	4.13
VIT_03s0063g00170	basic helix-loop-helic (bHLH) family	-4.45	Y	1.18		3.78
VIT_08s0040g01710	phenylalanine ammonia-lyase (VvPAL1)	-12.77	Y	-3.56	Y	3.58
VIT_05s0051g00690	no hit	-10.18	Y	-3.18	Y	3.20
VIT_00s2849g00010	phenylalanine ammonia-lyase (VvPAL1)	-8.32	Y	-2.75	Y	3.03
VIT_16s0039g01240	phenylalanine ammonia-lyase (VvPAL1)	-7.59	Y	-2.52	Y	3.01
VIT_19s0027g01820	potassium transporter (KUP1)	-39.66	Y	-13.21	Y	3.00
VIT_06s0061g01300	prephenate dehydratase	-2.94	Y	-1.04		2.82
VIT_14s0060g02630	unknown protein	-5.32	Y	-1.89		2.81
VIT_14s0068g00930	chalcone synthase (VvCHS)	-4.03	Y	-1.48	Y	2.72
VIT_16s0039g01280	phenylalanine ammonia-lyase (VvPAL1)	-5.10	Y	-1.92	Y	2.66
VIT_16s0039g01300	phenylalanine ammonia-lyase (VvPAL1)	-6.47	Y	-2.44	Y	2.65
VIT_04s0008g03640	FAD-binding domain-containing protein	-5.16	Y	-1.95	Y	2.64
VIT_11s0052g00870	IAA33	-4.76	Y	-1.96	Y	2.43

Table 2.2 **continued**

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)#	significant (SAM)^	berry/skin ratio
VIT_05s0049g01800	histone-lysine N-methyltransferase (SUVR4)	-2.60	Y	-1.07		2.42
VIT_12s0057g01030	glucose-6-phosphate 1-dehydrogenase 2, chloroplast precursor	-4.08	Y	-1.70		2.40
VIT_16s0039g01360	phenylalanine ammonia-lyase (VvPAL1)	-3.95	Y	-1.70	Y	2.32
VIT_16s0039g01120	phenylalanine ammonia-lyase (VvPAL1)	-3.78	Y	-1.65	Y	2.28
VIT_02s0154g00010	auxin-responsive SAUR11	-4.37	Y	-1.93	Y	2.27
VIT_01s0011g02960	Leucoanthocyanidin reductase 1 (VvLAR)	-4.69	Y	-2.09	Y	2.24
VIT_03s0091g00750	beta-1,4-xylosidase	-3.08	Y	-1.38		2.23
VIT_16s0039g02040	4-coumarate-CoA ligase 3	-3.71	Y	-1.67		2.22
VIT_08s0007g03050	E8 protein	-5.83	Y	-2.66		2.19
VIT_13s0320g00030	unknown protein	-3.65	Y	-1.67		2.18
VIT_04s0008g01800	myb domain protein 7	-3.21	Y	-1.47		2.18
VIT_18s0001g14780	lipase 3 (EXL3) family II extracellular	-4.07	Y	-1.87	Y	2.18
VIT_16s0039g01170	phenylalanine ammonium lyase (VvPAL1)	-4.84	Y	-2.22	Y	2.18
VIT_17s0000g04150	leucoanthocyanidin reductase (VvLAR)	-6.20	Y	-2.87	Y	2.16
VIT_09s0002g05990	6-4 photolyase	-5.45	Y	-2.54	Y	2.14
VIT_06s0004g00220	Protein kinase (APK1B)	-2.41	Y	-1.12		2.14

Table 2.2 **continued**

Microarray gene ID	Annotation/Blast Hits	FC in skin ('red' Chardonnay/control)*	significant (SAM)^	FC in whole berry ('red' Chardonnay/control)[#]	significant (SAM)^	berry/skin ratio
VIT_01s0010g02320	vinorine synthase	-14.40	Y	-6.90	Y	2.09
VIT_14s0006g00140	no hit	-3.49	Y	-1.69		2.06
VIT_15s0046g00490	wax synthase	-4.05	Y	-1.98	Y	2.04
VIT_05s0020g03000	2-hydroxyisoflavanone dehydratase	-5.23	Y	-2.61	Y	2.00

Transcript levels determined using microarrays

*'red' Chardonnay contained a *35S:VvMYBA1* construct and expressed the *VvMYBA1* gene. Non-transgenic un-pigmented Chardonnay berries were the control for these experiments

[#]'white' Shiraz contained a *VvMYBAsi* construct which completely silenced the expression of *VvMYBA1/2* genes. Non-transgenic red/black Shiraz berries were used as the control for these experiments.

^SAM = Significance Analysis of Microarray. Y indicates that the fold change ratio was significant as determined by a SAM

FC = Fold change

2.3.4 Analysis of gene expression changes in red, rose and white Shiraz berries

When analysing the microarray data obtained from transgenic ‘rose’, ‘white’, and non-transgenic control (red) Shiraz berries it was noticed that some genes had unexpected expression patterns such as those which had altered expression in the transgenic ‘rose’ berries but not in the ‘white’ berries. This led to a more in-depth analysis of all the genes with significant expression changes in the Shiraz microarray dataset. In this analysis, genes were sorted into different expression patterns in relation to berry colour. Twelve expression trends were discovered and these are explained in Figure 2.3. Table 2.3 shows the genes sorted into their expression trends 1 – 8 (trends 9-12 can be found in Appendix C).

Figure 2.3: Representation of gene expression trends of genes with altered transcription in transgenic 'rose' and 'white' Shiraz compared to control berries

Transgenic 'rose and 'white' Shiraz contained the *VvMYBAsi* construct designed to silence both *VvMYBA1* and *VvMYBA2* genes. Gene expression was determined by microarray analysis using the NimbleGen microarray 090818 Vitis exp HX12. Within each trend, boxes with the same colour indicate that there was no significant difference in gene expression between those samples. A different coloured box indicates a significant difference in gene expression between samples differing in berry colour as determined by a Significance Analysis of Microarray (SAM) test. Red indicates the highest gene expression, orange an intermediate expression level and yellow the lowest.

Table 2.3: Genes with altered expression in transgenic 'white' or 'rose' Shiraz separated into gene expression trends (1-8, see Figure 2.3)

Trend 1 (Red > Rose > White)		**'rose' Shiraz/control***		**'white' Shiraz/ control***		**'rose' Shiraz/'white' Shiraz***	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_04s0079g00690	Glutathione S-transferase (VvGST)	-2.85	*	-12.29	*	-4.31	*
VIT_16s0039g02230	UDP-glucose:flavonoid 3-*O*-glucosyltransferase (VvUFGT)	-5.15	*	-21.18	*	-4.12	*
VIT_03s0017g00870	BAHD acyl transferase	-7.93	*	-23.81	*	-3.00	
VIT_01s0010g03490	Flavonoid and anthocyanin-*O*-methyltransferase (VvFAOMT)	-12.55	*	-33.84	*	-2.70	
VIT_06s0009g02920	flavonoid 3',5'-hydroxylase (VvF3'5'H)	-31.05	*	-65.42	*	-2.11	*
Trend 2 (White > Rose > Red)							
VIT_10s0092g00310	unknown	1.75	*	35.77	*	20.49	*
VIT_10s0003g01880	receptor-like kinase in flowers 1 (RKF1)	1.44	*	3.78	*	2.62	*
VIT_00s0878g00020	stachyose synthase precursor	1.64	*	4.01	*	2.44	*
VIT_00s1530g00010	stachyose synthase precursor	1.51	*	3.48	*	2.31	*
VIT_04s0008g02670	cryptochrome DASH	1.60	*	3.32	*	2.07	*
Trend 3 (Red > White & Rose)							
VIT_06s0009g02880	Flavonoid 3',5'-hydroxylase (VvF3'5'H)	-20.11	*	-38.93	*	-1.94	*
VIT_06s0009g03000	Flavonoid 3',5'-hydroxylase (VvF3'5'H)	-14.67	*	-27.63	*	-1.88	*
VIT_01s0010g03510	Flavonoid and anthocyanin -O-methyltransferase (VvFAOMT)	-7.36	*	-13.42	*	-1.82	
VIT_06s0009g02830	Flavonoid 3',5'-hydroxylase (VvF3'5'H)	-24.26	*	-43.02	*	-1.77	
VIT_06s0009g02810	Flavonoid 3',5'-hydroxylase (VvF3'5'H)	-26.27	*	-46.55	*	-1.77	
VIT_06s0009g02970	Flavonoid 3',5'-hydroxylase (VvF3'5'H)	-28.14	*	-49.74	*	-1.77	

Table 2.3 continued

Trend 3 (Red > White & Rose) cont...		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_06s0009g02840	Flavonoid 3',5'-hydroxylase (VvF3'5'H)	-37.86	*	-65.96	*	-1.74	
VIT_06s0009g03050	Flavonoid 3',5'-hydroxylase (VvF3'5'H)	-18.63	*	-32.17	*	-1.73	*
VIT_06s0009g03040	Flavonoid 3',5'-hydroxylase (VvF3'5'H)	-17.09	*	-29.14	*	-1.71	*
VIT_06s0009g03110	Flavonoid 3,5'-hydroxylase (VvF3'5'H)	-13.12	*	-22.11	*	-1.68	*
VIT_18s0001g09400	cytochrome b5 DIF-F	-5.74	*	-9.55	*	-1.66	
VIT_05s0136g00260	chalcone synthase (VvCHS)	-2.71	*	-4.35	*	-1.61	*
VIT_02s0012g01170	pyruvate kinase	-1.08	*	-1.71	*	-1.58	*
VIT_02s0012g01570	cinnamoyl-CoA reductase	-1.46	*	-2.26	*	-1.55	*
VIT_06s0009g02860	flavonoid 3',5'-hydroxylase (VvF3'5'H)	-18.95	*	-29.15	*	-1.54	
VIT_13s0019g04460	phenylalanine ammonia-lyase 2 (VvPAL2)	-1.43	*	-2.07	*	-1.45	*
VIT_05s0094g00360	chitinase class IV	-1.16	*	-1.52	*	-1.31	*
VIT_09s0002g02330	nucleosome/chromatin assembly factor group A	-1.11	*	-1.45	*	-1.31	*
VIT_04s0023g03370	Flavonone- 3-hydroxylase (VvF3H)	-1.97	*	-2.57	*	-1.30	*
VIT_16s0022g01140	chalcone synthase (VvCHS)	-34.74	*	-43.95	*	-1.26	
VIT_18s0001g00210	lysine decarboxylase	-2.02	*	-2.54	*	-1.26	*
VIT_10s0003g02810	WRKY DNA-binding protein 71	-1.68	*	-2.07	*	-1.24	
VIT_05s0020g03140	sugar transporter 13	-1.21	*	-1.49	*	-1.23	*
VIT_16s0050g01890	unknown	-2.34	*	-2.87	*	-1.23	
VIT_10s0003g01160	basic helix-loop-helix (bHLH) family	-1.80	*	-2.15	*	-1.19	
VIT_16s0050g00910	anthoMATE1 transport protein (VvanthoMATE1)	-2.78	*	-3.29	*	-1.18	
VIT_16s0022g01000	chalcone synthase [Vitis vinifera]	-10.43	*	-12.22	*	-1.17	

Table 2.3 continued

Trend 3 (Red > White & Rose) cont...		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_19s0014g04660	flavodoxin-like quinine-reductase-1	-1.05	*	-1.21	*	-1.16	*
VIT_06s0004g08150	trans-cinnamate 4-monooxygenase	-2.10	*	-2.42	*	-1.15	
VIT_16s0022g01190	chalcone synthase (VvCHS)	-24.10	*	-26.55	*	-1.10	
VIT_06s0004g02620	phenylalanine ammonia-lyase (VvPAL1)	-4.40	*	-4.83	*	-1.10	
VIT_16s0022g01020	chalcone synthase (VvCHS)	-28.34	*	-30.73	*	-1.08	
VIT_13s0067g03820	Chalcone--flavonone isomerase (VvCHI)	-1.68	*	-1.79	*	-1.07	
VIT_18s0072g00160	peroxidase 12	-1.57	*	-1.64	*	-1.04	
VIT_11s0016g04330	no hit	-5.23	*	-5.07	*	1.03	
VIT_08s0007g03560	anthocyanin membrane protein 1 (VvAnm1)	-7.4706	*	-7.237	*	1.0323	
VIT_02s0025g04720	leucoanthocyanidin dioxygenase (VvLDOX)	-2.14	*	-2.01	*	1.06	
VIT_06s0061g00460	lactoylglutathione lyase	-1.44	*	-1.31	*	1.10	
VIT_03s0091g01240	serine carboxypeptidase-like gene	-11.49	*	-10.43	*	1.10	
VIT_02s0033g00390	myb (VvMYBA2)	-2.93	*	-2.66	*	1.10	
VIT_08s0007g05430	pyruvate kinase	-2.69	*	-2.43	*	1.11	
VIT_14s0128g00160	protein kinase CDG1	-3.28	*	-2.92	*	1.13	
VIT_13s0067g01080	protein kinase	-1.62	*	-1.44	*	1.13	
VIT_07s0005g06090	pore-forming toxin-like protein Hfr-2	-2.13	*	-1.88	*	1.13	
VIT_05s0020g03710	GCN5 N-acetyltransferase (GNAT)	-1.52	*	-1.31	*	1.16	*
VIT_12s0059g00640	beta-1,3-galactosyltransferase sqv-2	-1.34	*	-1.09	*	1.24	*
VIT_18s0001g12340	unknown protein	-1.83	*	-1.48	*	1.24	*
VIT_00s0391g00070	3-deoxy-D-arabino-heptulosonate 7-phosphate synthase	-1.67	*	-1.34	*	1.24	*

Table 2.3 continued

Trend 3 (Red > White & Rose) cont...		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_14s0060g01130	no hit	-1.77	*	-1.42	*	1.25	*
VIT_14s0068g00920	chalcone synthase (VvCHS)	-2.50	*	-2.01	*	1.25	
VIT_14s0068g02240	HcrVf1 protein	-1.98	*	-1.59	*	1.25	
VIT_16s0039g01300	phenylalanine ammonia-lyase (VvPAL1)	-2.04	*	-1.60	*	1.28	*
VIT_12s0057g00420	auxin-responsive protein (AIR12)	-1.85	*	-1.42	*	1.31	*
VIT_14s0060g02090	copper chaperone (CCH)	-2.49	*	-1.88	*	1.32	*
VIT_16s0022g01070	chalcone synthase (VvCHS)	-6.44	*	-4.84	*	1.33	
VIT_17s0000g03160	no hit	-2.54	*	-1.91	*	1.33	*
VIT_13s0158g00270	no hit	-1.84	*	-1.37	*	1.34	*
VIT_02s0033g00380	myb (VvMYBA1)	-3.18	*	-2.37	*	1.34	
VIT_16s0050g02310	no hit	-1.69	*	-1.24	*	1.36	*
VIT_11s0016g05530	plastocyanin domain-containing protein	-1.61	*	-1.15	*	1.40	*
VIT_16s0039g01170	phenylalanine ammonium lyase (VvPAL1)	-2.17	*	-1.55	*	1.40	*
VIT_16s0100g00830	stilbene synthase	-2.66	*	-1.89	*	1.41	*
VIT_02s0087g00330	glycosyl transferase family 1 protein	-2.42	*	-1.69	*	1.43	*
VIT_10s0116g00820	adenosine/AMP deaminase	-2.29	*	-1.53	*	1.49	*
VIT_16s0039g01320	phenylalanine ammonia-lyase (VvPAL1)	-2.62	*	-1.64	*	1.60	*
VIT_13s0074g00400	petal loss-like protein	-2.51	*	-1.56	*	1.61	*
VIT_03s0091g00500	unknown protein	-3.71	*	-2.26	*	1.64	*
VIT_03s0091g00510	unknown	-4.35	*	-2.63	*	1.65	*

Table 2.3 continued

Trend 4 (White & Rose > Red)		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_00s0480g00030	polyphenol oxidase	-2.50	*	-1.49	*	1.67	*
VIT_05s0049g01020	VvMybB1	-2.52	*	-1.47	*	1.71	*
VIT_11s0016g04920	early nodulin 93	-2.27	*	-1.32	*	1.72	*
VIT_02s0025g01080	no hit	-3.13	*	-1.72	*	1.81	*
VIT_00s0480g00040	polyphenol oxidase II, chloroplast precursor	-3.96	*	-2.01	*	1.97	*
VIT_16s0100g00770	stilbene synthase	-3.56	*	-1.79	*	1.99	*
VIT_00s0131g00060	annexin (ANN3)	4.37	*	2.25	*	-1.94	*
VIT_00s0131g00010	annexin (ANN3)	3.97	*	2.16	*	-1.84	*
VIT_15s0024g01910	no hit	2.28	*	1.37	*	-1.67	*
VIT_00s1206g00010	aspartic proteinase nepenthesin-1 precursor	2.88	*	1.77	*	-1.63	*
VIT_01s0011g02260	metal-nicotianamine transporter YSL7	1.95	*	1.39	*	-1.41	*
VIT_01s0011g02260	metal-nicotianamine transporter YSL7	1.95	*	1.39	*	-1.41	*
VIT_03s0017g01740	annexin (ANN3)	2.23	*	1.61	*	-1.39	*
VIT_05s0020g00130	unknown protein	1.73	*	1.25	*	-1.38	*
VIT_17s0000g02370	receptor protein kinase	1.94	*	1.52	*	-1.27	*
VIT_02s0025g04850	CYP76B1	1.59	*	1.31	*	-1.22	*
VIT_05s0020g00050	no hit	2.20	*	1.86	*	-1.19	*
VIT_06s0004g08080	zinc finger (C3HC4-type RING finger)	1.73	*	1.46	*	-1.18	*
VIT_03s0063g01630	CYP82C1p	2.59	*	2.19	*	-1.18	
VIT_06s0004g00610	accelerated cell death 1 (ACD1)	2.16	*	1.84	*	-1.17	*
VIT_18s0001g02050	tolB protein-related	1.35	*	1.16	*	-1.17	*

Table 2.3 continued

Trend 4 (White & Rose > Red) cont...		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_13s0067g01310	disease resistance protein (NBS-LRR class) Hom-B	2.41	*	2.08	*	-1.16	*
VIT_14s0060g00160	nucleoporin	1.29	*	1.13	*	-1.14	*
VIT_06s0004g04460	albino 3 (ALB3)	1.26	*	1.11	*	-1.14	*
VIT_16s0050g02480	ABC transporter C member 15	1.28	*	1.13	*	-1.13	*
VIT_02s0025g03530	gamma-glutamylcysteine synthetase	1.33	*	1.21	*	-1.10	*
VIT_08s0007g00840	ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor	1.60	*	1.49	*	-1.08	*
VIT_05s0020g04110	aarly light-inducible protein (ELIP1)	1.56	*	1.47	*	-1.07	
VIT_05s0094g00750	stearoyl-CoA 9-desaturase	1.15	*	1.08	*	-1.06	*
VIT_03s0017g02360	aarF domain containing kinase	1.25	*	1.18	*	-1.06	*
VIT_02s0012g01910	unknown protein	1.55	*	1.48	*	-1.05	
VIT_14s0068g00500	indole-3-acetate beta-glucosyltransferase	1.34	*	1.30	*	-1.03	
VIT_19s0140g00210	SOUL heme-binding	1.82	*	1.82	*	-1.00	
VIT_04s0008g05130	TSO1	5.56	*	5.57	*	1.00	
VIT_11s0118g00130	disease resistance protein	2.64	*	2.68	*	1.02	
VIT_02s0025g04730	glyoxylate reductase	1.62	*	1.66	*	1.02	
VIT_16s0148g00090	GASA4	8.24	*	8.42	*	1.02	
VIT_14s0006g00910	ATA15 protein	1.30	*	1.34	*	1.03	
VIT_08s0032g00760	eukaryotic translation initiation factor 2B family protein, putative, expressed	1.11	*	1.15	*	1.04	*

Table 2.3 continued

Trend 4 (White & Rose > Red) cont...		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_15s0107g00120	MADS-box protein short vegetative phase (SVP)	2.91	*	3.05	*	1.05	
VIT_01s0127g00310	Unknown protein	4.58	*	4.96	*	1.08	
VIT_06s0004g00260	SHOOT1 protein	1.62	*	1.79	*	1.10	
VIT_19s0027g00130	translation initiation factor IF-2, chloroplast	1.31	*	1.51	*	1.16	*
VIT_17s0000g08970	proline-rich family protein	1.16	*	1.38	*	1.19	*
VIT_18s0001g02460	unknown protein	1.20	*	1.44	*	1.20	*
VIT_19s0093g00510	S-2-hydroxy-acid oxidase, peroxisomal	1.30	*	1.56	*	1.20	*
VIT_06s0004g05180	ribulose bisphosphate carboxylase/oxygenase activase, chloroplast precursor	1.33	*	1.62	*	1.21	*
VIT_12s0034g02470	disease resistance protein (CC-NBS-LRR class)	1.17	*	1.43	*	1.22	*
VIT_12s0034g02470	disease resistance protein (CC-NBS-LRR class)	1.17	*	1.43	*	1.22	*
VIT_04s0023g03510	ferredoxin:nadp+ Oxidoreductase (PETH)	1.13	*	1.39	*	1.23	*
VIT_05s0049g00220	2-oxoglutarate-dependent dioxygenase	1.26	*	1.56	*	1.24	*
VIT_11s0065g00520	small G protein / RhoGAP	17.01	*	21.19	*	1.25	
VIT_03s0180g00290	gibberellin-regulated protein 4 (GASA4)	4.15	*	5.23	*	1.26	*
VIT_18s0001g05780	leucine-rich repeat family protein	1.07	*	1.34	*	1.26	*
VIT_00s0131g00210	annexin (ANN3)	1.93	*	2.51	*	1.30	*
VIT_11s0016g03830	protein kinase	1.35	*	1.77	*	1.31	*
VIT_11s0065g00560	small G protein / RhoGAP	4.50	*	5.92	*	1.32	*
VIT_16s0022g01650	receptor serine/threonine kinase (PR5K)	1.25	*	1.67	*	1.33	*
VIT_08s0056g01070	MATE efflux family protein	2.62	*	4.04	*	1.54	*

Table 2.3 continued

Trend 4 (White & Rose > Red) cont...		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_15s0024g01270	CLAVATA1 receptor kinase (CLV1)	3.26	*	5.67	*	1.74	*
VIT_04s0008g00290	CLAVATA1 receptor kinase (CLV1)	2.74	*	4.78	*	1.74	*
VIT_10s0003g01920	receptor-like kinase in flowers (RKF1)	1.64	*	2.96	*	1.81	*
VIT_00s0125g00140	SHR5-receptor-like kinase	1.54	*	2.87	*	1.87	*
VIT_04s0044g01860	auxin efflux carrier	1.40	*	2.78	*	1.99	*
Trend 5 (Red & Rose > White)							
VIT_00s0125g00310	unknown protein	1.09		-4.75	*	-5.17	*
VIT_08s0040g03170	no hit	-1.10		-2.84	*	-2.57	*
VIT_16s0100g01260	no hit	1.02		-1.78	*	-1.81	*
VIT_03s0038g04280	protein BCCIP homolog	1.05		-1.45	*	-1.51	*
Trend 6 (White > Red & Rose)							
VIT_13s0064g00980	telomerase reverse transcriptase (TERT)	1.17		85.24	*	72.76	*
VIT_05s0051g00050	myosin-like protein (XIB)	1.29		8.34	*	6.45	*
VIT_17s0000g06900	DNA helicase SNF2 domain-containing protein	-1.07		6.03	*	6.45	*
VIT_10s0092g00300	unknown	1.40		6.67	*	4.76	*
VIT_11s0149g00040	adenylate kinase	-1.19		3.09	*	3.67	*
VIT_18s0041g02180	LIM domain containing protein-like	1.49		4.58	*	3.07	*
VIT_03s0038g04210	phototropin-2	-1.10		2.10	*	2.31	*
VIT_11s0016g02740	beta-carotene 15,15'-monooxygenase	1.17		2.38	*	2.03	*

Table 2.3 continued

Trend 6 (White > Red & Rose) cont...		**'rose' Shiraz/control***		**'white' Shiraz/ control***		**'rose' Shiraz/'white' Shiraz***	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_16s0050g02320	tRNA-splicing endonuclease positive effector	-1.04		1.87	*	1.94	*
Trend 7 (Rose > White & Red)							
VIT_01s0011g00850	unknown protein	1.07		2.08	*	1.94	*
VIT_18s0001g14450	ferredoxin:nadp+ Oxidoreductase PETH	-1.02		1.88	*	1.92	*
VIT_16s0022g01670	indole-3-acetate beta-glucosyltransferase	1.09		1.96	*	1.79	*
VIT_16s0022g01670	indole-3-acetate beta-glucosyltransferase	1.09		1.96	*	1.79	*
VIT_08s0007g08540	Mg-chelatase subunit XANTHA-F	-1.06		1.68	*	1.77	*
VIT_18s0041g01170	lectin protein kinase	1.25		2.07	*	1.66	*
VIT_01s0011g03910	protein phosphatase 2C	-1.02		1.44	*	1.46	*
VIT_10s0003g02860	UDP-N-acetylglucosamine transferase subunit ALG14, related	1.06		1.55	*	1.46	*
VIT_09s0002g09200	CYP82M1v3	17.27	*	1.04		-16.54	*
VIT_15s0046g00680	wax synthase	6.78	*	-1.90		-12.92	*
VIT_09s0002g09210	CYP82M1v3	12.22	*	-1.06		-12.90	*
VIT_09s0002g09270	CYP82M1v4	9.84	*	-1.01		-9.94	*
VIT_18s0072g00450	unknown protein	4.37	*	-1.70		-7.44	*
VIT_13s0067g00090	pinene synthase	14.74	*	2.22		-6.65	*
VIT_00s2271g00010	pinene synthase	9.19	*	1.44		-6.40	*
VIT_16s0100g00250	mandelonitrile lyase-like protein	11.42	*	2.14		-5.34	*
VIT_08s0040g00490	caspase	3.38	*	-1.56		-5.28	*

Table 2.3 continued

Trend 7 (Rose > White & Red) cont...		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_18s0001g04780	(-)-germacrene D synthase	8.24	*	1.64		-5.03	*
VIT_16s0100g00230	glucose-methanol-choline (GMC) oxidoreductase family protein	10.76	*	2.23		-4.82	*
VIT_13s0067g00250	(-)-a-terpineol synthase	8.00	*	1.72		-4.65	*
VIT_09s0096g00210	coniferyl alcohol acyltransferase	4.53	*	1.02		-4.46	*
VIT_19s0014g04880	(-)-germacrene D synthase	4.02	*	-1.03		-4.15	*
VIT_18s0001g04560	Germacrene-D synthase	10.10	*	2.56		-3.94	*
VIT_19s0015g02080	(-)-germacrene D synthase	5.89	*	1.50		-3.93	*
VIT_19s0014g04840	(-)-germacrene D synthase	4.77	*	1.24		-3.84	*
VIT_16s0100g00240	mandelonitrile lyase-like protein	6.79	*	1.77		-3.83	*
VIT_01s0011g03530	lateral organ boundaries protein 41	3.84	*	1.02		-3.78	*
VIT_19s0090g01380	no hit	3.91	*	1.07		-3.65	*
VIT_19s0014g04850	germacrene-D synthase	3.89	*	1.07		-3.64	*
VIT_15s0048g02640	no hit	3.68	*	1.07		-3.46	*
VIT_02s0025g01090	unknown protein	3.24	*	-1.06		-3.42	*
VIT_00s0724g00010	pinene synthase	3.64	*	1.12		-3.24	*
VIT_17s0000g09770	cysteine endopeptidase	4.29	*	1.34		-3.20	*
VIT_19s0014g04930	germacrene-D synthase	3.99	*	1.30		-3.07	*
VIT_09s0096g00630	unknown	3.47	*	1.14		-3.03	*
VIT_07s0005g00870	erg-1	2.18	*	-1.35		-2.94	*
VIT_16s0050g00540	no hit	3.04	*	1.05		-2.89	*
VIT_05s0094g00740	stearoyl-acyl-[acyl-carrier-protein] desaturase	8.07	*	2.80		-2.88	*
VIT_18s0001g05000	(-)-germacrene D synthase	5.84	*	2.06		-2.84	*

Table 2.3 continued

Trend 7 (Rose > White & Red) cont...		**'rose' Shiraz/control***		**'white' Shiraz/ control***		**'rose' Shiraz/'white' Shiraz***	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_19s0014g04370	no hit	3.00	*	1.08		-2.78	*
VIT_09s0096g00720	C2-HC type zinc finger protein C.e-MyT1	3.42	*	1.23		-2.77	*
VIT_02s0025g03630	Cu2+-exporting ATPase, Heavy metal ATPase (HMA5)	2.56	*	1.05		-2.45	*
VIT_18s0001g14920	unknown protein	3.32	*	1.38		-2.41	*
VIT_13s0320g00020	signal transducer	2.75	*	1.14		-2.41	*
VIT_05s0094g00580	stearoyl-CoA 9-desaturase	3.10	*	1.35		-2.30	*
VIT_00s1216g00020	CYP82C1p	3.29	*	1.43		-2.30	*
VIT_17s0000g06550	no hit	3.04	*	1.39		-2.18	*
VIT_04s0008g04840	ABC transporter g family pleiotropic drug resistance 12 PDR12	2.50	*	1.17		-2.13	*
VIT_15s0046g01590	acidic chitinase III	1.95	*	-1.03		-2.02	*
VIT_18s0001g09000	no hit	3.32	*	1.68		-1.98	*
VIT_01s0026g02120	microtubule end binding-protein 1 (EB1)	2.18	*	1.11		-1.96	*
VIT_06s0004g07230	indole-3-acetate beta-glucosyltransferase	1.62	*	-1.19		-1.92	*
Trend 8 (White & Red > Rose)							
VIT_05s0094g00560	stearoyl-ACP desaturase	2.28	*	1.20		-1.90	*
VIT_17s0000g03900	no hit	1.80	*	-1.03		-1.86	*
VIT_19s0014g04800	(-)-germacrene D synthase	2.64	*	1.55		-1.70	*
VIT_02s0087g00930	9-cis-epoxycarotenoid dioxygenase	1.53	*	-1.06		-1.62	*
VIT_05s0020g05060	cellulose synthase (CSLG2)	1.64	*	1.08		-1.52	*

Table 2.3 continued

Trend 8 (White & Red > Rose) cont...		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_04s0210g00110	AMP-dependent synthetase and ligase	1.68	*	1.13		-1.48	*
VIT_14s0128g00720	long-chain-fatty-acid--CoA ligase.	1.60	*	1.14		-1.40	*
VIT_04s0008g02830	galactokinase like protein	1.37	*	1.00		-1.37	*
VIT_11s0016g00470	sucrose synthase	1.29	*	1.05		-1.24	*
VIT_14s0128g00750	aminopeptidase	1.25	*	1.05		-1.19	*
VIT_10s0116g01910	ABC transporter I member 15	1.23	*	1.04		-1.18	*
VIT_00s0160g00110	TIR-NBS-TIR type disease resistance protein	-4.88	*	1.11		5.40	*
VIT_00s0160g00100	disease resistance protein (TIR-NBS-LRR class)	-3.95	*	-1.19		3.32	*
VIT_14s0128g00790	lipoxygenase (LOX1)	-3.02	*	-1.01		2.99	*
VIT_18s0164g00100	Laccase	-3.15	*	-1.06		2.99	*
VIT_14s0128g00780	lipoxygenase	-2.47	*	1.14		2.80	*
VIT_14s0081g00010	IAA16	-2.56	*	1.05		2.68	*
VIT_19s0177g00230	no hit	-2.21	*	1.20	*	2.64	*
VIT_07s0031g02060	cellulose synthase (CESA2)	-2.98	*	-1.16		2.56	*
VIT_18s0001g10310	protein kinase family	-2.27	*	1.12		2.55	*
VIT_18s0001g11590	dual-specific kinase (DSK1)	-2.26	*	1.13		2.55	*
VIT_06s0004g01580	lipoxygenase	-2.54	*	-1.01		2.53	*
VIT_02s0025g03420	unknown protein	-2.70	*	-1.09		2.48	*
VIT_00s0160g00310	disease resistance protein (TIR-NBS-LRR class)	-2.06	*	1.19		2.46	*
VIT_01s0010g01850	tRNA-splicing endonuclease positive effector	-2.02	*	1.09		2.20	*

Table 2.3 continued

Trend 8 (White & Red > Rose) cont...		'rose' Shiraz/control*		'white' Shiraz/ control*		'rose' Shiraz/'white' Shiraz*	
Microarray gene ID	**Annotation/ BLAST hits**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**	**FC**	**significant (SAM)^**
VIT_03s0063g00750	carboxylesterase (CXE)	-2.35	*	-1.13		2.08	*
VIT_13s0139g00100	disease resistance protein (CC-NBS-LRR class)	-2.33	*	-1.18		1.98	*
VIT_14s0108g00190	aldose 1-epimerase	-2.14	*	-1.09		1.97	*
VIT_10s0003g04540	cationic amino acid transporter 1	-1.99	*	-1.03		1.92	*
VIT_16s0013g01120	ethylene-responsive transcription factor (ERF105)	-1.67	*	1.09		1.82	*
VIT_13s0019g02210	sterol 4-alpha-methyl-oxidase 1 (SMO1)	-1.63	*	1.10		1.79	*
VIT_00s0324g00050	UDP-glucose glucosyltransferase	-1.84	*	-1.05		1.75	*
VIT_09s0096g00760	disease resistance protein (NBS-LRR class)	-1.75	*	-1.03		1.70	*
VIT_12s0035g01900	pectinesterase family	-1.47	*	1.04		1.54	*
VIT_08s0056g00290	calcium-binding allergen Bet v 3 (Bet v III)	-1.59	*	-1.09		1.47	*
VIT_18s0122g00630	cinnamoyl-CoA reductase	-1.31	*	1.07		1.40	*
VIT_12s0028g03650	two-pore calcium channel (TPC1)	-1.28	*	1.05		1.35	*
VIT_08s0007g06040	beta-1,3-glucanase	-1.34	*	-1.02		1.32	*
VIT_03s0038g04670	isoflavone reductase	-1.17	*	1.03		1.21	*
VIT_05s0020g00930	soluble diacylglycerol acyltransferase	1.57	*	-1.08		-1.69	*

*'white' and 'rose' Shiraz contained a *VvMYBAsi* construct which completely (white) or partially (rose) silenced the expression of *VvMYBA1* and *VvMYBA2* genes. Non-transgenic red/black Shiraz berries were used as the control for these experiments. Transcript levels were determined by microarrays

^SAM = Significance Analysis of Microarray. Y indicates that the fold change ratio was significant as determined by a SAM FC = Fold change

2.3.4.1 Expression trends 1 and 2

Expression trends 1 and 2 represent genes that had expression patterns that followed the trend of anthocyanin concentration in the berries. That is, gene expression in red berries was > in rose berries which was > in white berries (trend 1) or gene expression in white berries was > in rose berries which was > in red berries (trend 2). Of the 305 genes that had significantly altered expression in the 'rose' and/or 'white' Shiraz berries compared to controls, only 5 genes followed expression patterns represented by trend 1 and another 5 fell into trend 2. Four of the 5 genes in trend 1 are flavonoid pathway genes *VvFAOMT, VvF3'5'H* and *VvUFGT* and the anthocyanin transport gene *VvGST*. The other gene in this group was the putative BAHD acyltransferase gene. Trend 2 did not contain any flavonoid related genes, and there were no links between their annotated functions. They code for, or have homology to the following proteins: a receptor-like kinase in flowers-1, two stachyose synthases, cryptochrome DASH and an unknown protein.

2.3.4.2 Expression trends 3 and 4

In expression trends 3 and 4 were genes with transcription levels that were significantly altered when comparing 'red' berry transcriptomes to those of 'rose' and 'white', but there was no significant difference between the latter two colour groups. That is, gene expression in red berries was > in rose and white (trend 3) or gene expression in rose and white berries was > in red berries (trend 4). These two categories had the largest number of genes in them (74 and 85 genes respectively) compared to the other trends.

Many of the genes that fell into trend 3 have known or putative roles in anthocyanin and flavonoid biosynthesis or transport. These were *VvPAL, VvPAL2, VvCHS, VvCHI, cinnamoyl-CoA reductase, trans-cinnamate-4-monooxygenase, leucoanthocyanidin dioxygenase (VvLDOX),* a putative anthocyanin membrane protein and *VvanthoMATE1*. *VvF3'5'H* and *VvFAOMT* were represented by multiple microarray gene IDs due to the presence of multiple copies of these genes in the grapevine genome. A number of these gene copies of both *VvF3'5'H* and *VvFAOMT* were categorised into expression trend 3 as well as trend 1. The *VvMYBA1* and *VvMYBA2* genes were in trend 3, both genes being the silencing targets in the transgenic Shiraz plants, as were a number of uncharacterised genes belonging to the WRKY (microarray gene ID VIT_10s0003g02810) and bHLH (microarray gene ID

VIT_10s0003g01160) TF families. The putative SCPL acyltransferase gene (discussed in section 2.3.2.3) was also sorted into trend 3. Other genes in this category coded for proteins with a range of functions including pyruvate kinase, protein kinases, an auxin-responsive protein, a stilbene synthase and an adenosine/AMP deaminase.

Unlike in trend 3, the genes in trend 4 were not associated with anthocyanin and flavonoid synthesis expect for *VvMYBB1* whose expression has been correlated to anthocyanin accumulation in berries previously (Kobayashi et al., 2002). In this trend there were genes with a variety of known or putative functions. More common functions included receptor kinase genes, a number of disease resistance genes and genes coding for proteins involved in transport, particularly across membranes, or that are associated with membranes, such as annexin, a metal nicotianamine transporter, a nucleoporin, an ABC transporter, and a MATE efflux protein.

2.3.4.3 Expression trends 5 and 6

Trends 5 and 6 contained genes that were expressed to significantly different levels in white berries compared to red and rose berries but with no significant difference between the latter two colour groups. That is, red and rose berry gene expression is > in white berries (trend 5), or white berry gene expression is > in red and rose berries (trend 6). There were only a small number of genes sorted into these categories, four in trend 5 and nine in trend 6. Of the four genes in trend 5, three of them were annotated as unknown functions. The other was homologous to a BCCIP (BRCA2 and CDKN1A-interacting protein**)** gene which codes for a nuclear protein involved in chromosomal recombination and cytokinesis during cell replication in humans (Meng et al., 2007). The genes in trend 6 were annotated to code for proteins of diverse functions but included three proteins associated with nucleic acids: a telomerase reverse transcriptase, a DNA helicase and a tRNA-splicing endonuclease.

2.3.4.4 Expression trends 7 and 8

Trends 7 and 8 represented genes which had significantly higher (trend 7) or lower (trend 8) expression levels in rose berries compared to white and red berries (which had no significant difference when compared). There were quite a large number of genes in these categories, 56 in trend 7 and 29 in trend 8. Many of the genes which were expressed more highly in rose berries (trend 7) are associated with the synthesis of flavour/aroma compounds such as *pinene*

synthase, *germacerene D synthase*, *terpineol synthase* and *coniferyl alcohol acyltransferase* genes. Genes also coding for proteins involved in fatty acid synthesis such as stearoyl desaturases and wax synthases were in this category. In trend 8 there were a number of disease resistance and lipoxygenase genes which were down-regulated in rose berries compared to red and white berries. The other genes in this category had a range of possible functions from which less obvious links could be drawn.

2.3.4.5 Expression trends 9-12

Trends 9-12 contained genes which had expression levels significantly different in all three berry colour categories (red, rose and white) but did not follow the trend of anthocyanin concentration or *VvMYBA* gene expression (Appendix C). The number of genes in these trends were small (7 genes in trend 9, 20 genes in trend 10, 2 genes in trend 11 and 10 genes in trend 12) and they had a range of different functions which could not be easily grouped.

2.4 Discussion

2.4.1 Over-expression of *VvMYBA* in white berries has greater effects on berry transcription than its silencing in red berries

A *Vitis* microarray chip was used to analyse the transcriptomes of transgenic Chardonnay and Shiraz berries with altered *VvMYBA* gene expression and compare them to controls. A large number of genes were found to have significantly altered expression levels in the transgenic berries. Over-expression of *VvMYBA1* in the white grape cultivar Chardonnay had a greater effect, in terms of the number of genes that were transcriptionally up-regulated, than did the knockout of its expression in the red grape background Shiraz. Previous to this PhD project, qPCR studies showed the levels of *VvMYBA* transcripts were many times higher than controls in berries from the transgenic Chardonnay lines used in this study. Anthocyanin concentrations were estimated to be four to five times higher in these transgenic berries compared to those found in wild-type Shiraz (Walker, personal communications). Such high levels were achieved due to the use of the 35S constitutive promoter from the *Cauliflower Mosaic Virus* to drive *VvMYBA* gene expression, the levels of which would be much higher than the natural expression levels of this TF in red berries. In comparison, *VvMYBA* expression in transgenic 'white' Shiraz berries is only reduced from its naturally occurring level in red Shiraz berries. A direct comparison between expression levels of *VvMYBA1*

between 'red' Chardonnay and 'white' Shiraz cannot be made due to the expression of non-functional *VvMYBA* genes that do not lead to anthocyanin accumulation (e.g. *VvMYBA2* and *VvMYBA3*). However it is very likely that *VvMYBA* gene expression changes would be much greater in transgenic 'red' Chardonnay berries compared to 'white' Shiraz berries when compared to their non-transgenic controls. This could explain why a higher number of genes had altered expression levels in the 'red' Chardonnay berries, and why much larger expression FCs were detected for them. For example *VvGST* was expressed at levels 806 fold higher in 'red' chardonnay berries compared to controls, while in 'white' Shiraz the expression of this gene was only decreased 12 fold compared to controls (Table 2.1). The fact that *VvMYBA* was constitutively expressed in all tissues of the transgenic 'red' berries and only expressed in the skins of Shiraz berries could also contribute to this.

2.4.2 *VvMYBA* gene expression has a large impact on global transcription

An analysis of the annotated functions of genes up- and down-regulated in 'red' Chardonnay berries showed that *VvMYBA* gene expression affected a large number of metabolic pathways involved in both primary and secondary metabolism. Genes involved in signalling pathways, transcription, and stress and defence responses also had changes to their expression levels (Figure 2.1). In these transgenic plants, very high concentrations of anthocyanins are synthesized post-veraison, which requires a large re-arrangement of global metabolic flux within the cell. In studies aimed at producing bacterial strains highly efficient at producing flavonoids, the rate of flavonoid synthesis has been shown to be limited by both malonyl-CoA and NADPH availability (Wang et al., 2011). In *Escherichia coli*, down-regulation of primary metabolic pathways such as the citric acid (TCA) cycle (Fowler et al., 2009), fatty acid synthesis (Leonard et al., 2008), and carbohydrate metabolism (Chemler et al., 2010), resulted in increased malonyl-CoA and/or NADPH substrate availability and carbon re-allocation, which increased metabolic flux through the flavonoid pathway. This illustrates how the regulation of flavonoid synthesis is linked to that of other metabolic pathways in the cell. Re-direction of carbon and increased metabolic flux through this pathway may therefore be responsible for some of the global transcriptomic changes in cell metabolism that were observed in 'red' Chardonnay berries with altered *VvMYBA* gene expression. However, studies have shown that metabolic flux is predominantly controlled post-translationally presumably by metabolites and their regulatory effects on metabolomic enzymes (Daran-Lapujade et al., 2004; Shlomi et al., 2007). For example, Daran-Lapujade et al. (2004)

measured genome-wide transcript changes in yeast cultures grown on two different carbon sources: glucose and maltose. It was found that only 180 genes responded to changes in the carbon source, despite major changes in central carbon metabolism. With this in mind, it is unlikely that the majority of transcriptomic changes identified in transgenic 'red' Chardonnay have occurred as a consequence of metabolic flux alone, but must be largely due to transcriptional activation/deactivation either directly or indirectly by VvMYBA.

Due to unnaturally high abundances of the VvMYBA TF in the transgenic 'red' Chardonnay berries, it is possible that some of the transcriptional changes detected in these microarray experiments would not occur naturally in wild-type red berries. Over-expression of transgenes can result in the production of neomorphs where the introduced protein confers a new function that is not present in wildtype (Zhang, 2003). The specificity of promoter binding by MYB TFs is determined by three factors: the presence of *cis*-elements in gene target promoters, the structure of the DNA binding domain, and the presence of co-factors (Lai et al., 2013). Recently, Lai et al. (2013) identified a number of potentially important amino acids in the binding domain of R2R3-MYBs that specifically regulate anthocyanin biosynthesis. In this study, they illustrated that there is little conservation of the known *cis*-elements that these MYBs bind. This is as expected, as MYB transcription factors must have high specificity to achieve fine-tuned regulation of metabolic pathways. On the other hand, there are examples where over-expression of anthocyanin related MYBs has been unreliable for determining their primary targets. For example, over-expression of *AtPAP1* in *Arabidopsis* resulted in up-regulation of the entire phenylpropanoid pathway, while silencing this gene only resulted in down-regulation of late anthocyanin biosynthetic genes (Borevitz et al., 2000). It is possible that the unnaturally high levels of VvMYBA in 'red' Chardonnay berries could result in non-specific binding of VvMYBA to gene promoters as a consequence of flooding the cell with such high levels of this TF. This may result in unusual gene expression patterns that are not indicative of the natural function of VvMYBA. This is why the comparison of transcriptomes from over-expression and silencing of *VvMYBA* genes in white and red grape backgrounds is so important in this study.

2.4.2.1 VvMYBA affects the expression of flavonoid biosynthetic genes and may do this in a cultivar-specific manner

In both the Chardonnay and Shiraz microarray datasets, the majority of genes which were most highly upregulated in berries expressing *VvMYBA* have functions in the flavonoid/anthocyanin biosynthetic pathways. Considering that VvMYBA is known to activate anthocyanin biosynthesis in grapevine (Kobayashi et al., 2002) this was an expected result. Those with the greatest FCs encode enzymes involved in either the later stages of anthocyanin biosynthesis, such as *VvUFGT* (Boss et al., 1996c), or code for enzymes involved in the modification (VvFAOMT, (Lücker et al., 2010)) or transport (VvGST and VvanthoMATE1, (Gomez et al., 2011)) of anthocyanins. Multiple genes belonging to the earlier anthocyanin biosynthetic gene families *VvCHS* and *VvF3'5'H* were also highly up-regulated in berries expressing *VvMYBA*. It has been previously shown that there are multiple functional copies of both *VvCHS* and *VvF3'5'H* genes in grapevine with the former having 3 copies (Sparvoli et al., 1994; Harris et al., 2013) and the latter shown to have 15 members (Falginella et al., 2010). Previous studies have shown that VvMYBA can activate the *VvUFGT* promoter and may be able to activate the transcription of other anthocyanin biosynthetic genes, including *VvCHS* and *VvF3'5'H* genes, but at much lower levels (Kobayashi et al., 2002; Bogs et al., 2007; Czemmel et al., 2009; Harris et al., 2013). Promoter activation of *VvGST* and *VvanthoMATEs* by VvMYBA has not been investigated. Further studies to determine if these genes are directly or indirectly up-regulated by VvMYBA, and, in the case of multiple gene families, if all or some of these family members are targets of VvMYBA, would provide further knowledge required to build a regulatory model for anthocyanin and flavonoid biosynthesis.

In the transgenic 'red' Chardonnay berries there was no increase in gene expression detected for the *VvMYBA2* gene compared to the non-transgenic control. This suggests that VvMYBA1 does not regulate the expression of *VvMYBA2*, but that the regulation of these two related genes are separate from one another. This is in contrast to what is seen in both Arabidopsis and apple.

There were a number of genes encoding flavonoid synthetic enzymes that were down-regulated in 'red' Chardonnay berries. These included *VvLAR* and *VvANR* genes which are involved in the PA biosynthesis branch of the flavonoid pathway (Figure 1.2). As anthocyanins and PAs are both synthesised through this pathway, they share common

metabolites (Anderson and Jordheim, 2006). It is possible that in the 'red' Chardonnay berries the activation of anthocyanin biosynthesis by VvMYBA may have directed the metabolic flux of the flavonoid pathway to anthocyanin synthesis, and as a consequence PA synthesis was down-regulated. During berry development in grapevine, the synthesis of anthocyanins and PAs, which both accumulate in berry skins, are mostly separated in time. PAs are synthesised pre-veraison while anthocyanins are synthesised post-veraison (Boss et al., 1996b; Downey et al., 2003a). Perhaps this switch in flavonoid synthesis is aided by the VvMYBA TF. However neither *VvLAR* or *VvANR* were found to have increased expression levels in the 'white' Shiraz berries even at levels lower than the initial ±1.5 FC cut off. In another transcriptomic study on grapevine hairy root cultures expressing *VlMYBA1*, they also found no significant differences in the transcription levels of *VvLAR* and *VvANR* in the transgenic tissue (Cutanda-Perez et al., 2009). Perhaps, down-regulation of *VvLAR* and *VvANR* in 'red' Chardonnay is a neomorphic consequence of over-expressing VvMYBA1 (as discussed in section 2.4.2) rather than a functional role of VvMYBA.

Another flavonoid gene *VvCHI* was significantly down-regulated in 'white' Shiraz berries but no significant difference in the expression of this gene was observed in 'red' Chardonnay berries. The phenylpropanoid pathway gene, *VvPAL*, had opposite trends in gene expression in relation to berry colour when comparing Chardonnay and Shiraz microarray datasets. It was up-regulated in both 'red' Chardonnay and 'white' Shiraz berries. This suggests that in Chardonnay the expression of this gene was down-regulated in response to increased *VvMYBA* gene expression whereas in Shiraz it was down-regulated when *VvMYBA* was silenced. These results indicate that VvMYBA may have some cultivar-specific effects on the expression of particular flavonoid genes, contributing to the differences that have been reported for flavonoid composition in berries from different cultivars (Mattivi et al., 2009; Ferrandino et al., 2012). More research is required to understand if these variety specific effects of VvMYBA on global transcription, and in particular flavonoid biosynthesis, are biologically significant.

2.4.2.2 VvMYBA may have a role in defence and stress responses

There were higher numbers of genes involved in defence/stress pathways which were up-regulated in 'red' Chardonnay berries compared to those which were down-regulated (Figure 2.1), suggesting that VvMYBA may have a role, whether direct or indirect, in up-regulating

these pathways. Anthocyanin accumulation has been shown to occur in some plant tissues when stressed due to abiotic stresses such as high light/UV, low temperature and low soil phosphate conditions (Dixon and Paiva, 1995). Their accumulation can sometimes be associated with pathogen infection sites (reviewed in Treutter, 2006) and in cotton leaves has been associated with resistance to blight disease caused by *Xanthomonas campestris* pv. *malvacearum* (Kangatharalingam et al., 2002). Expressing the anthocyanin regulatory gene *coloured-1* (C1) from maize in transgenic rice plants was shown to increase their resistance to the blast fungus *Magnaporthe grisea* (Gandikota et al., 2001). Considering the number of previously reported links between anthocyanins and plant responses to stress and pathogen attack it seems logical that the regulation of the pathways involved in these stresses be linked to anthocyanin biosynthesis regulation. The microarray results presented here suggest this may be the case. It is possible, however, that some berries used in these microarray experiments may have been diseased due to fungal infection problems that were encountered in the glasshouse in which these vines were grown. Care was taken to select berries for this experiment that did not appear diseased, yet some berries, particularly black coloured berries, could still have been infected. If so then gene expression changes, particularly in relation to defence and stress, may be as a result of this rather than the expression or silencing of *VvMYBA* genes.

2.4.3 VvMYBA affects global transcription in a tissue-specific manner

There were a number of genes which were differentially expressed in response to the presence of VvMYBA in skins compared to whole berries of transgenic ‘red’ Chardonnay berries, when compared to controls (Table 2.1). Flavonoid biosynthetic genes were expressed more highly in whole berries compared to skins, and in some cases this difference was very large. Many of these genes, such as *VvCHS*, *VvF3’5’H* and *VvFAOMT*, are involved in the synthesis of other flavonoids such as PAs and flavonols as well as anthocyanins. PAs and flavonols are synthesised in the skins and seeds of both red and white berries, but not in the pulp (Downey et al., 2003b; Bogs et al., 2005). The skin of wild-type Chardonnay berries would already contain transcripts of flavonoid biosynthetic genes involved in the synthesis of other flavonoids besides anthocyanins, but the pulp would not, as has been shown previously (Boss et al., 1996c). Therefore the difference in transcript levels of flavonoid biosynthetic genes when comparing ‘red’ Chardonnay skins to control berry skins would be smaller compared to the same comparison in whole berry samples as these genes would be expressed in both the

skin and pulp of transgenic 'red' Chardonnay berries due to the constitutive expression of *VvMYBA*.

A number of disease resistance genes were more highly up-regulated in skins expressing VvMYBA compared to whole berries. The fact that anthocyanins and disease resistance have been linked in the past was discussed in section 2.4.2.1. Considering that the skin would often come into contact with pathogens first, it seems logical that disease resistance pathways would be more active in this tissue. It is possible that the presence of VvMYBA, or of anthocyanins, may have an effect on the transcription or activity of other skin specific defence related TFs, which could then alter gene transcription of defence pathways in this tissue.
Photosynthesis genes, such as those coding for photosystem proteins, were down-regulated more in whole berries expressing VvMYBA compared to skins. This may be caused by differences in light absorption between these tissues caused by anthocyanins. Anthocyanins are thought to attenuate green/yellow and possibly blue light (Manetas et al., 2003) and therefore due to the presence of these compounds in red berry skin, it is possible that these light wavelengths will have been mostly absorbed in the skin resulting in a shading effect on the pulp of the berry. As a result there may be reduced photosynthesis in the flesh of red berries compared to their skin, which would not be observed in white berries due to their lack of anthocyanins, but at present this is only speculation.

2.4.4 Discussion on expression trends of genes in Shiraz microarray data in relation to berry colour

2.4.4.1 Genes whose expressions were altered in a manner consistent with *VvMYBA* gene expression and anthocyanin concentrations

At the commencement of this study, it was expected that the expression of genes affected by *VvMYBA* gene expression would change in a way consistent with anthocyanin concentration in the berries. For example, genes up-regulated by the presence of VvMYBA were expected to have the highest expression FC in red berries, followed by a lower but still positive FC in rose berries, when compared to white berries. The Shiraz microarray dataset was analysed to determine if these expected expression patterns were in fact followed (Table 2.3). Expression trends 1 and 2 (i.e. gene expression in red berries was > in rose berries which was > in white berries (trend 1), or gene expression in white berries was > in rose berries which was > in red berries (trend 2)) represent these expected expression patterns, but a total of only 10 genes

(from 305) were represented in these two categories. A previous analysis of transgenic shiraz lines showed that the anthocyanin concentrations of the 'rose' Shiraz berries, with partially silenced *VvMYBA* gene expression, were approximately only 10% of that of the wild-type (Walker, personal communication). This means that the white and rose berries are more closely related in regards to anthocyanin content than the red berries. It is possible that genes whose transcription is only slightly modulated in response to the presence/absence of VvMYBA might only have been detected as such in the red berries, and not the rose ones. Considering this, expression trends 3 and 4 (i.e. gene expression in red berries was > in rose and white (trend 3), or gene expression in rose and white berries was > in red berries (trend 4)) are not too far removed from the originally expected trends and are the two categories that contained the highest numbers of genes within them. This means that the majority of gene expression changes modulated in Shiraz berries with altered *VvMYBA* gene expression occurred on a scale where the differences between rose and white berries was not great enough to be detected as significantly different.

Genes which were therefore up-regulated by the presence of VvMYBA (i.e. in red berries) in a manner consistent with anthocyanin concentration were found in trends 1 and 3, while trends 2 and 4 contained the genes which were conversely down-regulated. The majority of genes that were up-regulated coded for enzymes involved in the anthocyanin, flavonoid or general phenylpropanoid biosynthetic pathways. This result is as expected as flux through theses pathways determines the final anthocyanin concentration in berries. Flavonoid related genes were not represented in trends 2 and 4. This suggests that VvMYBA functions as a positive regulator of anthocyanin biosynthesis and does not do this through negative regulation of competing flavonoid branches, at least in Shiraz. This finding will be discussed further in section 2.4.5. The only exception to this was that a MYB TF, VvMYBB1, was down-regulated in red Shiraz berries and represented in trend 4. The expression of the gene coding for this TF has been previously shown to correlate with anthocyanin accumulation in post-veraison berries (Kobayashi et al., 2002); however the targets of these genes are currently unknown. The closest characterised homologue of this protein, based on a BLAST search in NCBI, was *myb1* from *Nicotiana tabacum* (50% identity) which is involved in the regulation of pathogenesis-related proteins and defense responses involving salicylic acid signalling (Yang and Klessig, 1996). The *VvMYBA1* and *VvMYBA2* genes were present within trend 3 (which were the targeted silencing genes in the transgenic Shiraz plants), as were a number of uncharacterised genes with homology to WRKY and bHLH TFs. In plants, bHLH

TFs are known to be involved in the regulation of flavonoid biosynthesis, as well as in other metabolic pathways, within transcriptional complexes with WD40 and MYB TFs as discussed previously in section 1.4.3. In species such as *Arabidopsis* and petunia, multiple MYB and bHLH TFs have been identified with such roles. Different combinations of these TFs within the tri-protein transcriptional complex can regulate anthocyanin and flavonoid synthesis in different ways, sometimes in combination with other cell processes, allowing for temporal and spatial regulation of the flavonoid pathway (Hichri et al., 2011). For example, in petunia, four MYB TFs, ANTHOCYANIN 2 (PhAN2), PhAN4, PURPLE HAZE (PhPHZ), and DEEP PURPLE (PhDPL), and two bHLH TF, PhAN1 and PhJAF13, have been characterised. The combination of these, present within the transcriptional complex with the common WD40 TF AN11, is responsible for activating anthocyanin biosynthesis in different floral tissues at different developmental stages (Albert et al., 2011). A WRKY TF known as Transparent Testa Glabra 2 (TTG2) has also been identified in *Arabidopsis*, which is up-regulated in plants ectopically expressing the AtPAP1 regulator, suggesting an involvement in anthocyanin biosynthesis regulation (Tohge et al., 2005). *AtTTG2* has also been shown to be involved in PA biosynthesis in the seed coat (Johnson et al., 2002). It possible that the uncharacterised WRKY and bHLH TFs, and the VvMYBB1 TF, which were up- and down-regulated in 'red' Shiraz berries respectively, may be involved in anthocyanin or flavonoid regulation in grapevine. In the case of VvMYBB1, this gene has been shown to co-segregate with berry colour through QTL mapping (Salmaso et al., 2008), providing further support to this hypothesis.

The expression patterns of genes in trends 5 and 6 were a little altered from what would be expected of genes that had expression patterns correlated to anthocyanin concentration. However if only low levels of anthocyanins or VvMYBA were required to highly activate (or repress) the expression of a particular gene then such a pattern may be expected. In saying this, there were no genes in either of these trends relating to anthocyanin, phenylpropanoid or aroma/flavour compound synthesis.

2.4.4.2 Some genes were modulated but did not follow *VvMYBA* gene expression and anthocyanin concentrations

Trends 7-12 represented genes with expression patterns which did not follow that of *VvMYBA* gene expression or anthocyanin concentration in the berries (Table 2.3 and Appendix C). Of the 305 genes that had altered expression levels in the transgenic Shiraz berries, 123 of them

belonged to these categories. This was an unexpected result, which is difficult to interpret. Fifty six of these genes were sorted into trend 7 and were expressed more highly in transgenic rose berries compared to red and white berries, which had no significant differences. Genes involved in aroma/flavour compound synthesis were predominant in this category, including those involved in terpenoid production (the monoterpenes pinene and terpineol, and the sesquiterpene germacerene) and the production of phenylpropenes [e.g. coniferyl alcohol acyltransferase, (Koeduka et al., 2006)]. Why lower levels of *VvMYBA* gene expression, such as those in rose coloured berries, would alter flavour metabolism, but higher *VvMYBA* expression levels, such as those in red berries, would not, is hard to understand.

One possible explanation could be differences in the ripeness between berries used in these experiments. While it was aimed to collect berries from individual transgenic grapevine lines (as well as from non-transgenic controls) with a similar TSS (measured in ^{o}Brix) this was not always possible. Berry TSS were measured from a small subsample of berries from each bunch before they were collected, after which a larger subsample was used to more accurately determine this. For some samples, the initial predicted ^{o}Brix measurements were different to those obtained from the larger subsample. Unfortunately, due to low numbers of bunches, there was limited transgenic material, so there are instances where bunches which had higher or lower brix readings than the optimum target (20-24 ^{o}Brix) had to be used in these experiments. There was a larger range of TSS measurements between the four biological replicates of the control (red) and transgenic 'white' Shiraz berries (27–19.5 ^{o}Brix and 24.2–19.2 ^{o}Brix respectively) compared to the transgenic 'rose' shiraz replicates (21.9–24.2 ^{o}Brix, Appendix A). Yet the average ^{o}Brix of the 4 replicates from control, 'rose' and 'white' Shiraz vines were similar (23.8, 23.1 and 21.7 ^{o}Brix respectively). Even so, it is possible that more variation in ripeness between the replicates of the red and white berry samples, compared to the rose ones, may have resulted in altered gene expression in the rose berries that was more related to the ripeness of the berries rather than *VvMYBA* gene expression. Further research is required to investigate if this is the case or to determine if VvMYBA has concentration specific affects on gene transcription.

2.4.5 VvMYBA regulates a narrow set of genes consistently across cultivars and is a positive regulator of anthocyanin biosynthesis and transport

When the Shiraz and Chardonnay microarray datasets were compared to one another there were only 26 genes that had expression levels altered in response to *VvMYBA* gene expression in a consistent manner in both varieties (Table 2.1). Of these genes, those with the highest FCs were up-regulated in red berries and have characterised roles in anthocyanin biosynthesis (except for the uncharacterised BAHD and SCPL acyltransferase genes which are discussed in section 2.4.6). Only 11 genes were down-regulated consistently with *VvMYBA* gene expression in both varieties, and none of these genes have roles in anthocyanin biosynthesis. This suggests that VvMYBA activates the expression of a narrow set of genes and is a positive regulator of anthocyanin biosynthesis. Transcriptome studies in other plants expressing VvMYBA homologues, such as in tomatoes and *Arabidopsis,* have shown similar results to this (Borevitz et al., 2000; Tohge et al., 2005). On the other hand, the expression of *VvMYBA* was observed to have a large effect on global transcription within the individual cultivars used in this study. As discussed previously, some of those differences in the transgenic 'red' Chardonnay over-expressing *VvMYBA1* may be due to the ectopic and unnaturally high levels of expression of the VvMYBA TF and may not represent its wildtype function. Gene silencing of *VvMYBA* in Shiraz resulted in fewer transcriptomic changes but the list of genes with altered transcription were still much larger than the 26 genes affected in both cultivars. It is possible that VvMYBA has a conserved role to regulate a narrow set of genes, but altering the expression of these genes may have other variety specific effects on global transcription.

In grapevine VvUFGT catalyses the final step in producing stable anthocyanins by adding a glucose molecule to anthocyanidins (Ford et al., 1998). The core anthocyanins can then be further modified by methyltransferases and acyltransferases before they are transported into the vacuole (Mazza and Francis, 1995; Fournier-Level et al., 2011). *VvUFGT* and genes involved in anthocyanin methylation (*VvFAOMT*) and transport (*VvGST* and *VvanthoMATE1*), plus two putative acyltransferase genes (discussed further in section 2.4.6), were all up-regulated in berries expressing VvMYBA. These results show that VvMYBA specifically up-regulates the later steps of the anthocyanin biosynthetic pathway and their modification. This has been shown previously by Cutanda-Perez et al. (2009) who used microarrays and transgenic grapevine hairy root cultures to examine the role of *VlMYBA1* in gene transcription. They also found that *VvMYBA* gene expression resulted in an up-

regulation of several earlier flavonoid pathway genes; *VvPAL*, *VvCHS*, *VvF3H* and *VvLDOX*. In this respect their results differed to those presented here, as the only early flavonoid pathway genes up-regulated in our berries containing VvMYBA were *VvPAL2*, *VvCHS* and *VvF3'5'H*. In grapes, anthocyanins can be either di- or tri-hydroxylated at the 3'4' (cyanidin and peonidin) or the 3'4'5' (malvidin, delphinidin and petunidin) positions of the B ring (Figure 1.1). The VvF3'H and VvF3'5'H enzymes catalyse these reactions (Bogs et al., 2006), the result of which can determine the hue and stability of the compound (Forkmann, 1991). A previous analysis of the anthocyanin composition of the transgenic 'red' Chardonnay berries revealed that they had a significantly high proportion of 3'4'5' hydroxylated anthocyanins compared to 3'4' hydroxylated anthocyanins (Walker, personal communication). The microarray results would suggest this was a consequence of increased expression of *VvF3'5'H* in these berries. These results suggest that VvMYBA preferentially up-regulates the synthesis of tri-hydroxylated anthocyanins in berries.

2.4.6 The identification of two putative anthocyanin acyltransferases up-regulated by VvMYBA

The microarray results revealed that two previously uncharacterised genes, belonging to the BAHD and SCPL gene families, were both up-regulated in transgenic Chardonnay and Shiraz berries expressing *VvMYBA* (Table 2.1). Members of both of these gene families code for enzymes which catalyse the addition of acyl groups to molecules. BAHD acyltransferases utilise CoA thioesters as their acyl donors while SCPL proteins utilise sugar conjugated donors (Milkowski and Strack, 2004; D'Auria, 2006). In grapevine, anthocyanins can be conjugated to acyl groups attached to the C6'' position of the glucose molecule (Mazza and Francis, 1995); however the acyltransferases responsible for these reactions have not been identified. In other plant species, anthocyanin acyltransferases have been characterised that belong to the BAHD and the SCPL protein families (Nakayama et al., 2003; Fraser et al., 2007). The expression of the putative BAHD and SCPL genes in the microarray results presented in this chapter were up-regulated to levels similar to those of genes known to be involved in anthocyanin biosynthesis. It seemed possible that one or both of these genes may function as anthocyanin acyltransferases. This prediction formed a hypothesis from which the experimental work presented in the later Chapters 4 and 5 was based.

2.4.7 The role of VvMYBA in flavour/aroma metabolism

One of the research questions of this PhD project was 'Does VvMYBA have a role in flavour and aroma metabolism in berries of grapevine?' Of the 26 genes which were regulated in a consistent manner in relation to the presence of VvMYBA in both Chardonnay and Shiraz cultivars, 2 of these have characterised or putative roles in flavour metabolism. The monoterpene synthase, *E-beta-ocimene synthase* (Martin et al., 2010) and an uncharacterised p450 gene annotated as belonging to a monoterpene synthesis gene network, were both down-regulated in red berries. This suggests that VvMYBA may have a negative effect on monoterpene production in grapes. Monoterpenes are important wine flavour compounds that possess floral/citrus aromas which are more commonly associated with white or rosé wines (Ballester et al., 2009). No other flavour associated genes were found to be consistently up- or down-regulated by VvMYBA in Chardonnay and Shiraz. There were, however, terpenoid synthesis genes, discussed in section 2.4.4.2, that were up-regulated in the transgenic 'rose' Shiraz berries but not in the transgenic 'white' or control (red) Shiraz berries. These results suggest that VvMYBA may have a role in regulating terpenoid production in grapes. Further research into how this regulation may occur will be presented in Chapter 3.

In both Chardonnay and Shiraz berries, altering *VvMYBA* gene expression had an effect on expression levels of genes involved in fatty acid metabolism, although these were in a cultivar specific manner. Fatty acids and fatty acid esters, the latter often synthesised by yeast from grape precursors during fermentation, are volatile aroma compounds which form a large component of wine flavour (Suomalainen and Lehtonen, 1979). It is possible that the changes in fatty acid metabolism, as a consequence of *VvMYBA* gene expression, could change the levels of these aroma compounds or their precursors in red grapes which could contribute to the differences of flavour between red and white wine. It is possible that VvMYBA may be able to directly regulate the transcription of fatty acid metabolism genes, as a way to co-regulate pigmentation and aroma synthesis. It is perhaps more probable that fatty acid metabolism has been altered as a consequence of a global re-direction of carbon to the anthocyanin biosynthesis pathway within grape cells. A link between these two metabolic pathways has been shown before. Down-regulating fatty acid synthesis in *E. coli* cells, engineered to contain flavonoid structural genes, resulted in an increased production of flavonoids due to increased carbon channelling through this pathway (Leonard et al., 2008; Santos et al., 2011). In *Arabidopsis*, over-expression of the anthocyanin biosynthesis MYB regulator *AtPAP1* resulted in up-regulation of a putative long-chain-fatty-acid CoA ligase

gene suggesting a link between fatty acid metabolism and anthocyanin biosynthesis in this plant species also (Tohge et al., 2005).

2.5 Conclusion

In this chapter, results have been presented from a study comparing the transcriptome of Chardonnay and Shiraz berries with altered *VvMYBA* gene expression to their controls. The results show that VvMYBA has a conserved function as a positive regulator of anthocyanin biosynthesis, and specifically activates the later stages of anthocyanin synthesis, modification and transport. VvMYBA altered the expression of 26 genes in a consistent manner in both Chardonnay and Shiraz, but had many more cultivar specific effects on global transcription which included some differences in the regulation of flavonoid biosynthesis between the cultivars. Two putative acyltransferases were up-regulated in red berries to levels similar to those of other anthocyanin biosynthetic genes. It was hypothesised that these may function as anthocyanin acyltransferases. A link between VvMYBA and the regulation of terpene synthesis genes was also established. The following chapters will present results from a flavour/aroma analysis of wines made from these transgenic grapes along with research into the link between VvMYBA and terpene biosynthesis. Experimental work aiming to study the function of the two aforementioned acyltransferases will also be presented.

Chapter 3: An investigation into the role of VvMYBA in the synthesis of flavour and aroma compounds in the cultivated grapevine *Vitis vinifera L.*

Note about the experimental work in this Chapter

The bulk of the exerimental work presented in this chapter was carried out by Amy Rinaldo (the author of this thesis), but there were a couple of aspects of the work that other people contributed to (see below). The interpretation of all the results and preparation of this chapter was done entirely by Amy Rinaldo.

The qPCR experiments analysing the MEP pathway gene transcript levels in berry skins of Cabernet Sauvignon, Malian and Shalistin developmental series (Section 3.3.6 and Figure 3.8) were carried out by Lucy Arrowsmith, a CSIRO Summer Student. Amy Rinaldo was responsible for the design of her project, which she completed under Amy's direct supervision. Amy carried out the qPCR analysis of the expression of these genes in the whole berry series of these varieties (section 3.3.6 and Figure 3.7).

3.1 Introduction

Colour and flavour are two of the most important attributes of grapes and wine. Flavour can be defined as the interaction of the taste, odour and texture of a food substance (Belitz and Grosch, 1999). Aroma compounds are small molecules (generally with molecular weights < 300) that are volatile enough to be transported to the olfactory system, either through the mouth or nose, where they are detected by odour receptors (Noble, 1996). They make up only around 1% of the ethanol concentration of wine (approximately 0.8 – 1.2 g/L) but play a definitive role in wine flavour (Rapp, 1998). PAs (or condensed tannins) are non-volatile compounds that contribute to the textural properties of wine and are responsible for their astringency, or coarse mouthfeel (Jackson, 2000).

Colour and scent traits have long been known to aid in plant reproductive success due to their roles in attracting pollinators and seed dispersers (Galen and Kevan, 1980; Majetic et al., 2009). In other plant species, researchers have shown that colour and scent combinations can be linked (Majetic et al., 2007; Salzmann and Schiestl, 2007), which could be explained by the presence of shared biochemical pathways for non-volatile colour pigments and volatile aroma compounds. However, there have been no studies on whether berry colour and aroma compound biochemical pathways may be interlinked in grapevine.

3.1.1 The phenylpropanoid pathway produces substrates used in the synthesis of volatile phenylpropanoids/benzenoids and flavonoids

Anthocyanins, are produced via the flavonoid biosynthetic pathway, a branch of the much larger phenylpropanoid pathway (see section 1.4.2) from which volatile phenylpropanoids and benzenoids are also derived (Schuurink et al., 2006; Majetic et al., 2010). Phenyl acetaldehyde, 2-phenylethanol and phenyl ethylacetate are some of the phenylpropanoids derived from the precursor phenylalanine. Benzenoids are derived from the next metabolite in the phenylpropanoid pathway, cinnamic acid (Moerkercke et al., 2009). Therefore, it is plausible that anthocyanin and volatile phenylpropanoid/benzenoid synthesis could be related due to metabolic flux through the phenylpropanoid pathway. Such a link was first demonstrated by Zuker et al. (2002) when they measured volatile release from transgenic carnations (*Dianthus caryophyllus* L.) in which they had silenced the expression of the

flavonoid structural gene *DcF3H*. They found an increased release of methyl benzoate that correlated with a decrease in anthocyanin accumulation in the transgenic flowers. Kessler et al. (2008) were able to abolish the release of benzyl acetone from tobacco flowers by silencing another flavonoid structural gene *NaCHS*. AtPAP1, a TF responsible for activating anthocyanin biosynthesis, was overexpressed in *Arabidopsis*, which resulted in up-regulated anthocyanin accumulation and an increased emission of volatile phenylpropanoid/benzenoid compounds (Zvi et al., 2008). These studies demonstrate that the synthesis of anthocyanins and volatile phenylpropanoids/benzenoids can be linked, but how one may affect the other may vary between different species. These differences, however, may also be a consequence of the different experimental systems used in these studies, i.e. a different response may be the result of whether the expression of a MYB TF or a flavonoid structural gene is altered, or whether this expression was constitutive.

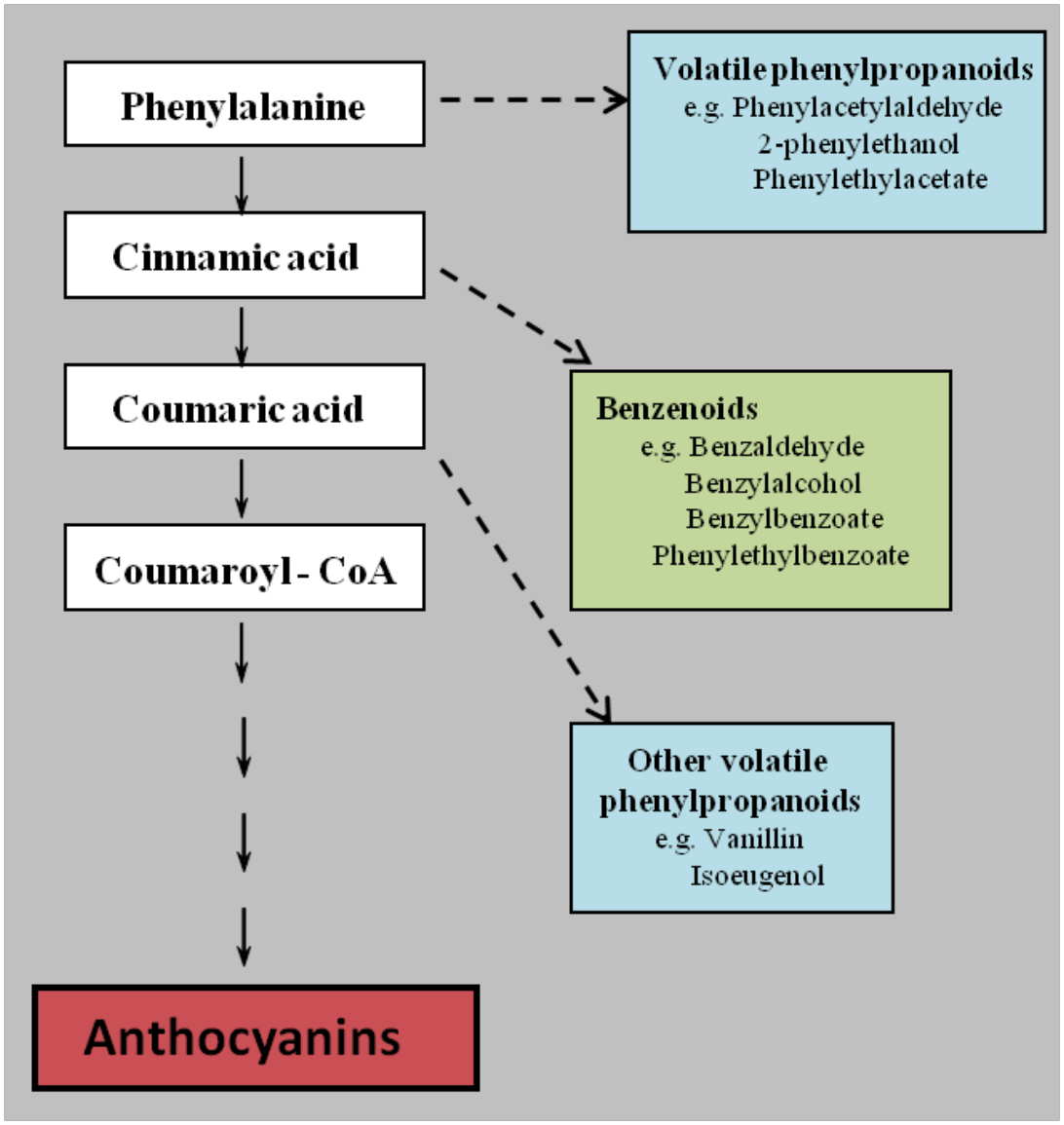


Figure 3.1: How the phenylpropanoid biosynthesis pathway links anthocyanin and volatile aroma compound biosynthesis

(after Moerkercke et al., 2009)

3.1.2 Links between flavonoid related MYB transcription factors and terpene synthesis

The link between the synthesis of anthocyanins and volatile phenylpropanoids/benzenoids is clear as they are both derived from intermediates of the same biochemical pathway. What is perhaps more unexpected are studies that link the presence of anthocyanins with terpenoid production. For example, Salzmann and Schiestl (2007) analysed the volatile release from red and yellow colour morphs of the orchid species *Dactylorhiza romana* and found that higher amounts of benzaldehyde were emitted from the yellow morphs, while red morphs released greater amounts of the monoterpene linalool. The genetic basis of these morphs has not been biochemically characterised. Regulation of the flavonoid biosynthesis pathway is controlled through the actions of MYB-R2R3 TFs as discussed previously in section 1.4.3. There are studies reported in which the expression of these TFs has been altered, resulting also in changes to the abundance of terpenes. For example, the grapevine flavonoid regulatory gene *VvMYB5A* was expressed in tomato plants which were subsequently shown to have both increased anthocyanin and terpenoid levels in the transgenic fruit (Mahjoub et al., 2009). In another study, overexpression of the *Pinus taeda* TF *PtMYB14* in *Picea glauca* resulted in an accumulation of anthocyanins along with an up-regulation of sesquiterpene synthesis (Bedon et al., 2010).

3.1.3 Terpene biosynthesis

Terpenoids are a large class of metabolites which include monoterpenes, diterpenes, carotenoids, sesquiterpenes and sterols, and all arise from isopentenyl prenyldiphosphate (IPP) and dimethylallyl diphosphate (DMAPP) precursors (Eisenreich et al., 2001). These precursors are synthesised through one of two biochemical pathways: the cytosolic mevalonate pathway (predominantly for sesquiterpenes and sterols) and the plastidic methylerythritol (MEP) pathway (for monoterpenes, diterpenes and carotenoids) (Wu et al., 2006). An array of terpenoid synthases (TPS) and other enzymes, such as cytochrome P450 dependent monooxygenases and various other transferases, utilise the IPP precursors to produce the large range of terpenoid secondary metabolites found in plants (Martin et al., 2010). They have a very broad range of functions, such as being essential for plant growth and development and playing important roles in plant-plant and plant-environment interactions (Yu and Utsumi, 2009). Many terpenoids, especially monoterpenoids and

sesquiterpenoids are known to have scent properties and are important aromas in the cut flower, food and wine industries. Many monoterpenes in particular have been shown to be responsible for varietal aroma characters of wines made from various grapevine cultivars (Mateo and Jiménez, 2000).

3.1.3.1 The MEP pathway

As mentioned in section 3.1.3, monoterpenes are produced from precursors derived from the plastidic MEP pathway. A schematic of the MEP pathway is shown in Figure 3.2. In the first step glyceraldehyde 3-phosphate (G3P) and pyruvate precursors are converted to 1-deoxy-D-xylulose 5-phosphate (DXP) by DXP synthase (DXS). Research in a number of plant species has shown that DXS catalyses a rate-limiting step of IPP and DMAPP synthesis as well as for the synthesis of various compounds made from these precursors (reviewed in Cordoba et al., 2009). For example, in grapevine the gene coding for this enzyme co-localises with a major quantitative trait locus (QTL) explaining monoterpene variation in berries (Battilana et al., 2009). The second and seventh MEP pathway enzymes, DXP reductoisomerase (DXR) and IPP/DMAPP synthase (IDS), have also been shown to have critical rate-limiting functions (Botella-Pavía et al., 2004; Carretero-Paulet et al., 2006). There are multiple levels of regulation of the MEP pathway including environmental cues such as light and circadian regulation, nutritional cues such as sugars, and post-translational modifications of MEP pathway enzymes (reviewed in Cordoba et al., 2009).

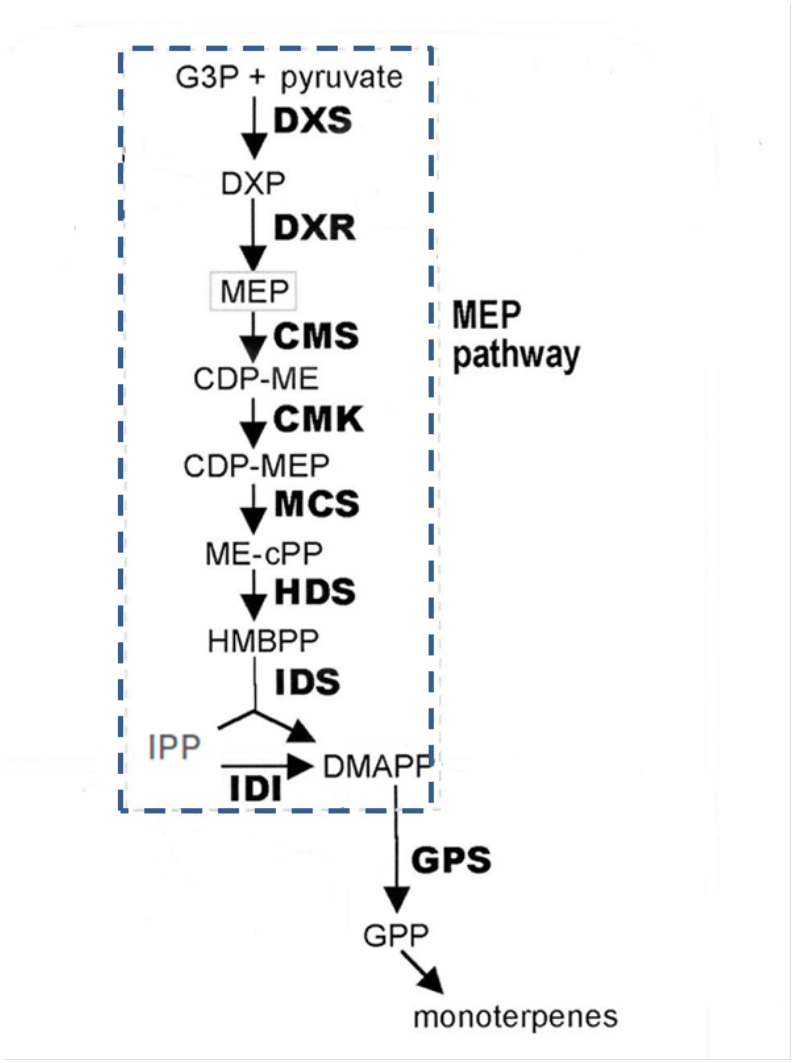


Figure 3.2: The methylerythritol phosphate (MEP) pathway

A schematic of the methylerythritol phosphate (MEP) pathway located in the cell plastid and responsible for producing precursors used to synthesise monoterpenes. The metabolites at each step are abbreviated: glyceraldehyde 3-phosphate (G3P), 1-deoxy-D-xylulose 5-phosphate (DXP), 2-*C*-methyl-D-erythritol 4-phosphate (MEP), 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol (CDP-ME), 2-phospho-4-(cytidine 5'-di-phospho)-2-*C*-methyl-D-erythritol (CDP-MEP), 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate (ME-cPP), 4-hydroxy-3-methylbut-2-enyl diphosphate (HMBPP), isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP). Enzymes responsible for catalysing each step in the pathway are in bold: DXP synthase (DXS), DXP reductoisomerase (DXR), CDP-ME synthase (CMS), CDP-ME kinase (CMK), ME-cPP synthase (MCS), HMBPP synthase (HDS), IPP/DMAPP synthase (IDS), IPP isomerase (IDI), GPP synthase (GPS). (after Rodríguez-Concepción and Boronat, 2002)

3.1.4 How can anthocyanin and terpene biosynthesis be co-ordinately regulated?

The anthocyanin biosynthesis pathway is not as closely related to terpenoid synthesis as it is with volatile phenylpropanoid/benzenoid production, and hence metabolic flux through either of these two pathways is less likely to have a direct effect on the other. If this is the case, why then are there reports, as discussed in section 3.1.2, of links between the expression of MYB TFs involved in anthocyanin biosynthesis affecting terpene production? One possibility is that the expression of these TFs in cells where they are not normally expressed might influence these pathways in ways that do not reflect their natural biological functions. Another explanation could be that some flavonoid related MYB TFs may play a regulatory role in terpenoid and perhaps other volatile aroma pathways. In an evolutionary perspective this could make sense, as being able to co-ordinately control both colour and scent in flowers and fruits may be an advantage when trying to attract pollinators and seed dispersers.

3.1.5 The research question: Does VvMYBA have a role in flavour/aroma biosynthesis in grapevine?

In this study we wished to investigate the possible link between anthocyanin and flavour/aroma compound synthesis in grapevine. In section 1.6, the natural colour sports of Cabernet Sauvignon, Malian and Shalistin, and the transgenic Chardonnay and Shiraz grapevines with altered *VvMYBA* gene expression were described. These mutant grapevines provided berry material with almost identical genetic backgrounds but differing in *VvMYBA* gene expression and hence were essential tools in this investigation. An analysis of volatile aroma compounds in wines made from these mutants, and their controls, was used to answer the research question '*Does VvMYBA have a role in flavour/aroma biosynthesis in grapevine?*'

3.2 Materials and methods

3.2.1 Chemicals

Unless otherwise stated all chemicals and reagents were purchased from Sigma-Aldrich (Castle Hill, Sydney, Australia) and used without further preparation.

3.2.2 Plant material

3.2.2.1 Transgenic berries used in microfermentations

Transgenic Chardonnay and Shiraz vines with altered *VvMYBA* gene expression and their controls were grown as previously described in section 2.2.1. One to three bunches at the same ripening stage (i.e. same flowering date and amount of TSS (^{o}Brix) were collected from single vines, and the berries from these were pooled. It was aimed to collect bunches with an average ^{o}Brix of between approximately 20 and 24. Berries from different vines were collected on different days due to unsynchronized flowering of the vines when grown in glasshouse conditions. TSS data were recorded from a small representative subsample (~5 berries) of each pooled berry sample. All berries were frozen in liquid N_2 before storing at -80^{o}C.

3.2.2.2 Cabernet Sauvignon, Malian and Shalistin used in microfermentations

Berries from Cabernet Sauvignon, Malian and Shalistin varieties were collected from grapevines grown in a commercial vineyard at Langhorne Creek, South Australia (35° 17’ 30” South, 139° 2’ 33” East). In the 2010/11 season, 20 bunches from each of the three varieties were selected with an average TSS of between 20 – 22 ^{o}Brix. These measurements were obtained from the average TSS of 5 berries randomly selected from each bunch. All berries from the 20 bunches were pooled. Microfermentations carried out on pressed berries (see section 3.2.3.2) were started immediately on fresh berries while berries used in microfermentations containing only free-run juice (‘see section 3.2.3.3) were stored at 4^{o}C for 16 hours before processing.

3.2.2.3 Shade and light experiment for microfermentations

In the 2012/13 season opaque boxes were applied to whole bunches of Cabernet Sauvignon, Malian and Shalistin berries pre-veraison, at approximately the peppercorn size stage (EL stage 29, (Coombe, 1995)) and were left on the berries until harvest (22 ^{o}Brix). These boxes were designed to exclude light while maximising airflow, thus reducing any temperature or relative humidity differences between the bunches inside the boxes and bunches in the canopy (Downey et al., 2004). When these berries were harvested, unboxed control bunches were also collected from the same vines as the boxed samples. All berries of each bunch were

pooled, brix readings recorded from a small representative subsample, and frozen in liquid N_2 before storing at -80^{o}C.

3.2.2.4 Cabernet Sauvignon, Malian and Shalistin developmental series for RNA extractions

A berry developmental series was also collected from Cabernet Sauvignon, Malian and Shalistin varieties from the same vineyard and in the same season as described in section 3.2.2.2. Samples were collected every week from flowering (defined as 50% capfall) till the berries reached ~22 ^{o}Brix in TSS. For each variety, approximately 5 bunches were randomly selected from the same 20 vines. Berries were pooled, and berry weight and TSS measurements were taken from a subsample of these. For berry skin samples, the skins were removed from the fresh berries. All samples were frozen in liquid N_2 and stored at -80^{o}C. Those collected 2, 4, 6, 8, 9, 10, 12, 15 and 18 wpf were selected as representing a typical grape berry developmental series. Veraison occurred at 9wpf.

3.2.3 Microfermentations

3.2.3.1 Transgenic grapes

Frozen whole berries were ground to a powder using a chilled grinding mill (IKA®, Germany). Total fructose and glucose concentrations of berry material were determined from a subsample of frozen powder using the Megazyme K-FRUGL kit (Megazyme, Co. Wicklow, Ireland, Appendix D). 25 g of frozen powder was added to a 125 ml conical flask to which 25mL of a sugar/water solution was added to make it up to a final volume of 50 ml. To ensure each ferment would have the same initial total sugar concentration (240 g/L), previously determined amounts of a sugar solution (240 g/L D-glucose, 240 g/L D-fructose), based on the initial total sugar concentration of the berry material in each ferment, were mixed with sterile water to make up the 25ml sugar/water solution that was then added to the berry powder. This also ensured that the liquid:solids ratio was the same in each ferment. Fermentations were then carried out as described in 3.2.3.4 and 3.2.3.5.

3.2.3.2 Pressed berry fermentations

This fermentation style was designed to represent a typical ‘red’ winemaking style where fermentation occurs in the presence of the skins, seeds and pomace of the berries. 100 g of

berries were pressed by hand, using a large rubber bung, in a strong plastic bag. The juice, skins, seeds and pomace was transferred to a 125 mL flask and fermentations carried out as described in 3.2.3.4 and 3.2.3.5. Total fructose and glucose concentrations of berries were determined from a subsample of the juice using the Megazyme K-FRUGL kit (Appendix D).

3.2.3.3 Fermentations on free-run juice

This fermentation style was designed to represent a typical 'white' winemaking style where fermentation occurs on free-run juice of the berries. 200 g of berries were pressed in the same way as described in section 3.2.3.2. The juice was then filtered through miracloth into a beaker resting on ice. 50 mL of juice was transferred into 125 mL conical flask and fermentations carried out as described in 3.2.3.4 and 3.2.3.5. Total fructose and glucose concentrations of berries were determined from a subsample of the juice using the Megazyme K-FRUGL kit (Appendix D).

3.2.3.4 Yeast preparation

Yeast starter cultures were prepared by adding approximately 0.25 g of dry yeast (*Saccharomyces cerevisiae bayanus,* strain EC1118, Prise de Mousse, AB Mauri, Australia) to 25 mL of model grape juice medium (MGJM). MGJM (pH 3.2) was prepared as described by Keyzers and Boss (2010) with slight alterations. 100 g *D*-Glucose, 100 g *D*-fructose, 5 g *D/L*-malic acid, 5 g tartaric acid, 0.2 g citric acid, 1.7 g yeast nitrogen base (YNB) without ammonium sulphate (1000 mg/L KH_2PO_4, 2 mg/L *myo*-inositol, 0.04 mg/L $CuSO_4$, 500 mg/L $MgSO_4$, 0.4 mg/L niacin, 0.1 mg/L KI, 100 mg/L NaCl, 0.2 mg/L *para*-aminobenzoic acid, 0.2 mg/L $FeCl_3$, 100 mg/L $CaCl_2$, 0.4 mg/L pyridoxine, 0.4 mg/L $MnSO_4$, 0.002 mg/L biotin, 0.2 mg/L riboflavin, 0.2 mg/L Na_2MoO_4, 0.4 mg/L calcium pantothenate, 0.4 mg/L thiamine, 0.4 mg/L $ZnSO_4$, 0.002 mg/L folic acid, 0.5 mg/L H_3BO_3; MP Biomedicals, Santa Ana, CS, USA) were dissolved in 1 L water. Media was sterilized by filtration through a 0.2 µm filter unit (Nalgene, Rochester, NY, USA). Culture was incubated overnight at 28^{o}C with shaking. Yeast was pelleted by centrifugation at 2600 *g* for 10 mins, and then resuspended in 20 mL of sterile water to wash. Centrifugation and resuspension in water was repeated two more times. The culture was then diluted to 1.0 AU at 600 nm with sterile water for inoculation.

3.2.3.5 Fermentation conditions

All fermentations were set up in a laminar flow hood (Gelman Sciences Australia, Melbourne, Australia). To each flask 500 µl of a 60 g/L NH_4Cl solution and 1 ml of yeast starter culture (see section 3.2.3.4) was added. Air-locks were used to seal the flasks and maintain anaerobic fermentation conditions. Fermentations were carried out in the dark and flasks were swirled and weighed twice daily until mass loss stabilised. This indicated that CO_2 production and release (as a product from the conversion of sugar to ethanol during fermentation) had ceased. Yeast was removed from the wine through centrifugation at 2600 *g* for 5 min, and clarified wines were stored in glass under N_2 gas at 4^oC prior to analysis.

In all fermentation experiments four replicates were carried out, except those using transgenic and control Chardonnay berries where only three replicates were used. In the case of transgenic Shiraz and Chardonnay berries and their controls, these replicates contained berries from independent transformation lines.

3.2.4 Determination of wine anthocyanin concentrations

3.2.4.1 Spectrophotometric assay

Total anthocyanin concentrations within wines were determined using a UV-1601 spectrophotometer (Shimadzu Scientific Instruments, Kyoto, Japan) and methods described by Iland et al. (2000). This method estimates total anthocyanin concentration based on the wine's spectrophotometric absorbance properties of the wine and the extinction co-efficient (at A_{520nm}) of malvidin-3-glucoside.

3.2.4.2 HPLC

Wine anthocyanins of a 25 µl sample were separated and quantified using a Hewlett Packard 1100 HPLC system with a Wakosil C18 analytical column (3µm, 150mm x 4.6mm, SGE, USA) protected by an C18 guard column (SGE), following the method described by Downey and Rochfort (2008). Anthocyanins were identified based on their retention times previously reported using this method (Downey and Rochfort, 2008). Total anthocyanin concentration was determined by comparing total anthocyanin peak area to a standard curve of known concentrations of malvidin-3-glucoside.

3.2.5 Initial analysis of volatile wine compounds

Headspace solid phase microextraction followed by gas chromatography and mass spectrometry (HS-SPME-GC/MS) was used to analyse volatile constituents of the wine. In initial experiments, wine samples were diluted 1:2 and 1:100 in nanopure® H_2O to final volumes of 10 mL. 3 g of NaCl was added to each SPME vial. Samples were spiked with D_{13}-hexanol as an internal standard (1:2 samples with 9.20 µg and 1:100 samples with 1.15 µg). HS-SPME-GC/MS was carried out using an Agilent 7890A gas chromatograph equipped with a Gerstel MP2 auto-sampler and a 5975C mass spectrometer (Agilent Technologies, Mulgrave, Australia) using conditions described previously by Dennis et al. (2012).

The identity of detected volatiles was determined using AnalyzerPro software (SpectralWorks Ltd, Runcorn, UK). This software utilised a library of 263 known wine volatile compounds containing known mass spectral data and retention times for each compound. The program scanned for the presence of these compounds in the wine GC/MS chromatograms based on the library parameters, and then determined volatile peak areas based on a single extracted ion. These were then normalised to the D_{13}-hexanol standard ion peak area within the sample. Most volatile compounds were analysed from chromatograms obtained from the 1:2 diluted wine sample but some highly abundant compounds were measured in the 1:100 samples.

A one-way analysis of variance (ANOVA) was used within the SPSS 16.0 statistical software package (SPSS Inc., Chicago, IL) to determine which volatile compounds had mean normalised peak areas that were significantly different when comparing wines between different colour categories i.e. between red, rose and white wines. Wine colour categories were defined by their total anthocyanin concentration as determined by the method in 1.2.4. Red wines had total anthocyanins > 220 mg/L, rose wines were between 20 – 100 mg/L, and whites had values < 7 mg/L (Appendix E). A p-value of < 0.05 was considered significant and in this case Duncan's multiple range tests were performed to determine significant differences ($p< 0.05$) between the wine colour groups.

3.2.6 Analysis of monoterpenes in wine

A more targeted approach was used to analyse the presence of specific monoterpenes in the wines which were found to have significant differences in relation to wine colour in the initial screen. HS-SPME-GC/MS was carried out on 1:2 dilutions of wines as described in 3.2.5;

however wines were spiked with 1.76 pg of a D_3-linalool standard in place of D_{13}-hexanol. Peak areas of single ions of linalool (93), hotrienol (82), geranyl acetate (136) and β-citronellol (123) were determined manually within MSD Productivity ChemStation (Agilent Technologies) and normalised to the D3-linalool ion (74) peak area. Statistical analyses were then carried out as described in section 3.2.5.

3.2.7 RNA extractions and cDNA synthesis

RNA extractions, using 50 – 100 mg of powder from frozen whole berries of Cabernet Sauvignon, Malian and Shalistin were carried out using a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, MO, USA) and an On-column DNase I Digestion Kit (Sigma-Aldrich) according to the manufacturer's instructions. A NanoDrop® 1000 spectrophotometer (V3.7.1, Thermo Fisher Scientific) was used to determine RNA quantity and ensure absorbance ratios (A260/280) were between 1.8 and 2.0. RNA integrity was analysed by agarose gel electrophoresis to assess the presence of intact ribosomal bands. The Phusion ® RT-PCR Kit (Finnzymes, Massachusetts, USA) was used to synthesize cDNA from RNA samples according to their instructions.

3.2.8 qPCR analysis

Specific primers were designed to amplify 100 - 200 bp products from genes of interest and the housekeeping gene *Ubiquitin* (Appendix F). The specificity of each primer pair was confirmed by sequencing of the subsequent PCR product, and detection of a single peak of fluorescence from melt curves from both the samples and the standard (purified PCR product). cDNA was diluted 1:40 in sterile Nanopure® water (Thermo Fisher Scientific) before use. qPCR experiments were conducted using a LightCycler® 480 II instrument (Roche). Each sample was assayed in triplicate in a reaction volume of 15 µl made up of 5 µl of diluted cDNA and 0.5 µM of each primer in 1x LightCycler® 480 SYBR Green I Master Mix (Roche). Thermocycling conditions were as follows: initial activation at 95^{o}C for 5 mins followed by 45 cycles of 95^{o}C for 20 sec, 58^{o}C for 20s and 72^{o}C for 20s, then final extension at 72^{o}C for 5 mins. Reactions were then heated to 95^{o}C for 5 mins, cooled to 50^{o}C for 45s then heated to 95^{o}C at a 0.11^{o}C/sec ramping rate to produce melt curves. For each gene, standard curves were produced from a linear dilution series of target DNA fragments created

by PCR. Mean Cp values (cycle threshold values) were plotted against DNA concentration and this was used to determine the DNA concentration within cDNA samples. These concentrations were normalised against the value obtained from the housekeeping genes *Ubiquitin* (Genbank accessions CF406001) and are reported as relative transcript levels.

qPCR transcript analysis of genes in Cabernet Sauvignon, Malian and Shalistin whole berries was carried out by Amy Rinaldo (the author of this thesis) except for the analysis of MEP pathway transcripts in berry skins (see section 3.3.6.2), which was carried out by a CSIRO summer student, Lucy Arrowsmith, under Amy Rinaldo's supervision. The MEP pathway genes from grapevine were previously isolated by a visiting postgraduate student Maryham Pezhmanmehr, except for *VvIDI* which was not isolated (Pezhamnmehr, unpublished).

3.3 Results

3.3.1 Analysis of flavour and aroma compounds in wines made from berries with altered *VvMYBA* gene expression and colour

In order to study the effect of MYBA on flavour/aroma compound synthesis, berries from grapevines with altered *VvMYBA* gene expression, and hence colour, were used in microfermentations to produce wines. Transgenic Shiraz and Chardonnay berries, alongside non-transgenic controls, and Cabernet Sauvignon and its colour sports Malian and Shalistin were used in these experiments. Two methods were tested to determine total anthocyanin concentration in wines: separation of anthocyanins by HPLC and comparison of total anthocyanin peak areas to a standard curve, and a spectrophotometric assay estimating anthocyanin concentration based on wine absorbance properties at 520_{nm} (see section 3.2.4). The HPLC method was deemed unsuitable due to only very low anthocyanin concentrations being detected in rose wines, despite a clear difference in their red pigmentation compared to white wines (Figure 3.3). It was hypothesised that this may be due to the association of anthocyanins with other compounds in the wine hindering their detection using this method. The spectrophotometric assay was therefore used to determine the approximate anthocyanin concentrations in these wines (Appendix E), which were used to group the wines into three colour categories. In the following sections these colour categories will be referred to as red, rose and white wines. It is important to note that all three of these wine categories have been

named due to their colour. They have all been made in the same way and hence their names are not representative of the wine making style.

Red wines (from Cabernet Sauvignon, Shiraz, and Chardonnay containing the 35S:VvMYBA construct) had concentrations of total anthocyanins > 220 mg/L, in rose wines (from Malian, transgenic Chardonnay containing the VvMYBA1Pr:VvMYBA1 construct, and transgenic rose Shiraz containing the VvMYBAsi construct) these values were between 20 – 100 mg/L, and whites (from Shalistin, Chardonnay, and transgenic white Shiraz containing the VvMYBAsi construct) had values < 7 mg/L. Even though the white wines were made from white berries with no anthocyanins, they had low absorption readings at 520_{nm} resulting in low concentrations of anthocyanins being calculated to be in them using the spectrophotometric assay. No anthocyanins were observed in the white wines in their HPLC chromatograms (Figure 3.3). It was therefore concluded that the white wines likely contained other compounds with similar absorbance properties to anthocyanins which were detected in the spectrophotometric assay.

A comparison of average anthocyanin concentrations in wines from wildtype and transgenic Shiraz calculated via the HPLC and spectrophotometric methods shows similar values obtained from red wines by these two methods (274.8 mg/L and 257.7 mg/L respectively) but a significant difference in the values obtained from the rose (5.1 mg/L and 27.6 mg/L) and white (0 mg/L and 6.2 mg/L) wines. This illustrates that these two methods are different and that neither of them were perfect methods to determining the anthocyanin concentrations of the wines. Based on visual inspection of the wines the HPLC method seemed to underestimated anthocyanin concentration in rose wines while the spectrophotometric assay detected small concentrations of anthocyanins in white wines. However, the spectrophotometric assay values allowed us to classify the wines into the three colour groups (red, rose and white) and hence served its purpose for this study.

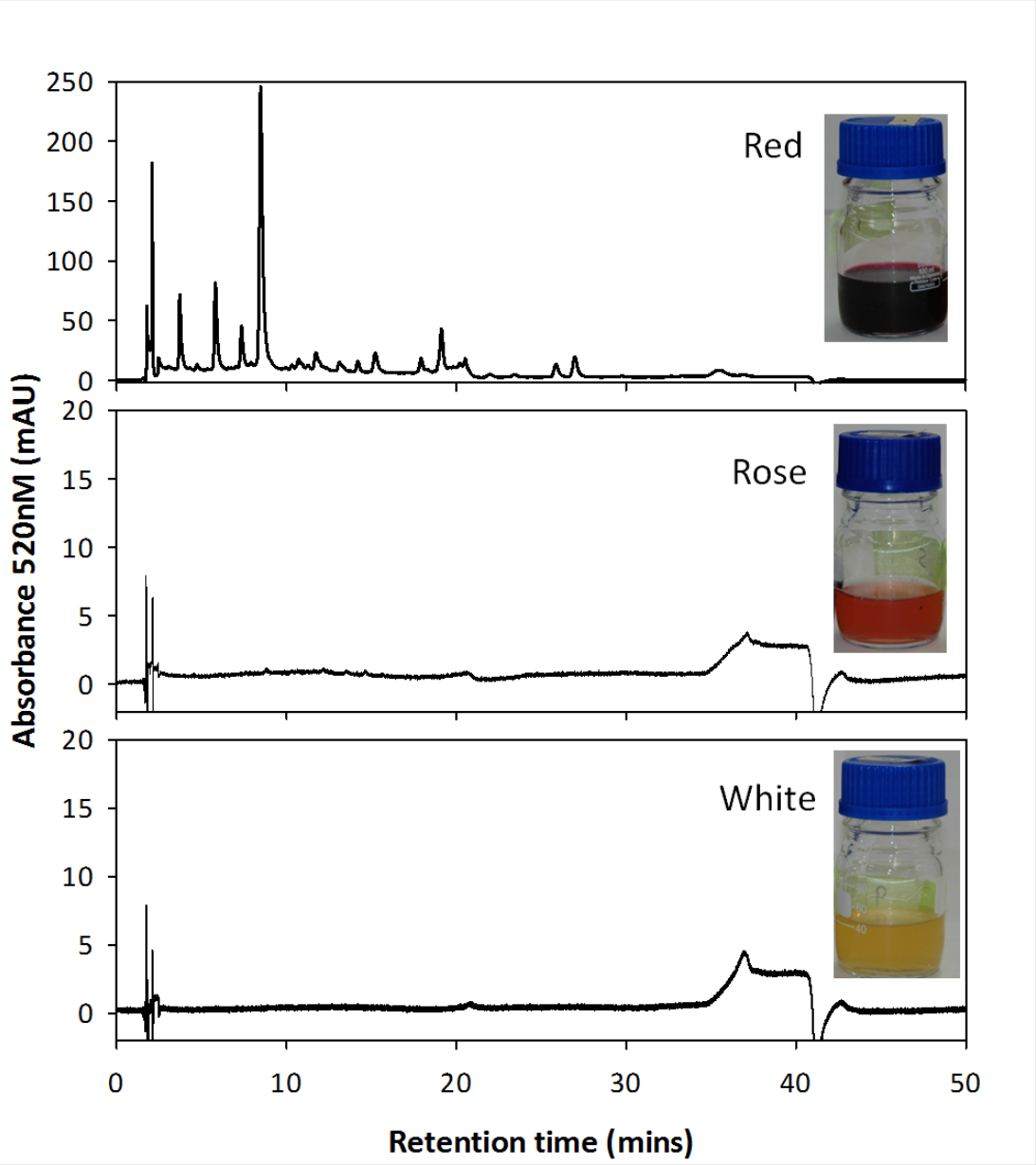


Figure 3.3: Anthocyanin differences in wines made from berries with altered *VvMYBA* gene expression

HPLC chromatograms separating anthocyanins in wines made from control Shiraz berries (red), and transgenic Shiraz berries expressing the *VvMYBA* silencing construct (*VvMYBAsi*) where gene expression is partially (rose) or completely (white) silenced. Pictures of wines are presented within the chromatograms.

Wines were analysed for the presence of 263 previously identified wine flavour/aroma compounds and their relative amounts were compared between the three wine colour categories (red, rose and white) within the same cultivar. Volatiles with significantly differing amounts (p-value > 0.05) in relation to wine colour are listed in Appendices G-J. Wines made from transgenic Chardonnay (red) berries differed from its non-pigmented controls by 56 compounds with significantly altered abundances, the highest of the three cultivars. Cabernet Sauvignon, Malian and Shalistin wines made from pressed berry fermentations (see section 3.2.3.2) had 51 significantly altered compounds, more than twice the number of 25 found in those made from grapes of the same cultivars fermented on free-run juice only (see section 3.2.3.3). The transgenic and control Shiraz wines had 21 compounds with significantly altered amounts in relation to wine colour. In all varieties, the types of compounds were varied and included a range of alcohols, esters and terpenes. Some compounds changed in only one variety (Appendices G-J) but we were particularly interested in those that were altered in at least two of the three varieties as this had the potential to reveal any conserved links between levels of VvMYBA transcripts and the biosynthesis of volatile compounds. There were 24 compounds which significantly differed in relation to wine colour in more than one of the three varieties (Table 3.1).

3.3.1.1 Flavour compounds altered by *VvMYBA* gene expression with similar trends in different cultivars

As described above, 24 flavour compounds were identified for which a significant variation in abundance correlated with berry colour and hence *VvMYBA* gene expression, and that also followed similar trends in at least two of the three cultivars used to make the wines (Table 3.1).

Linalool was the only volatile which had significantly differing amounts in relation to wine colour and followed a similar trend across the three cultivars used in this study. In all cases there was a significantly higher amount of linalool in white wines compared to reds. In Cabernet Sauvignon linalool levels in the rose wines were statistically similar to the red wines, whereas in Shiraz and Chardonnay rose coloured wines levels were similar to whites.

There were higher amounts of 3-ethoxypropyl alcohol in red wines compared to whites when made from Shiraz and transgenic Chardonnay berries, with rose wines having intermediate amounts in Shiraz but having no significant difference to whites in Chardonnay. There was a

similar trend with the volatile dihydro-2-methyl-3(2H)-thiophenone, but this was only significantly different in Cabernet Sauvignon wines made from ferments on free run-juice (and not in those made from pressed berry ferments) and in Chardonnay wines.

Ethyl phenylacetate was significantly higher in white wines compared to reds made from Cabernet Sauvignon (both fermentation styles) and Chardonnay berries, although the amounts of this volatile were not consistent in rose wine. Phenyl ethyl alcohol was highest in white wines, lowest in red wines and intermediate in rose wines from Cabernet Sauvignon (but only in pressed berry ferments) and Chardonnay.

Isobutyl acetate was significantly higher in red and rose wines made from Cabernet Sauvignon (both fermentation styles) and higher in only red wines of Chardonnay. Similarly, there were greater amounts of benzyl acetate in the red wines made from Cabernet Sauvignon (free-run juice ferments) and Chardonnay berries compared to the rose and white wines.

3.3.1.2 Flavour compounds altered by *VvMYBA* gene expression with different trends in different cultivars

There were a number of flavour compounds for which the abundances were significantly different in relation to wine colour in more than one variety, but that had dissimilar trends when comparing between them (Table 3.1). Some of these had similar trends in two varieties but then a different trend in the third. This included isoamyl acetate and ethyl butanoate, which were both significantly higher in rose wines compared to whites and reds from Cabernet Sauvignon and Shiraz, but higher in red wines, compared to roses and whites, from Chardonnay. Nerol acetate was highest in red wines, intermediate in rose wines and lowest in white wines from both Cabernet Sauvignon (from ferments on free-run juice) and Chardonnay, whereas in Shiraz wines it was highest in rose, followed by red then lowest in whites.

Other compounds had dissimilar trends in all of the varieties in which they were significantly different. In some cases their trends were completely opposite. For example, n-propyl acetate, 3-hexen-1-ol, ethyl hexanoate and isoamyl caproate were significantly higher in white wines (compared to rose and red) in one variety but then higher in red wines (compared to rose and white) in the other. It was often also the case that a particular volatile would be significantly

higher in rose wines (compared to red and white) in one variety and then higher in either red or white wines in another (e.g. hotrienol, ethyl octanoate and propyl hexanoate).

Table 3.1: Normalised peak areas of flavour compounds with significant differences in the different coloured wines of Cabernet Sauvignon, Chardonnay and Shiraz, and their colour mutants with altered *VvMYBA* gene expression

Chemical Name	Flavour/Aroma	CS skins wine colour†			CS juice only wine colour‡			Shiraz wine colour*			Chardonnay wine colour^		
		Red	Rose	White	Red	Rose	White	Red	Rose	White	Red	Rose	White
3-ethoxypropyl alcohol	?	NSD	NSD	NSD	NSD	NSD	NSD	0.0190^{c}	0.0161^{b}	0.0120^{a}	0.0216^{b}	0.0107^{a}	0.126^{a}
3(2H)-thiophenone, dihydro-2-methyl-	sulphur, fruity, berry	NSD	NSD	NSD	0.0456^{c}	0.0393^{b}	0.0332^{a}	NSD	NSD	NSD	0.0072^{b}	0.0050^{a}	0.0030^{a}
propyl octanoate	coconut, gin	0.0062^{c}	0.0045^{b}	0.0028^{a}	NSD	NSD	NSD	0.0017^{a}	0.0028^{b}	0.0016^{a}	0.0058^{b}	0.0020^{a}	0.0012^{a}
n-propyl acetate	pear, raspberry, melon, strawberry	0.0062^{a}	0.01^{b}	0.0149^{c}	0.0093^{b}	0.0091^{b}	0.0066a	NSD	NSD	NSD	0.0229^{b}	0.0081a	0.0053a
3-hexen-1-ol	grassy, green	0.0028^{a}	0.0035^{b}	0.007^{c}	0.0077^{b}	0.0052^{a}	0.0045^{a}	NSD	NSD	NSD	0.0058^{b}	0.0051^{b}	0.0031^{a}
ethyl phenylacetate	sweet, floral, honey, rose, balsamic	0.0178^{a}	0.0303^{b}	0.038^{c}	0.0433^{a}	0.0382^{a}	0.0560^{b}	NSD	NSD	NSD	0.0065^{a}	0.0176^{b}	0.0173^{b}
ethyl hexanoate	sweet, fruity, pineapple, banana, green	0.0915^{a}	0.0990^{b}	0.1223^{c}	NSD	NSD	NSD	NSD	NSD	NSD	0.2420^{b}	0.0857^{a}	0.0768^{a}
phenylethyl alcohol	rose, honey, floral	0.2455^{a}	0.3987^{b}	0.4259^{c}	NSD	NSD	NSD	NSD	NSD	NSD	0.2549^{a}	0.03154^{ab}	0.03708^{b}
hotrienol	sweet, tropical	0.0005^{a}	0.0013^{ab}	0.002^{b}	0.0004^{a}	0.0011^{b}	0.0012^{b}	0.0002^{a}	0.0027^{b}	0.0004^{a}	NSD	NSD	NSD
isoamylacetate	sweet, fruity, banana	0.0390^{a}	0.0557^{b}	0.0351^{a}	NSD	NSD	NSD	0.2081^{a}	0.4148^{b}	0.2233^{a}	0.6319^{b}	0.214^{a}	0.1712^{a}
ethyl octanoate	fruity, (banana, apricot, pear), waxy, wine	0.0448^{a}	0.0540^{b}	0.0454^{a}	NSD	NSD	NSD	NSD	NSD	NSD	0.1348^{b}	0.0568^{a}	0.0363^{a}
ethyl butanoate	fruity, pineapple, apple	0.1382^{a}	0.1554^{b}	0.1375^{a}	NSD	NSD	NSD	0.1045^{a}	0.1637^{b}	0.1222^{a}	0.3170^{b}	0.1751^{a}	0.1619^{a}

Table 3.1 continued

Chemical Name	Flavour/Aroma	CS skins wine colour†			CS juice only wine colour‡			Shiraz wine colour*			Chardonnay wine colour^		
		Red	Rose	White	Red	Rose	White	Red	Rose	White	Red	Rose	White
ethyl acetate	ethereal, fruity, sweet (grape, cherry)	0.2520^{b}	0.2482^{b}	0.2065^{a}	0.2092^{b}	0.2258^{b}	0.1633^{a}	0.1535^{a}	0.2157^{b}	0.2029^{b}	0.3903^{b}	0.2293^{a}	0.2028^{a}
isobutyl acetate	sweet, fruity, ethereal, banana, tropical	0.0258^{b}	0.0241^{b}	0.0162^{a}	0.0214^{b}	0.0221^{b}	0.0136^{a}	NSD	NSD	NSD	0.0290^{b}	0.0098^{a}	0.0083^{a}
propyl hexanoate	pineapple, fruity, sweet, green	0.0055^{b}	0.0033^{a}	0.0028^{a}	NSD	NSD	NSD	0.0015^{a}	0.0036^{b}	0.0014^{a}	NSD	NSD	NSD
2-ethyl-4-butanol	alcohol	0.0449^{a}	0.0808^{b}	0.0758^{b}	NSD	NSD	NSD	0.0572^{a}	0.0878^{b}	0.0546^{a}	NSD	NSD	NSD
ethyl isovalerate	sweet, fruity, pineapple, apple	0.0046^{a}	0.0068^{b}	0.0061^{b}	0.0027^{a}	0.0024^{a}	0.0036^{b}	NSD	NSD	NSD	0.0020^{a}	0.0029^{b}	0.0031^{b}
linalool	citrus, floral	0.0086^{a}	0.0091^{a}	0.0129b	0.0112^{a}	0.0102^{a}	0.0148^{b}	0.0153^{a}	0.0235^{b}	0.0216^{b}	0.0053^{a}	0.0102^{b}	0.0102^{b}
2-furancarboxylic acid, ethyl ester	floral, plum, raisin, balsamic	NSD	NSD	NSD	0.0046^{a}	0.0064^{ab}	0.0073^{b}	0.0046^{b}	0.0022^{a}	0.0042^{b}	0.0004^{a}	0.0021^{b}	0.0012^{ab}
nerol acetate	sweet, rose, orange, blossom	NSD	NSD	NSD	0.0023^{b}	0.0016^{ab}	0.0012^{a}	0.0014^{b}	0.0019^{c}	0.0009^{a}	0.0009^{b}	0.0006^{ab}	0.0005^{a}
6-tridecane	?	NSD	NSD	NSD	NSD	NSD	NSD	0.0003^{a}	0.0008^{b}	0.0006^{ab}	0.0004^{a}	0.0003^{a}	0.0012^{b}
isoamyl caproate	fruity, apple, banana, peach, plum	NSD	NSD	NSD	NSD	NSD	NSD	0.0335^{a}	0.0429^{ab}	0.0483^{b}	0.0675^{b}	0.0367^{a}	0.0299^{a}
ethyl nonanoate	fruity, rose, waxy, wine, grape	NSD	NSD	NSD	NSD	NSD	NSD	0.0211a	0.0269b	0.0165a	0.0217b	0.0141a	0.0078a
benzyl acetate	floral, fruity (apple, banana, apricot)	NSD	NSD	NSD	0.0021^{b}	0.0011^{a}	0.0009^{a}	NSD	NSD	NSD	0.0018^{b}	0.0007^{a}	0.0009^{a}

†CS skins – wines were made with skins, seeds and pomace present during the fermentation. ‡CS juice – wines were made from fermentations of the free-run juice of the berries

*Wines made from control Shiraz (Red) and transgenic shiraz expressing a *VvMYBA* silencing construct (VvMYBAsi) (see section 1.6) with rose (Rose) or white (White) berry phenotypes. ^ Wines made from control Chardonnay (White) and transgenic Chardonnay expressing the *VvMYBA1* gene under the control of its own promoter [(VvMYBA1Pr:VvMYBA1), Rose)] or a 35S promoter [(35S:VvMYBA1), Red] (see section 1.5).

Means followed with the same letter do not significantly differ by Duncan's test at $P \leq 0.05$. Where there is a significant difference between the means the boxes are shaded in different colours. a's are shaded in blue, b's in orange and c's in red. p-value determined by one way ANOVA test, NSD = no significant difference

3.3.2 Further analysis of monoterpenes in wines using a linalool standard

As described previously, linalool was the only volatile for which the abundance varied in line with wine colour across the three varieties used in this study. Linalool belongs to the monoterpene family (Mateo and Jiménez, 2000) and several other monoterpenes including hotrinol, geranyl acetate and β-citronellol also had significantly altered amounts in some of the wines, albeit in a sometimes inconsistent manner between varieties (Appendices G-J). In the initial screening of these volatiles, total peak areas were normalised to a D_{13}-hexanol standard. It was possible that the monoterpene differences detected in the wines may have been caused by a wine matrix effect which did not affect the alcohol standard. To test this, the wine samples were reanalysed with the addition of a D_3-linalool standard to which monoterpene peak areas were normalised (Table 3.2). Using this standard, linalool was still significantly higher in white wines of all varieties compared to reds. Rose wines had intermediate levels of linalool compared to red and white wines of Cabernet Sauvignon (both fermentation styles), but were not statistically different from white wines of Chardonnay and Shiraz. There were significantly higher amounts of hotrienol in white and rose wines of Cabernet Sauvignon (both fermentation styles) and in Chardonnay wines, hotrienol was higher in rose wines. Geranyl acetate was higher in rose and red wines made only from Chardonnay, and also β-citronellol was higher in white and rose Chardonnay wines. These results showed that the differences observed in monoterpenes in the initial flavour/aroma compound analysis of the wines were not due to a matrix effect.

Table 3.2: Monoterpenes in wines made from Cabernet Sauvignon, Chardonnay and Shiraz and their colour mutants with altered *VvMYBA* gene expression

	CS skin				CS juice				Chard MYBA				Shiraz MYBAsi			
	Mean normalised peak area				Mean normalised peak area				Mean normalised peak area				Mean normalised peak area			
Sample	**White**	**Rose**	**Red**	**p-value**	**White**	**Rose**	**Red**	**p-value**	**White**	**Rose**	**Red**	**p-value**	**White**	**Rose**	**Red**	**p-value**
Linalool	6.5763^{b}	5.1908^{ab}	4.2528^{a}	**0.015**	8.3313^{b}	6.3367^{ab}	6.0271^{a}	**0.032**	4.9778^{b}	5.9259^{b}	3.1946^{a}	**0.034**	12.9985^{b}	14.9424^{b}	8.8682^{a}	**0.03**
Hotrinol	1.0596^{b}	0.9184^{b}	0.2987^{a}	**0.01**	1.0210^{b}	0.8594^{b}	0.391^{a}	**0.023**	0.1544^{a}	0.1093^{a}	0.1575^{a}	0.474	0.3629^{a}	2.2205^{b}	0.0785^{a}	**0.004**
Geranyl Acetate	0.0106^{a}	0.0192^{a}	0.0173^{a}	0.458	0.0622^{a}	0.0800^{a}	0.1147^{a}	0.169	0.2123^{a}	0.3645^{b}	0.3600^{b}	**0.036**	0.1269^{a}	0.1523^{a}	0.1388^{a}	0.861
β-citronellol	6.0259^{a}	5.7517^{a}	5.2903^{a}	0.31	6.2372^{a}	7.0163^{a}	7.1450^{a}	0.266	3.4189^{b}	3.9350^{b}	1.8687^{a}	**0.009**	4.40473^{a}	5.3103^{a}	5.6702^{a}	0.181

†CS skins – wines were made with skins, seeds and pomace present during the fermentation.

‡CS juice – wines were made from fermentations of the free-run juice of the berries

*Wines made from control Shiraz (Red) and transgenic shiraz expressing a *VvMYBA* silencing construct (VvMYBAsi) (see section 1.6) with rose (Rose) or white (White) berry phenotypes.

^ Wines made from control Chardonnay (White) and transgenic Chardonnay expressing the *VvMYBA1* gene under the control of its own promoter [(VvMYBA1Pr:VvMYBA1), Rose)] or a 35S promoter [(35S:VvMYBA1), Red] (see section 1.5).

Means followed with the same letter do not significantly differ by Duncan's test at $P \leq 0.05$

p-value determined by one way ANOVA test. Where there is a significant difference between the means the boxes are shaded in different colours. a's are shaded in blue, b's in orange and c's in red

3.3.3 Gene expression analysis of monoterpene biosynthesis genes identified from analysis of microarrays experiments

The results from the flavour and aroma analysis of wines made from grapes with altered *VvMYBA* gene expression, indicated that there was a link between the expression of this TF and the synthesis of monoterpenes. The microarray results discussed in chapter 2 identified two genes with links to monoterpene biosynthesis which had consistently altered gene expression in relation to the presence of VvMYBA in both Chardonnay and Shiraz berries (Table 2.1). One of these was the characterised *V. vinifera E-β-ocimene synthase* (*VvbOci*) gene (microarray ID VIT_12s0134g00030), encoding a functional monoterpene synthase, which produces the monoterpene ocimene in *in vitro* assays (Martin et al., 2010). The other gene (microarray ID VIT_18s0001g13790, Table 2.1), is uncharacterised, but has homology to cytochrome P450 genes from other plant species, and was annotated as belonging to the 'vv10902Monoterpenoid_biosynthesis' network in the relevant column within the microarray dataset provided to us (see raw dataset at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=cnmxwmmsfjctvgt&acc=GSE56915). The transcription of both genes was down-regulated in red grapes expressing VvMYBA and up-regulated in white grapes lacking this TF. To investigate whether either of these genes could be contributing to the monoterpene differences seen in the wines made from grapes with altered *VvMYBA* gene expression, their transcript levels were analysed in the skins of Cabernet Sauvignon, Malian and Shalistin berries collected from just before veraison through to harvest (Figure 3.4).

VvbOci was not expressed in the berry skins until between 11-13 wpf, from which point there was an increase in transcript levels through to harvest in all three varieties. Transcription of this gene was highest in Malian berry skins, followed by that of Shalistin, and was the lowest in Cabernet Sauvignon. At harvest (18 wpf) Cabernet Sauvignon transcript levels were almost 6 fold lower than Shalistin and > 11 fold lower than Malian. A similar trend was observed in the expression patterns of the putative P450 gene but low levels of transcripts of this gene were detected earlier at 9 wpf, which then increased post-veraison. At harvest the P450 transcript levels were highest in Malian, then Shalistin and lowest in Cabernet Sauvignon. These differences were smaller compared to the *VvbOci* gene with Malian and Shalistin transcript levels 2.5 fold and 1.6 fold higher than Cabernet Sauvignon respectively.

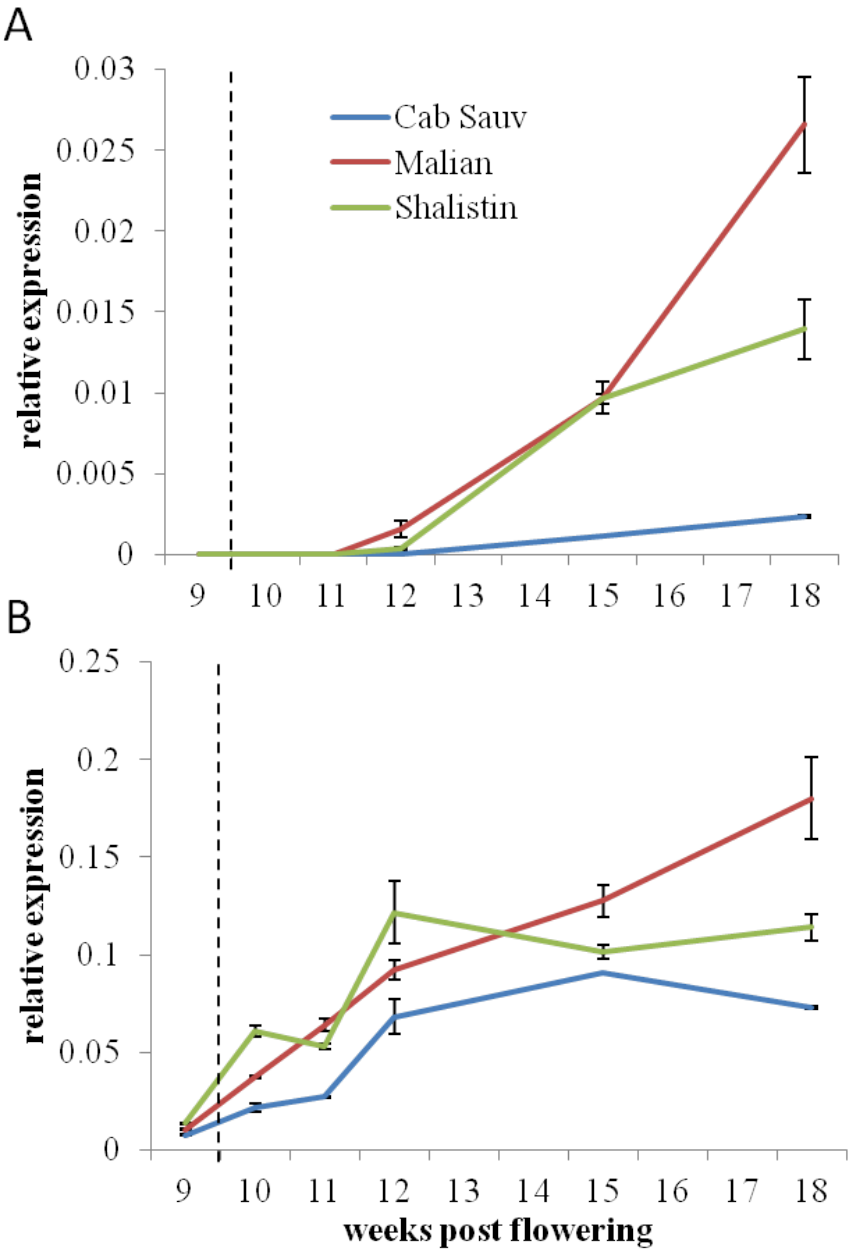


Figure 3.4: Transcript levels of genes with links to monoterpene biosynthesis in berry skins of Cabernet Sauvignon, Malian and Shalistin

Transcript levels in berry skins of **A)** the characterised *Vitis vinifera E-β-ocimene synthase* gene and **B)** a putative P450 gene throughout berry development of Cabernet Sauvignon (Cab Sauv), Malian and Shalistin varieties. Vertical dashed line represents the time when veraison occurred. Gene expression was determined by qPCR and is shown relative to expression levels of the housekeeping gene *VvUbiquitin*. All data is presented as a mean of three replicates with standard error bars.

3.3.4 Linalool synthase is down-regulated in red grapes

The only volatile that was present in altered amounts in relation to wine colour in a consistent manner in all three of the cultivars used was linalool. Wines made from white grapes not expressing *VvMYBA* contained higher amounts of linalool compared to red wines. It seemed possible that lower levels of linalool in red wines could be a consequence of reduced transcription of linalool synthase genes in red berries. In a recent study of the terpene synthase family in grapevine, seven linalool synthase genes were identified and biochemically characterised from both Pinot Noir and Cabernet Sauvignon (Martin et al., 2010). In a follow-up study the expression of these genes from Pinot Noir was analysed in parallel with the accumulation of linalool in berries and two *(3S)- Linalool/(E)- Nerolidol synthase* genes (*VvPNRLinNer1* and *VvPNLinNer2*) were discovered to have the highest expression levels in post-veraison berries which most closely correlated with linalool accumulation (Matarese et al., 2013). The homologous gene from Cabernet Sauvignon (*VvCSLinNer*) therefore seemed the most logical choice for gene expression studies in Cabernet Sauvignon, Malian and Shalistin berries.

Transcript levels of *VvCSLinNer* were measured in whole berries of Cabernet Sauvignon, Malian and Shalistin throughout berry development (Figure 3.5). This gene was most highly expressed in 2 wpf berries with little differences between the three cultivars. Transcript levels were then low from 4 – 8 wpf after which they increased followed by a second decrease in expression between 12 – 15 wpf. Gene expression differences between the three varieties were observed shortly after veraison and were largest at 12 wpf when Shalistin had a 4.5 fold higher transcript level than Cabernet Sauvignon and slightly more than this compared to Malian. There was very little or no *VvCSLinNer* transcript detectable in all three varieties at 15 wpf through till harvest.

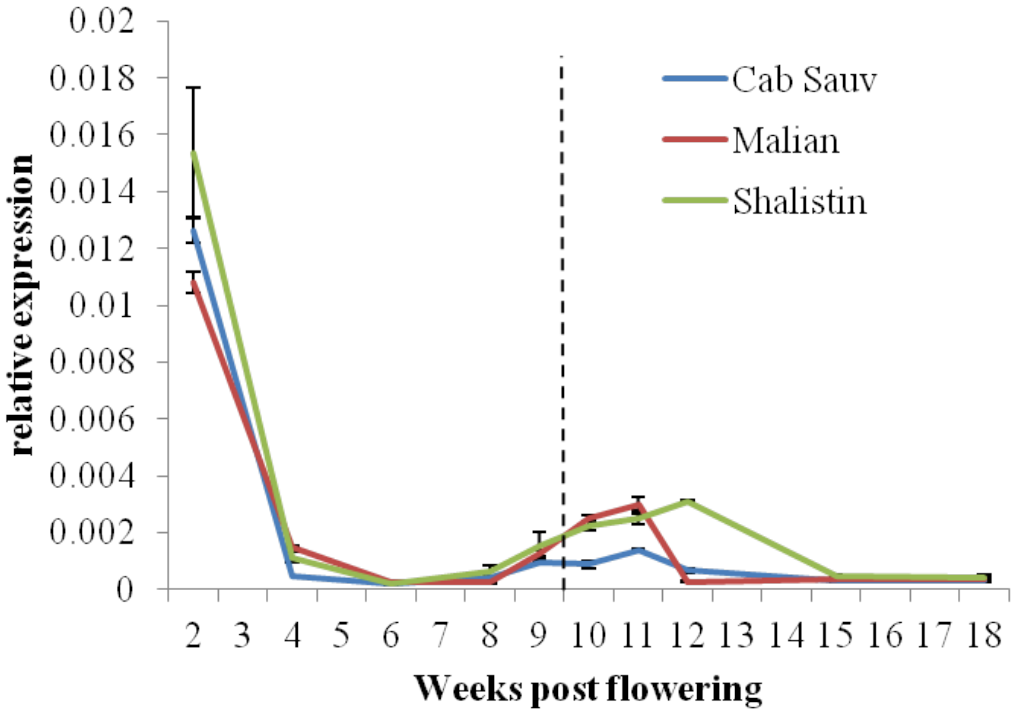


Figure 3.5: Transcript levels of a linalool synthase gene in berries of Cabernet Sauvignon, Malian and Shalistin

Transcript levels of the Cabernet Sauvignon *(3S)-linalool/(E)- nerolidol synthase* sp. *Vitis vinifera* (*VvCSLinNer*) in whole berries of Cabernet Sauvignon (Cab Sauv), Malian and Shalistin throughout development. Vertical dashed line represents the time when veraison occurred. Transcript levels were determined by qPCR and are shown relative to transcript levels of the housekeeping gene *VvUbiquitin*. All data is presented as a mean of three replicates with standard error bars.

3.3.5 The effect of light exclusion from Cabernet Sauvignon, Malian and Shalistin berries on wine linalool abundance

Due to the presence of anthocyanins, red grapes may absorb light differently to white grapes, which may alter light sensitive secondary metabolite pathways, including monoterpene production. To test whether light absorption may have contributed to lower abundances of linalool in wines made from red grapes a light exclusion experiment was set up where Cabernet Sauvignon, Malian and Shalistin grape bunches were covered with a box from the pre-veraison period through to harvest. The levels of linalool present in wines made from boxed berries and non-boxed controls were measured (Figure 3.6). As seen previously, there were significantly higher amounts of linalool in wines made from non-boxed control Shalistin berries compared to Cabernet Sauvignon, with Malian having mean linalool levels intermediate between the other two varieties but not significantly different to Shalistin wines. Wines made from shaded berries with no light exposure did not have this trend but instead there were no significant differences in the abundance of linalool across all three varieties. Furthermore the levels of linalool in shaded berries were similar to those in wines made from control Cabernet Sauvignon berries.

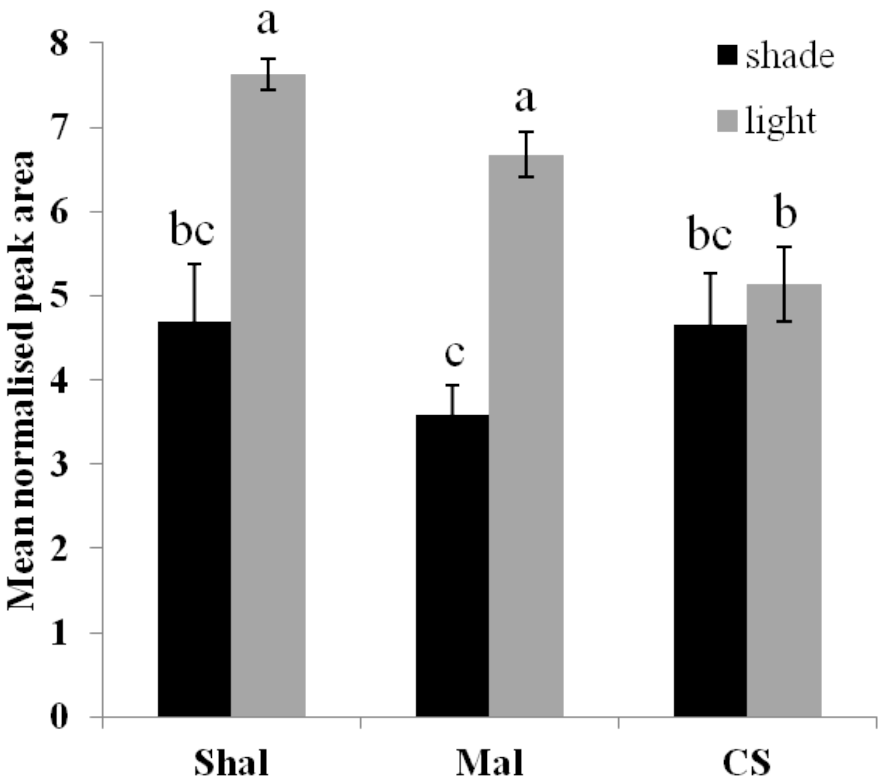


Figure 3.6: Shade effect on linalool abundance in wines made from Cabernet Sauvignon, Malian and Shalistin berries.

Abundance of linalool in wines made from Cabernet Sauvignon (CS), Malian (Mal) and Shalistin (Shal) berries grown in ambient light conditions or in the shade. Berries grown in the shade were boxed pre-veraison to eliminate light exposure throughout veraison to harvest (when berries reached 22 ^{o}Brix). Wines were produced from berries using microfermentations and linalool abundance was measured using head-space SMPE-GC/MS. Means were calculated from 4 biological replicates.

3.3.6 The expression of monoterpene precursor biosynthesis genes in Cabernet Sauvignon, Malian and Shalistin berries

Monoterpenes, including linalool, are synthesised from isopentenyl diphosphate (IPP) precursors which are derived from the methylerythritol phosphate (MEP) pathway located within plastids (reviewed in Yu and Utsumi, 2009). Figure 3.2 summarises the MEP pathway intermediates and the enzymes involved in their synthesis. Transcript levels of MEP pathway genes were measured in berries of Cabernet Sauvignon, Malian and Shalistin cultivars throughout fruit development and ripening. Monoterpenes are produced in both the flesh and skin of berries (Luan and Wüst, 2002); hence gene expression was analysed in both whole berries (throughout development from 2 wpf to harvest, Figure 3.7) and in skins only (from 9 wfp to harvest, Figure 3.8). The *IDI* gene from grapevine has not been isolated and so the expression of this gene was not measured in this project.

3.3.6.1 MEP pathway gene expression in whole berries

In general there were no significant differences between transcript levels of MEP pathway genes in Cabernet Sauvignon, Malian and Shaistin berries. Transcript levels of *VvDXR*, *VvMCS*, *VvHDS*, and *VvIDS* were slightly higher in Shalistin berries compared to Cabernet Sauvignon post-veraison, but the greatest difference observed was for *VvIDS* at 11wpf and was only 1.4 fold higher than in Shalistin berries. Whether transcript levels in Malian were highest, intermediate between the two varieties, or lowest, was inconsistent and differed between the genes. The transcript levels of some MEP pathway genes in whole berries of Malian collected at 4 and 12 wpf, were often an outlier when compared to the overall trend of expression in Malian, and also when compared to the trend in the other two varieties.

3.3.6.2 MEP pathway gene expression in skins

For all genes except *VvDXS* and *VvCMS*, transcript levels in Shalistin berry skins were significantly higher post-veraison than in Cabernet Sauvignon. They remained higher in Shalistin till harvest except in the case of *VvCMK* where Shalistin transcripts decreased after 15 wpf to levels similar to those in the skins of Cabernet Sauvignon. The largest difference was observed in *VvHDS* transcript levels 18 wpf, which were 1.8 fold higher in Shalistin than Cabernet Sauvignon. The second largest differences were observed for *VvMCS* and *VvIDS* transcript levels which were both 1.6 fold higher in Shalistin at 18 wpf and 12 wpf respectively. Malian berry skin transcript levels were usually similar to that of Shalistin.

These results clearly demonstrate differential expression of MEP pathway genes in the berry skins of Cabernet Sauvignon, Malian and Shalistin cultivars.

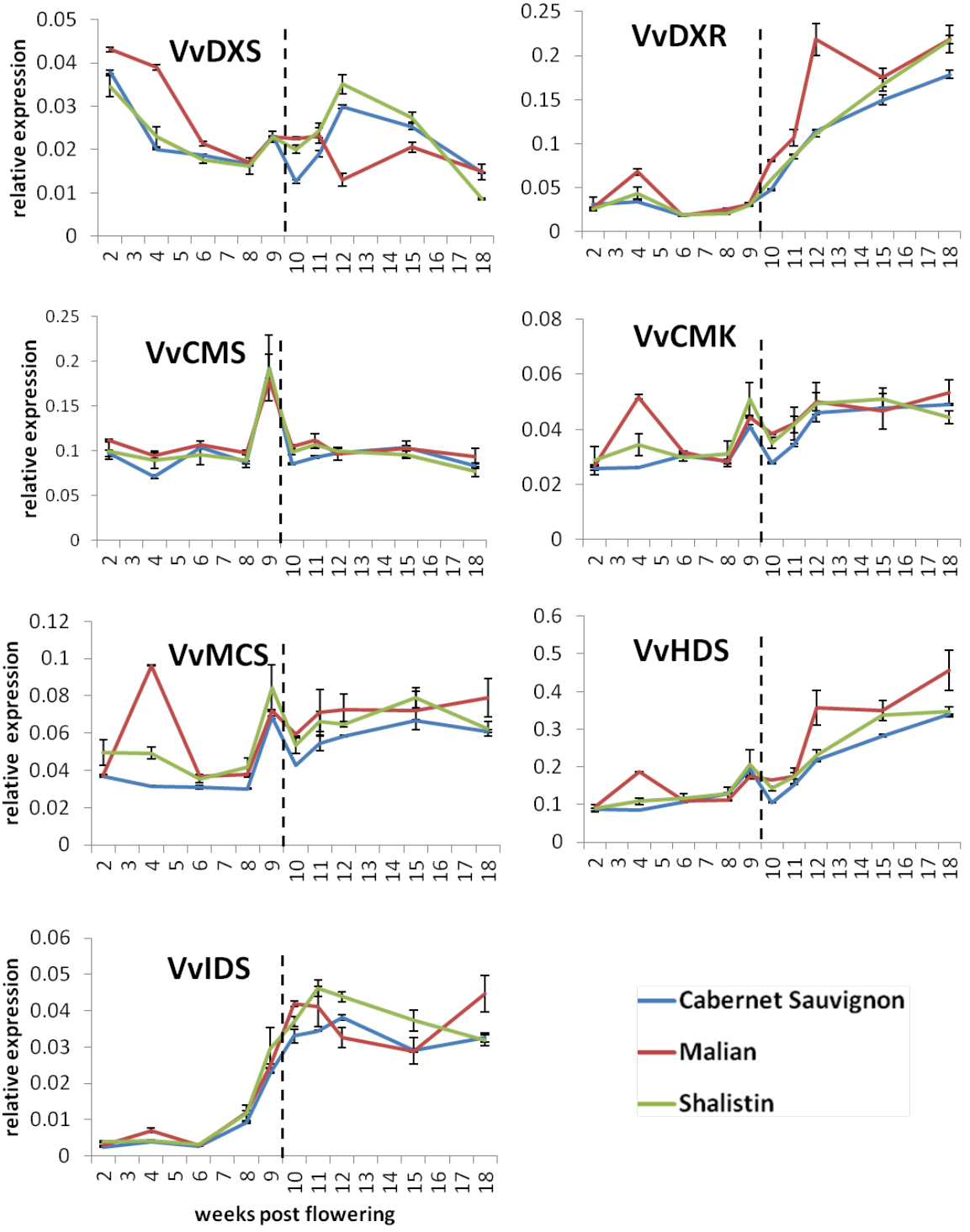

VvDXS
VvDXR
VvCMS
VvCMK
VvMCS
VvHDS
VvIDS
relative expression
weeks post flowering
Cabernet Sauvignon
Malian
Shalistin

Figure 3.7: Transcript levels of MEP pathway genes in whole berries of Cabernet Sauvignon and its colour sports

Relative gene expression of putative *Vitis vinifera* MEP pathway genes over the development of Cabernet Sauvignon, Malian and Shalistin whole berries. The vertical dashed line represents the onset of ripening (veraison). Gene expression was determined by quantitative PCR and is shown relative to the expression of the housekeeping gene *VvUbiquitin*. All data are presented as the mean of three technical replicates, with standard error bars. Gene names are as follows: *1-deoxy-D-xylulose 5-phosphate synthase* (*VvDXS*), *1-deoxy-D-xylulose 5-phosphate reductoisomerase* (*VvDXR*), *4-diphosphocytidyl methylerythritol* synthase (*VvCMS*), *4-(cytidine 5′ -diphospho)-2-C-methyl-D-erythritol kinase* (*VvCMK*), *2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase* (*VvMCS*), *4-hydroxy-3-methylbut-2-enyl diphosphate synthase* (*VvHDS*), *1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase* (*VvIDS*)

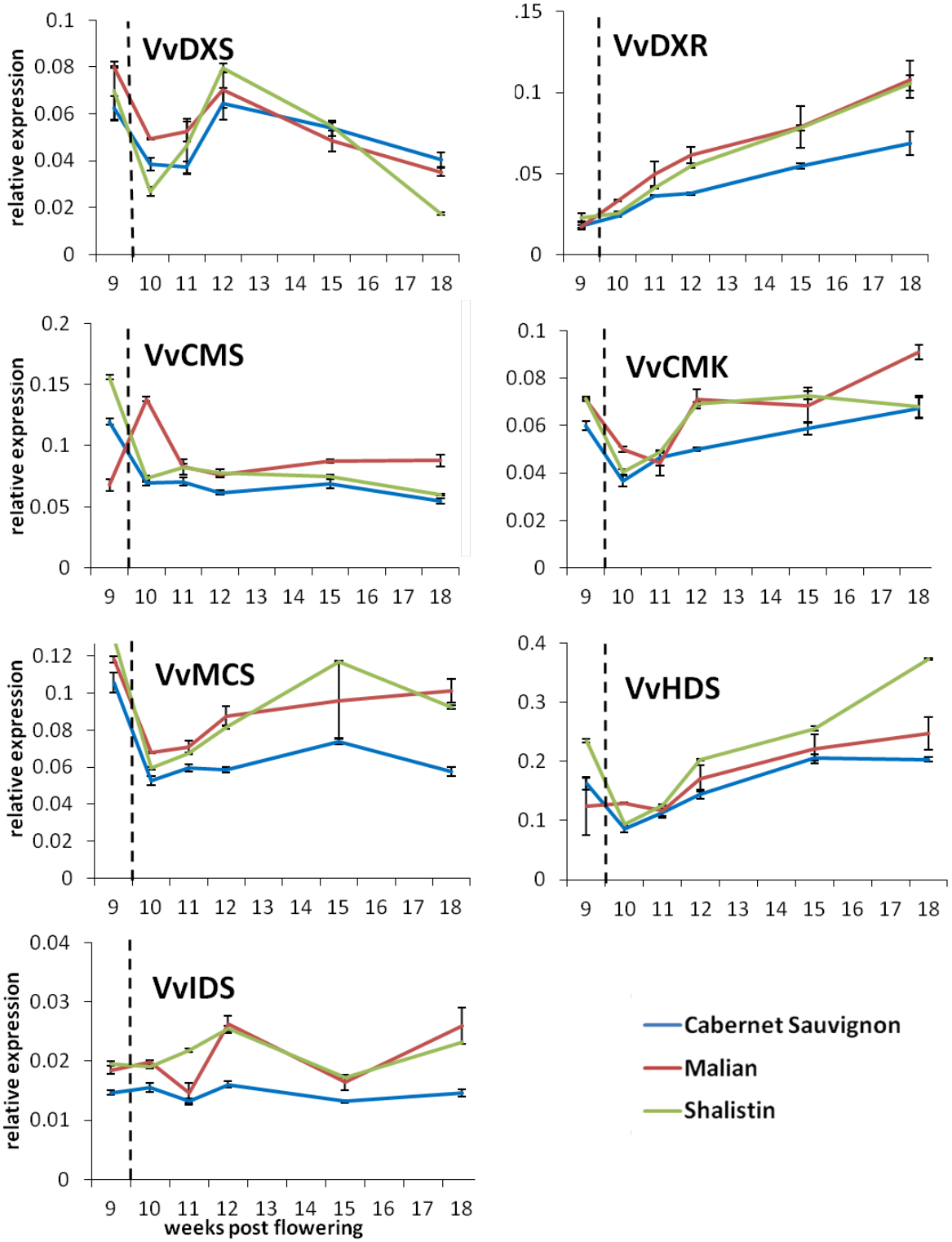

VvDXS
VvDXR
VvCMS
VvCMK
VvMCS
VvHDS
VvIDS
relative expression
weeks post flowering
Cabernet Sauvignon
Malian
Shalistin

Figure 3.8: Transcript levels of MEP pathway genes in berry skins of Cabernet Sauvignon and its colour sports

Relative gene expression of putative *Vitis vinifera* MEP pathway genes in berry skins over the development of Cabernet Sauvignon, Malian and Shalistin berries. The vertical dashed line represents the onset of ripening (veraison). Gene expression was determined by quantitative PCR and is shown relative to the expression of the housekeeping gene *VvUbiquitin*. All data are presented as the mean of three technical replicates, with standard error bars. Gene names are as follows: *1-deoxy-D-xylulose 5-phosphate synthase* (*VvDXS*), *1-deoxy-D-xylulose 5-phosphate reductoisomerase* (*VvDXR*), *4-diphosphocytidyl methylerythritol* synthase (*VvCMS*), *4-(cytidine 5′ -diphospho)-2-C-methyl-D-erythritol kinase* (*VvCMK*), *2-C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase* (*VvMCS*), *4-hydroxy-3-methylbut-2-enyl diphosphate synthase* (*VvHDS*), *1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase* (*VvIDS*)

3.4 Discussion

3.4.1 Altering *VvMYBA* gene expression in berries changes flavour compounds present in their wines

In this study, wines were made from mutant Cabernet Sauvignon, Shiraz and Chardonnay berries with altered *VvMYBA* gene expression, and analysed for flavour and aroma compounds in order to assess if the colour regulator VvMYBA also has a role in regulating flavour metabolism. There were 24 volatiles found to be significantly altered in the different coloured wines in at least two of the three varieties (Table 3.1). In some cases the volatile amounts were altered in relation to wine colour in the same way between the different varieties, and in other cases they were not.

It is important to note that the identification of wine volatiles was reliant on a library of 263 previously identified wine compounds. As a consequence volatiles not present within this library were not analysed. It is also important to keep in mind that while berry bunches from the transgenic Chardonnay and Shiraz vines, and their non-transgenic controls, were collected at the same approximate 'ripeness', they were not collected at the same time, as flowering in the glasshouse was not synchronized. This meant that berries were frozen and stored at -80^{o}C until all samples had been collected. Frozen berries then had to be ground to a powder before being used in the microfermentations. The consequence of the skins and seeds of the berries being ground to a fine powder may have affected the final volatile profile of the wines made from these berries and may not represent that of wines made from fresh berries. This is why being able to compare volatile profile changes to the wines obtained from fresh berries of Cabernet Sauvignon, Malian and Shalistin has been important in this study. However, a direct comparison cannot be made between the levels of volatiles in wines made from Cabernet Sauvignon, Malian and Shalistin to those made from transgenic Chardonnay and Shiraz berries because of the difference in the fermentation set up of the fresh and frozen berries. As a consequence of the frozen berries being finely ground, a sugar/water solution need to be added to the grape matter to ensure the ferments were liquid enough to facilitate thorough mixing throughout the fermentation. This means that the grape matter was diluted 1:2 in fermentations of frozen berry material, which was not the case in the fresh berry ferments. Even so, a comparison on the trends of volatile abundances in relation to wine colour can be compared between the different varieties, yet it is possible that some differences may have

been too diluted to be detected in the wines made from the transgenic Chardonnay and Shiraz berries and their controls.

3.4.1.1 VvMYBA has an effect on the presence of volatile phenylpropanoids and benzenoids

Levels of ethyl phenylacetate were significantly higher in white wines compared to reds except those made from Shiraz berries where there were no significant differences (Table 3.1). This compound is a volatile phenylpropanoid derived from a phenylalanine precursor which is converted via phenylacetaldehyde, and phenylacetate, to form ethyl phenylacetate. Phenylethyl alcohol, another volatile phenylpropanoid derived from phenylalanine, was also present in higher amounts in white wines, with rose wines having intermediate levels of this compound, and reds having the lowest levels (Table 3.1). Phenylalanine is also a precursor for the synthesis of anthocyanins as well as many other metabolites in plants (Colquhoun et al., 2010). It is possible that due to the rapid synthesis of anthocyanins in red grapes post-veraison, metabolic flux through the phenylpropanoid pathway is directed to flavonoid biosynthesis preferentially rather than to the synthesis of volatile phenylpropanoids. Previous research has demonstrated that the up- or down-regulation of one branch of the phenylpropanoid pathway can have an opposite effect on another branch of the pathway. For example, in *Arabidopsis* silencing *hydroxycinnamoyl CoA:shikimate hydroxycinnamoyl transferase*, a lignin biosynthetic gene, resulted in repression of lignin biosynthesis and re-direction of metabolic flux to flavonoid biosynthesis (Besseau et al., 2007). Similarly, overexpressing the regulator ZmMYB31 in maize reduced the synthesis of lignin, directing metabolic flux to the flavonoid branch of the phenylpropanoid pathway (Li et al., 2010).

The benzenoid, benzyl acetate, followed a different trend to the phenylpropanoids mentioned above as there were significantly higher amounts of this compound in red wines compared to whites (Table 3.1). Alcohol acyltransferases catalyse the conversion of benzyl alcohol [derived from *trans*-cinnamic acid, a phenylpropanoid pathway metabolite (Colquhoun et al., 2010)] to benzyl acetate (Shalit et al., 2001). One might expect that phenylpropanoid and benzenoid synthesis would be regulated in a similar manner in response to metabolic flux through these pathways. This was observed previously when the *Arabidopsis* MYB TF gene homologous to *VvMYBA, AtPAP1,* was expressed in petunia and roses (Zvi et al., 2008; 2012). In the petunia flowers, anthocyanin biosynthesis was up-regulated as were emissions of volatile phenylpropanoids and benzenoids. In the roses there was an increase in volatile phenylpropanoid emission but no difference in benzenoid release. These studies, in

conjunction with the research presented in this chapter, indicate that the effect of anthocyanin synthesis on the regulation of phenylpropanoid and benzenoid emissions may be species specific. It is important to note that this study was conducted in fruit rather than flowers which may also be contributing to the differences in our results from those previously reported. In apple *MdMYB10,* a functional homologue of *VvMYBA*, was ectopically expressed in the cultivar Royal Gala. The abundance of 45 volatile compounds was measured in the fruit and of which there were almost no significant differences compared to controls, with only two compounds, putatively identified as hexenoic acid and as methyl iso-eugenol, having slightly lower abundances in the *MdMYB10* overexpressing lines (Espley et al., 2013). No alteration in phenylpropanoid or benzenoid volatile abundanace was detected. This suggests that the effect of anthocyanin synthesis on volatile production in the fruit of grapevine is distinct to that seen in apple and hence is not necessarily conserved between different species.

3.4.1.2 VvMYBA has a negative effect on the abundance of linalool

Linalool was the only volatile identified in this study to have significantly altered amounts in the different wine colour categories with a similar trend seen across all three varieties (Table 3.1 and Table 3.2). White wines made from Chardonnay and mutant Shiraz and Cabernet Sauvignon berries lacking *VvMYBA* gene expression had consistently higher amounts of linalool compared to red wines of the same variety. Whether the rose wines had statistically similar linalool levels to the red or white wine categories was variety specific. Linalool is a common white wine flavour compound belonging to the monoterpene family (Mateo and Jiménez, 2000). It is considered to be one of the more important wine monoterpenes as it is often present in greater amounts than the others and has a lower flavour perception threshold (Pedersen et al., 2003). It possesses a floral/citrus aroma which is a descriptor more commonly associated with white wines (Ballester et al., 2009). It is therefore perhaps not surprising that these results suggest that berry colour may be linked to the amount of linalool present in their wines.

3.4.1.3 VvMYBA expression changed volatile production in a variety specific manner

Nineteen of the 24 compounds that had significantly altered levels in different coloured wines in at least two of the varieties used in this study, did not display consistent trends of

abundance with respect to wine colour across the different varieties (Table 3.1). In some cases their abundances were even the complete opposite in the different varieties, being highest in red wines of one variety and then highest in white wines of the other. Furthermore the majority of compounds that were identified as having significantly altered abundances due to wine colour were only detected as such in one variety. This was an unexpected result but may indicate that VvMYBA, or the presence of anthocyanins, may have cultivar-specific roles in flavour metabolism.

Another explanation for this is that there may have been slight differences in ripeness between the replicates and cultivars in the case of the transgenic Chardonnay and Shiraz berries. Due to small bunch sizes and a low number of transgenic bunches, TSS were measured from a small subsample of berries from each bunch before they were collected, with the aim of keeping TSS levels similar between replicates. In some cases the combined total sugars determined from a pooled juice sample from all berries of the bunch was different to the initial measurement, which meant that there were differences between replicates and varieties (Appendix D). Sugar levels were controlled in the fermentation but if the berries were at different developmental stages they may have differed in the abundance of grape-derived volatile precursors affecting the results. This may have contributed to the high number of volatiles which had different trends in relation to *VvMYBA* gene expression in only one cultivar.

3.4.1.4 Rose wines were different from red and white wines

At the commencement of this study, it was expected that rose wines would possess flavour characteristics intermediate between those of red and white wines. The results have suggested that this was not the case. Phenylethyl alcohol was the only volatile that had intermediate levels in rose wines and this was only seen in two of the three varieties tested (Table 3.1). There were many instances when volatile abundances were significantly higher or lower in rose wines when there was no significant difference between their levels in red and whites. It is difficult to understand how these differences could be related to their synthesis within the berries, considering *VvMYBA* gene expression levels were intermediate in the rose coloured berries compared to the white and red grapes. As the rose wines were made in exactly the same way as the white and red wines these differences are clearly not related to the winemaking strategy. It may be possible that low concentrations of anthocyanins, such as those found in the rose wines, may cause increased release or retention of volatiles from the

wine matrix, and that these effects are not imposed when there are no anthocyanins or higher concentrations of anthocyanins present. There have been no studies which have compared volatile release from wines with anthocyanin concentrations ranging from zero to high. One study analysed the sensory properties of Malbec wines which differed in their total polyphenol concentrations; one group of wines had low concentrations and the other high concentrations (Goldner et al., 2010). They found that the wines of low polyphenol concentration were described to have greater fruity, citrus, strawberry, cooked fruit and floral aromas compared to the high concentration group, but the study lacked a white wine comparison. Further research is required to understand why the abundances of volatiles in rose wines in this study were often different compared to the red and white wines.

3.4.1.5 Altering *VvMYBA* gene expression resulted in more flavour differences in wines from ferments on pressed berries

Cabernet Sauvignon, Malian and Shalistin wines were made from fermentations in the presence of skins, seeds and pomace (pressed berries ferments) as well as from ferments on juice only. The wines made from pressed berry ferments had over twice the number of flavour compounds with significantly altered abundances in relation to wine colour, compared to those made from free-run juice (51 to 25 compounds respectively) (Appendices G-J). *VvMYBA* is only expressed in the skins of Cabernet Sauvignon and Malian berries (Walker et al., 2006). If this TF had a role in regulating flavour metabolism in these berries then it would be expected that differences in volatile abundances would more likely reside within the skins of the berries. Due to a more complete extraction of flavour compounds from berry skins when they are present throughout fermentation it is not surprising that there are more flavour differences between the wines made in this style compared to the free-run juice fermentation style. Furthermore, in the pressed berry ferments, higher concentrations of PAs and anthocyanins would also be extracted from the skins and seeds of the berries, which would create greater differences in the non-volatile matrix between the Cabernet Sauvignon, Malian and Shalistin wines made in this style. A number of studies have shown that the presence of anthocyanins and PAs can affect the release of volatile flavour compounds from the wine matrix (Dufour and Sauvaitre, 2000; Goldner et al., 2010; Mitropoulou et al., 2011; Rodriguez-Bencomo et al., 2011; Sáenz-Navajas et al., 2012). The abundances of wine volatiles in this study were normalised to a D_{13}-hexanol standard, which belongs to the alcohol family of volatiles. This standard may interact with the wine matrix in a different

manner to other volatiles, particularly those belonging to a different family. Wine contains numerous classes of volatile aroma compounds including esters, alcohols, acids, lactones, carbonyl compounds, and phenols (Rapp and Mandery, 1986). Rocha et al. (2001) studied the behaviour of nine different wine aroma compounds, belonging to a number of these families, and their sorption onto an SPME fibre from the headspace of model wine matrices. Their results illustrated that different compounds were released from the matrix and absorbed by the SPME fibre at different rates. To understand whether volatiles with altered abundances in relation to wine colour that differed between the two fermentation styles in this study were due to wine matrix affects or a consequence of *VvMYBA* gene expression, further analyses of the wines using a number of different types of standards representing all of the wine aroma classes is required.

3.4.2 Considerations for further investigation into flavour differences of wine affected by VvMYBA

As outlined in the preceding discussion sections of this chapter, this study identified a number of alterations to wine volatile profiles of wines made from grapes with altered *VvMYBA* gene expression. One question that remains is whether these volatile differences actually have an effect on wine flavour. Many wine volatile compounds have reported odour thresholds, determined within wine or model wine matrices, which indicate the lowest concentration of that compound that has been perceived in wine (Czerny et al., 2008). If the volatiles were at concentrations below their odour thresholds then it is less likely that differences in their abundances would affect the flavour of the wine. Volatile abundances were qualitatively determined in this study by normalising their peak areas to the area of a standard. To determine the actual concentrations of the volatiles and therefore whether they are above or below their odour threshold, the GC/MS peaks areas would need to be compared to a standard curve of known concentrations of that same compound.

It has been shown that the final flavour and aroma of a wine is not only related to the concentrations of the individual volatiles within it, but is also determined by how these volatiles interact with one another. Escudero et al. (2007) spiked dearomatized wine with 10 μg/L of dimethyl sulphide (DMS) and demonstrated that at this low concentration the aroma of this compound could not be detected. Yet when a mixture of fruity esters was added to the

same wine matrix, the addition of DMS, at this low concentration, clearly increased the intensity of the fruity notes detected in those wines. Sensory studies of the wines made in our study, where the aroma of the different coloured wines are compared, could determine if the combined effect of the altered abundances of the volatiles in these wines have changed their overall aroma.

As discussed in section 3.4.1.5 it is possible that some of the volatile differences detected in this study were a result of alterations to the non-volatile wine matrix rather than due to the levels of their precursors within the berries. To determine whether the volatile differences originated within the berries as a consequence of *VvMYBA* transcription, the abundance of these compounds, or their precursors, needs to be measured within berry tissue.

3.4.3 Differential expression of genes involved in monoterpene biosynthesis due to VvMYBA may account for altered levels of monoterpenes in wines

As linalool was the only aroma compound found to be significantly altered in relation to grape and wine colour in the same manner across all three cultivars used in this study, a further analysis of the expression of genes involved in its synthesis was carried out. The results showed that the linalool synthase gene *VvCSLinNer* is differentially expressed post-veraison in Cabernet Sauvignon, Malian and Shalistin berries (Figure 3.2). The greatest difference in transcript levels were observed at 12 wpf where there were low levels in Cabernet Sauvignon and Malian, and at least 4.5 fold higher levels in Shalistin. The differences in the expression of this gene may explain why Shalistin wines contained higher amounts of linalool compared to Cabernet Sauvignon wines. Even though *VvCSLinNer* was expressed early in berry development, differential expression of the gene between the varieties was only observed from 10 wpf, just after veraison. This is when VvMYBA is expressed in red berries, suggesting that the *VvCSLinNer* gene expression differences can be correlated to the expression of this TF.

There were also two genes with known or putative roles in monoterpene biosynthesis that were identified through the microarray experiments discussed in Chapter 2. Both of these were more highly expressed in white grapes compared to red. The expression of these genes was analysed over the development of Cabernet Sauvignon, Malian and Shalistin berries to determine whether they too may be contributing to the differences in linalool abundance in their wines (Figure 3.4). One of these genes was the characterised *E-β-ocimene synthase*

(*VvbOci*), which was differentially expressed between the three varieties post-veraison, with greatest differences observed at 18 wpf. Malian had the highest transcript levels, followed by Shalistin, and Cabernet Sauvignon had the lowest. Even though transcript levels of this gene was greater in Shalistin than Cabernet Sauvignon, it seems unlikely that this could be contributing to the differences in linalool abundance in their wines as gene expression in Malian was significantly higher than Shalistin, while linalool levels were statistically similar in their wines. There were no monoterpenes analysed with highest levels in Malian, intermediate levels in Shalistin, and lowest levels in Cabernet Sauvignon, which would have matched the expression of *VvbOci*. Levels of β-ocimene were not signficanly different between the three varieties. While this thesis was being written, the uncharacterised p450 gene was re-annotated from being associated to a monoterpene biosynthesis gene network to belonging to a sugar metabolism network (Vv10966Glucosinolate biosynthesis). The expression of this gene follows a pattern that would be expected of one involved in sugar metabolism: it is expressed from just before veraison and transcript levels gradually increase till harvest.

3.4.4 Anthocyanins may cause a shading effect, down-regulating monoterpene accumulation

In order to determine if light may have played a role in the altered levels of linalool observed in the different wine colour categories, light was excluded from Cabernet Sauvignon, Malian and Shalistin berries throughout the entire post-veraison growth phase. Linalool levels in wines made from these shaded berries were not significantly different between the varieties, but light exposed controls had significantly higher linalool levels in white wines compared to red (Figure 3.6). This suggests that berry colour differences between the varieties could be causing the berries to respond to light stimuli differently, affecting linalool synthesis. Red berries will absorb light of different wavelengths compared to white grapes. From review of a combination of studies of light absorption properties of anthocyanins *in vitro* and *in vivo*, Manetas et al. (2003) concluded that *in vivo* anthocyanins attenuate green/yellow and possibly blue light, but not red light. This could mean that signalling through blue light sensitive cryptochromes (reviewed in Cashmore et al., 1999) may be reduced in grapes containing anthocyanins compared to those that do not. This may cause gene expression changes of metabolite pathways sensitive to blue light signalling. In fact, studies in leaves and fruit have shown that volatile production can be manipulated by altering the light wavelengths available

to plants (Loughrin and Kasperbauer, 2002, 2003) For example, the transcription of carotenoid biosynthesis genes have been shown to be up-regulated by blue light (Steinbrenner and Linden, 2003). Carotenoids are essential metabolites utilised in photosynthesis with many roles including light harvesting and photoprotection (Frank and Cogdell, 1996). If the synthesis of these compounds was reduced in red grapes then this may affect the synthesis of a number of important carotenoid-derived wine aroma compounds such as norisoprenoids (e.g. β-ionone and β-damascenone) (Winterhalter and Rouseff, 2001). Furthermore, carotenoids belong to the larger terpenoid family of compounds and hence share precursor metabolites, with other volatiles such as monoterpenes (e.g. linalool and geraniol) (Wu et al., 2006). Perhaps the down-regulation of linalool synthesis in red berries is a consequence of a reduced need of carotenoids due to the shading effect of anthocyanins. This may also explain other variety specific differences in volatile levels that we found in our red wines. In a study conducted by Bureau et al. (1998) it was shown that carotenoid levels of pre-veraison Syrah (Shiraz) berries grown in 90% shading bags were significantly lower than in non-shaded controls, 50% and 70% shaded berries, while at maturity these carotenoid levels were significantly higher compared to the same treatments. One possible explaination for this would be if the effect of light on carotenoid accumulation in berries was variable depending on the berry growth phase. It is important to note that this study was focused on the overall effect of light on carotenoids and not that of particular wavelengths. It is possible that anthocyanins shade red berries from particular wavelengths of light, as discussed above, which may affect carotenoid synthesis in a different manner compared to when all light wavelengths are shaded.

This is not the first study to show berry shading effects on wine flavour. Ristic et al. (2007) investigated the effect of sunlight exclusion from Shiraz berries on wine sensory properties. They found that wines made from boxed berries had significantly decreased overall fruit flavour compared to unboxed controls. The levels of C_{13}-norisoprenoids were examined in the wines and shaded fruit produced wines with lower levels of β-damascenone, but the presence of other volatiles such as monoterpenes was not analysed.

A link between the regulation of anthocyanin and carotenoid synthesis has been shown previously in both tomato (Davuluri et al., 2005) and apple (Espley et al., 2013). In tomato the *DET1* gene, involved in suppressing photoresponsive signalling pathways, was silenced which resulted in increased accumulation of both carotenoids and anthoycanins (Davuluri et

al., 2005). In apple, *MdMYB10* overexpressing lines accumulated higher levels of both anthocyanins and carotenoids (Espley et al., 2013). While this demonstrates that there may be some shared regulation between these pathways, these results contradict the hypothesis discussed above. One major difference between these two species and grapevine is that anthocyanin accumulation has been shown to be photoresponsive in tomato (Mustilli et al., 1999) and apple (Takos et al., 2006) whereas in grapevine there has been no clear evidence of this (Downey et al., 2004). Therefore it is not unreasonable to expect that any links between anthocyanins and carotenoid biosynthesis regulation relating to light stimuli would be different between these species.

An analysis of the microarray data presented in Chapter 2 for expression changes in genes involved in carotenoid biosynthesis revealed a few genes of interest. In Shiraz a transcript annotated as *beta-carotene 15,15'monooxygenase* was expressed 2.38 FC higher in transgenic white berries compared to non-transgenic red berry controls. In Chardonnay a gene annotated as *zeaxanthin epoxidase* was expressed 1.69 FC higher in white Chardonnay control berries compared to the transgenic red berries. Both of these genes are annotated to be involved in carotenoid biosynthesis and imply an upregulation of this pathway in white berries, a result which corresponds to the hypothesis that anthocyanin accumulation in berries may down-regulatate carotenoid synthesis. Yet in contrast, two other transcripts, annotated as *zeta-carotene desaturase* and *carotenoid isomerase* were up-regulated in 'red' Chardonnay berries (1.94 and 1.60 FC respectively) compared to their non-transgenic white berry controls. As this microarray analysis was carried out at only one time point in berry development, i.e. close to ripeness, this snapshot of gene expression does not necessarily provide much information of the effect of anthocyanins on carotenoid synthesis, particularly since Bureau et al. (1998) has showed the differences in carotenoid accumulation in response to light over berry development.

As linalool is synthesised from precursors derived from the MEP pathway, expression of these genes throughout development of Cabernet Sauvignon, Malian and Shalistin whole berries and berry skins was measured (Figure 3.7 and Figure 3.8). A number of these genes, particularly in berry skins, were up-regulated in Shalistin compared to Cabernet Sauvignon post-veraison. In skins, Malian usually had expression levels similar to those of Shalistin or intermediate between Shalistin and Cabernet Sauvignon. A number of studies have shown that some MEP pathway genes can be up-regulated in response to light stimuli (reviewed in

Cordoba et al., 2009). It is possible that the light effect discussed above, due to the accumulation of anthocyanins in red berries, could have altered MEP pathway gene expression and hence the amount of monoterpene precursors in red berries. This may have contributed to reduced linalool levels in red wines, although the levels of linalool in the berries should be measured to test this hypothesis.

Whole berries showed fewer differences in MEP pathway gene expression between Cabernet Sauvignon, Malian and Shalistin compared to those measured in berry skins. This is perhaps not surprising considering that VvMYBA is only expressed in the skin and so differences associated with the expression of this TF are more likely to be observed in this tissue. Yet linalool levels were significantly lower in Cabernet Sauvignon wines compared to Shalistin when made using both fermentation on free-run juice and in the presence of seeds and skins (Table 3.2). As this difference was no less pronounced when the wine was made from free-run juice this suggests that linalool synthesis was down-regulated to similar levels in the skin and the pulp of the berries. Thus, the MEP pathway transcript data cannot entirely explain the reduced levels of linalool in red wines compared to white wines. Furthermore, the MEP pathway synthesizes precursors used to produce all monoterpenes, but linalool was the only monoterpene that was detected to have a conserved pattern of abundance in relation to wine colour in the three varieties used in this study. The linalool synthase gene (*VvCSLinNer)* was clearly down-regulated in whole berries of Cabernet Sauvignon (compared to Shalistin) (Figure 3.5) so perhaps the differential expression of this gene is the main cause of the linalool difference observed in their wines.

3.5 Conclusion

In this chapter, results from a comparative screening of volatiles in wines made from grapes with altered *VvMYBA* gene expression and hence colour has been presented. This research has shown that the presence of VvMYBA in berries does have an effect on the abundance of volatile flavour/aroma compounds in their wines; however these differences were often in a cultivar specific manner. One conserved difference was a decrease in the abundance of linalool in red wines compared to white wines. Light exclusion studies and transcript analysis of genes associated with linalool metabolism have shown that the accumulation of anthocyanins in red grapes may cause a shading effect which down-regulates linalool synthesis.

Chapter 4:

Characterisation of a Serine Carboxypeptidase-like gene up-regulated in *Vitis vinifera* berries expressing *VvMYBA*

4.1 Introduction

In Chapter 2, the identification of a gene with homology to the serine carboxypeptidase-like (SCPL) acyltransferase family, that was up-regulated in berries expressing *VvMYBA* (see section 2.3.2.3), was described. Since the transcript levels of this gene were much higher in red berries (when compared to white berries) and were similar to *VvUFGT* and other anthocyanin biosynthesis genes, it was hypothesised that it too may function in anthocyanin biosynthesis. This Chapter will report experimental work aimed at understanding the function of this gene, which we named *VvSCPL1*.

4.1.1 Anthocyanin acylation in grapevine

Wine grapes (*V. vinifera* L.) contain both 3-*O*-monoglucoside and 3-*O*-acyl monoglucoside anthocyanins derived from 5 main anthocyanidin aglycones: delphinidin, cyanidin, peonidin, petunidin and malvidin (He et al., 2010). The acylated anthocyanins can be in the form of 3-*O*-acetyl, 3-*O*-coumaroyl, and 3-*O*-caffeoyl-monoglucosides (Mazza and Francis, 1995). Van Buren et al. (1968) showed that wines made from cultivars, with high proportions of acylated anthocyanins, for example Ives and Veeport, had greater colour stability when exposed to light compared to wines from grapes with no acylated anthocyanins. The increased stability of these compounds has been shown in the fruit and flowers of other species, and is likely to be due to increased intramolecular stacking of the anthocyanins (Yonekura-Sakakibara et al., 2008). Pinot Noir is a cultivar that lacks acylated anthocyanins (Van Buren et al., 1968) and produces red wines with low anthocyanin content and unstable colour (Smart, 1992). Despite the importance of red colour stability to wine quality, and the association between acylated anthocyanins and increased wine colour stability (Van Buren et al., 1968), anthocyanin acyltransferases from grapevine have not yet been identified.

4.1.2 The SCPL acyltransferase family

The SCPL protein family has only recently been discovered, some of the first members were characterised not much more than a decade ago. Li and coworkers (1999; 2000) characterised the first member of this family, a glucose acyltransferase from *Lycopersicon pennellii*, which has homology to the serine carboxypeptidase-type (SCP) of peptide hydrolases (such as barley carboxypeptidase I, II, and III, wheat serine carboxypeptidase II, and yeast

carboxypeptidase Y), but does not possess carboxylpeptidase activity. Instead this enzyme catalysed the conversion of two 1-*O*-β-acylglucose molecules to 1,2-di-*O*-acylglucose and glucose through acyltransferase activity. Soon after, several other SCP homologues involved in acyltransferase reactions in phenylpropanoid metabolism were discovered, including 1-*O*-β-sinapoylglucose:L-malate sinapoyltransferase (AtSMT) from *Arabidopsis* (Lehfeldt et al., 2000), 1-*O*-β-sinapoylglucose:choline sinapoyltransferase (AtSCT) from *Arabidopsis* (Shirley et al., 2001), and BnSCT from *Brassica napus* (Milkowski et al., 2004). This confirmed the existence of a new class of enzymes in plants, the serine carboxypeptidase-like (SCPL) acyltransferases.

SCPL acyltransferases catalyse the transfer of an acyl group from 1-*O*-β-glucose esters to the hydroxyl, amino or thiol groups of an acceptor molecule. The types of acceptor molecules identified so far for this group of enzymes have been broad, including low molecular weight compounds (such as choline and L-malate), flavonoids and terpenoids (Mugford and Milkowski, 2012). These genes seem to have evolved from a single common ancestor through the separation of mosses and algae to higher plants (Mugford and Osbourn, 2010). Why the SCPL enzymes lack peptidase activity, present in their SCP homologues, but instead have acyltransferase activity, has been an area of interest in recent studies of this gene family. Sequence alignments of SCP and SCPL proteins show that the Ser, His, Asp catalytic triad responsible for the peptidase activity of SCPs is conserved in SCPL enzymes (Milkowski and Strack, 2004). Further studies showed that the hydrogen-bonding network required for peptide binding to SCP active sites is modified in SCPL proteins to accommodate the acyl glucose substrate (Stehle et al., 2008).

4.1.2.1 How do the SCPL acyltransferases differ from other acyltransferases?

Besides the SCPL family, another better characterised class of enzymes, known as the BAHD family (named after the first four characterised enzymes, BEAT, benzylalcohol *O*-acetyltransferase; AHCT, anthocyanin *O*-hydroxycinnamoyltransferase; HCBT, anthranilate N-hydroxycinnamoyl benzoyltransferase; DAT, deacetylvindoline 4-O-acetyltransferase) are known to acylate secondary metabolites in plants. BAHD acyltransferases utilize coenzyme A thioesters as their acyl donors, in contrast to the donors used by the SCPL class of acyltransferases (St-Pierre and Luca, 2000; D'Auria, 2006). Another difference is found in their cellular compartmentalization. Most SCPL proteins have been shown to contain

predicted N-terminal signal peptides (Fraser et al., 2005) and AtSMT was found to be located within the central vacuole of mesophyll and epidermal cells in *Arabidopsis* leaves (Hause et al., 2002). This suggests that SCPL proteins are located within the vacuole (Mugford and Milkowski, 2012), whereas BAHD proteins have been shown or predicted to be cytosolic (Fujiwara et al., 1998; D'Auria, 2006; Yu et al., 2008).

4.1.3 SCPLs as anthocyanin acyltransferases

A number of SCPL genes have been identified that code for flavonoid acyltransferases. A *Glucose acyltransferases-like* (*VvGAT-like*) gene was discovered in grapevine whose expression is related to PA synthesis (Terrier et al., 2009; Grégory et al., 2013), and *DkSCPL1,* from *Diospyros kaki,* is also expressed during flavonoid production (Ikegami et al., 2007). A number of anthocyanin-specific acyltransferases have been identified including a sinapoyl-Glc:anthocyanin acyltransferase from *Arabidopsis* (Fraser et al., 2007), a 1-*O*-malylglucose:pelargonidin 3-*O*-glucose-6''-*O*-malyltransferase from carnation (Abe et al., 2008), a 1-*O*-acylglucose dependent anthocyanin acyltransferase from butterfly pea (*Clitoria ternatea*) (Noda et al., 2006), 1-*O*-hydroxycinnamoyl-β-D-glucose-dependent acyltransferases from carrot (*Daucus carota)* and silvertop (*Glehnia littoralis*) (Matsuba et al., 2008) and a *p*-hydroxybenzoyl-Glc-dependent anthocyanin acyltransferase from *Delphinium* (Nishizaki et al., 2013). Considering this, it was hypothesised that the *VvSCPL1* gene upregulated in Shiraz and transgenic Chardonnay berries expressing VvMYBA (see Chapter 2, Table 2.1, section 2.3.2.3) may function as an anthocyanin acyltransferase.

4.1.4 Specific aims of this research

The aim of the research outlined in this chapter was to characterise the *VvSCPL1* gene, and in particular to ascertain whether it functions as an anthocyanin acyltransferase. Bioinformatics, gene expression studies, transient promoter-binding luciferase activity assays and the use of transgenic tobacco plants constitutively expressing *VvSCPL1* were all used to investigate this.

4.2 Materials and Methods

4.2.1 Plant material

Berry samples of Cabernet Sauvignon, Malian and Shalistin varieties were collected as previously described in section 3.2.2.4.

Cabernet Sauvignon and Pinot Noir berry samples were collected during the 2007/08 season from a commercial vineyard at Slate Creek, Willunga, South Australia (35^{o} 15' South, 138^{o} 33' East) as previously described (Dunlevy et al., 2013). Samples from 5-10 bunches were taken in triplicate from randomly selected vines within the vineyard at 2, 4, 6, 8, 10, 12, and 14 wpf and pooled. Approximately 100g of detached berries were sampled from each replicate and frozen immediately in liquid N_2. These were deseeded by gently breaking frozen berries open and removing the seeds before the tissue thawed.

Young leaves for DNA extraction were collected from Coombe Vineyard, University of Adelaide, Urrbrae, South Australia (34^{o} 56' South, 138^{o} 36' East), frozen in liquid nitrogen, and stored at -80^{o}C (Walker et al., 2007).

4.2.2 Nucleic acid extraction and cDNA synthesis

RNA extractions and cDNA synthesis from frozen whole berries of Cabernet Sauvignon, Malian and Shalistin, and tobacco flowers, were carried out as described in section 3.2.7.

The RNA and cDNA synthesis from the Cabernet Sauvignon and Pinot Noir developmental series was conducted earlier by Dr. Jake Dunlevey. The RNA was extracted from deseeded berries using a perchlorate method described in Boss et al. (2001). DNA was removed from the samples using RNAse-free DNAse (Qiagen, Nimburg, Netherlands) in conjunction with the RNeasy Mini kit (Qiagen) according to their protocols. cDNA was synthesised using Superscript® III Reverse Transcriptase (Invitrogen, CA, USA) and an oligo$(dT)_{20}$ primer according to manufacturer's instructions.

Walker et al. (2007) extracted DNA from young leaves of Cabernet Sauvignon and Pinot Noir vines. DNA was extracted from young transgenic tobacco leaves using the ISOLATE Plant DNA mini kit (Bioline, London, UK) according to manufacturer's instructions.

4.2.3 Analysis of gene expression

Specific primers were designed (Appendix F) to amplify 100 - 200 bp products from *VvSCPL1* and housekeeping genes. The specificity of each primer pair was confirmed and qPCR analysis was carried out as previously described in section 3.2.8.

4.2.4 Obtaining the DNA sequences of *VvSCPL1*

To obtain the gene sequence of *VvSCPL1*, primers VvSCPLfor1 and VvSCPL_R4 (Appendix F) were used in a PCR reaction to amplify the gene from its start to stop codons based on the sequence in the 12X grapevine genome sequence database, V1 gene prediction version (http://genomes.cribi.unipd.it/grape/). Reactions consisted of 1 unit of Platinum Taq DNA Polymerase High Fidelity (Invitrogen), 1 x high fidelity PCR Buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.33 mM dNTPs (Roche), 25 ng of DNA, 0.5 µM of each primer in a total volume of 50 µl. Cycling conditions were as follows: 95^{o}C for 3 mins followed by 35 cycles of 95^{o}C for 30 sec, 50^{o}C for 30s and 72^{o}C for 4 min, then final extension at 72^{o}C for 10 mins. Fragments were purified using agarose gel electrophoresis and the QIAEX® II Gel Extraction Kit (Qiagen), and ligated to pDrive (Qiagen). Sequencing was done on both the purified PCR product and single clones using a number of primers (Appendix F) in order to obtain the full gDNA sequence.

The cDNA sequence of *VvSCPL1* was obtained using the VvSCPLfor1 and VvSCPL_R4 primers (Appendix F) and cDNA obtained from Cabernet Sauvignon and Pinot Noir berry skins harvested 2 weeks post veraison (wpv). Reactions consisted of 1 unit of Platinum *pfx* enzyme (Invitrogen), 1 x *pfx* amplification buffer (Invitrogen), 1 mM $MgSO_4$ (Invitrogen), 0.33 mM dNTPs (Roche), 5 µl of cDNA (previously diluted 1:40), 0.5 µM of each primer in a total volume of 50 µl. Cycling conditions were as follows: 94^{o}C for 3 mins followed by 35 cycles of 94^{o}C for 30 sec, 55^{o}C for 30s and 68^{o}C for 2 min, then final extension at 68^{o}C for 10 mins. Fragments were purified using agarose gel electrophoresis and the QIAEX® II Gel Extraction Kit (Qiagen), and ligated to the pDrive. Sequencing was done on both the purified PCR product and single clones.

4.2.5 Sequencing the 5' UTR of the *VvSCPL1* gene in CS and PN

The GeneRacer™ Kit (Invitrogen) was used, according to their protocol, to ligate the GeneRacer™ RNA oligo to the 5' end of full length mRNA transcripts from Cabernet

Sauvignon and Pinot Noir berry skins harvested at 10 wpf. Using the SuperScript™ III RT enzyme provided in the kit, the capped mRNA was reverse transcribed to produce cDNA. This was used in a PCR using the GeneRacer™ 5' Primer and the gene specific VvSCPL_R2 primer (Appendix F). Reactions consisted of 1 unit of Platinum® *Taq* DNA Polymerase (Invitrogen), 1 x PCR Rxn Buffer (Invitrogen), 1.5 mM $MgCl_2$ (Invitrogen), 0.33 mM dNTPs (Roche), 2 µl of reverse transcribed cDNA, 0.5 µM of each primer in a total volume of 50 µl. Cycling conditions were as follows: 95°C for 3 mins followed by 35 cycles of 95°C for 30 sec, 55°C for 30s and 72°C for 2.5 min, then final extension at 72°C for 10 mins. This PCR reaction was then used in a nested PCR using the GeneRacer 5' Nested and VvSCPL_R5 primers (Appendix F). Reactions and cycling conditions were as above but only 1 µl of the previous PCR reaction was used instead of the 2 µl of cDNA. Fragments were purified using agarose gel electrophoresis and the QIAEX® II Gel Extraction Kit (Qiagen), and ligated to pDrive. Sequencing was done on both the purified PCR product and single clones.

4.2.6 Transient promoter-binding luciferase activity assays

Primers were designed to amplify the first 1148 bp immediately 5' of the start codon of the *VvSCPL1* gene and to contain SacI and BglII restriction sites immediately 5' and 3' of this fragment respectively (Appendix F). Promoter regions were amplified from Cabernet Sauvignon and Pinot Noir DNA using these primers (VvSCPLPr_F2SacI and VvSCPLPr_R1BglII). PCR reactions contained 1 unit of Platinum® *Pfx* DNA Polymerase (Invitrogen), 1 x *Pfx* Amplification Buffer (Invitrogen), 1 mM $MgSO_4$ (Invitrogen), 0.33 mM dNTPs (Roche), 3 µl of gDNA (10 ng/µl), 0.5 µM of each primer in a total volume of 50 µl. Cycling conditions were as follows: 94°C for 3 mins followed by 35 cycles of 94°C for 15 sec, 52°C for 30s and 68°C for 2 min, then final extension at 68°C for 10 mins. Products were purified using the QIAEX® II Gel Extraction Kit (Qiagen), ligated to the pCR®-Blunt II-TOPO® Cloning Vector, using the Zero Blunt® TOPO® PCR Cloning Kit (Invitrogen), sequenced and then ligated to the firefly (*Photinus pyralis*) luciferase (LUC) plasmid pLUC (Horstmann et al., 2004) using the SacI and BglII restriction to produce the *VvSCPL1pr:LUC* constructs.

The other constructs used in the transient assays were *35S:VvMYBA1* in pART7 (Walker et al., 2007) to express the VvMYBA1 transcription factor, *pFF19:EGL3* (Ramsay et al., 2003), containing the bHLH transcription factor from *Arabidopsis* known to complex with

VvMYBA1 and required for transient activation of VvMYBA1 gene targets, and *VvUFGTpr:LUC* containing the *VvUFGT* gene promoter 5' to the firefly luciferase gene and used as a positive control for VvMYBA mediated transcriptional activation (Bogs et al., 2007). For normalisation of transfection efficiency *Renilla reniformis* LUC plasmid *pRluc* (Horstmann et al., 2004) was used. Gold particles were coated with a mixture of 500 ng each of *pFF19:EGL3*, *35S:VvMYBA1*, and either the *VvUFGTpr:LUC, VvSCPL1-CSpr:LUC* or *VvSCPL1-PNpr:LUC* construct, and 100 ng of *pRluc* using methods described in (Ramsay et al., 2003). Negative controls lacking *35S:VvMYBA1* were used to determine background fluorescence.

A suspension culture of grapevine Chardonnay petiole callus was grown in Grape Cormier (GC) medium (Do and Cormier, 1991) to a log phase, diluted to a packed cell volume of 0.6 cells/ml, filtered onto sterile Whatman® discs (5.5 cm), and placed on the surface of GC plates with 7 mg/ml agar (TC grade, *Phyto*Technology Laboratories^{TM}, KS, USA). The grape cells were bombarded with DNA-coated gold particles at 350 kPa helium in a vacuum of 75 kPa and a distance of 14 cm as described by Torregrosa et al. (2002). Cells were incubated in the dark for 48 h at 27 °C, harvested, and lysed by grinding on ice in 150 µl of Passive Lysis Buffer (Promega). Enzyme activities of both *P. pyralis* and *R. reniformis* LUC were determined using the Dual-Luciferase Reporter Assay system (Promega, WI, USA). Light emission was measured with a TD-20/20 Luminometer (Turner Biosystems, Promega).

4.2.7 Production of genetically modified tobacco containing the *VvSCPL1* gene

Primers were designed to the *VvSCPL1* cDNA sequence to include an XhoI restriction site immediately 5' of the start codon and an EcoRI site immediately 3' of the stop codon (VvSCPLXhoIFor2 and VvSCPLEcoRIRev, Appendix F). These were used to amplify the gene fragment by PCR. Reactions consisted of 1 unit of Platinum Taq DNA Polymerase High Fidelity (Invitrogen), 1 x high fidelity PCR Buffer (Invitrogen), 2 mM $MgCl_2$ (Invitrogen), 0.33 mM dNTPs (Roche), 10 µl of cDNA (1:40 dilution), 0.5 µM of each primer in a total volume of 50 µl. Cycling conditions were as follows: 95^{o}C for 3 mins followed by 35 cycles of 95^{o}C for 30 sec, 52^{o}C for 30s and 72^{o}C for 2 min, then final extension at 72^{o}C for 10 mins. Fragments were purified using agarose gel electrophoresis and the QIAEX® II Gel Extraction Kit (Qiagen), ligated to the vector pDrive (Qiagen) and sequenced. The gene was then inserted into the multiple cloning site of the pART7 cloning vector, which is loacted between

a cauliflower mosaic virus 35S promoter and an octopine synthase gene (OCE) transcriptional terminator (Gleave, 1992), using the XhoI and EcoRI restriction sites. This expression cassette was excised from pART7 using a NotI restriction enzyme and ligated to the plant expression vector pART27 (Gleave, 1992) to create the *35S:VvSCPL1* construct.

Agrobacterium tumefaciens (herein *Agrobacterium*) strain LBA4404 containing the *35S:VvSCPL1* construct was used to transform *N. tabacum* var. Samsun. This was grown on LB containing Bacto™ Agar (Jomar Bioscience), 25 µg/ml rifampicin, 50 µg/ml spectinomycin and 200 µM acetosyringone at 28°C for 4 days. Bacteria were resuspended in 30 ml of Murashige and Skoog (MS) medium [1x MS salts and 1x Gamborg's vitamins (*Phyto*Technology Laboratories®), 30 g/L sucrose] and adjusted to an OD_{600nm} of 0.8 – 1.0. Incisions were made on the underside of tobacco leaves parallel to the midrib and leaves were submerged in the MS/*Agrobacterium* mixture for 10 mins before blotting on sterile filter paper and transferring (adaxial side down) to MS plates [MS medium with 5 mg/ml Phytagel™ (Sigma-Aldrich)] containing 1 µM each of α-naphthaleneacetic acid (NAA) and 6-benzylamino purine (BAP) (Sigma). *Agrobacterium* was co-cultivated on leaves for 4 days at 20°C. Leaf pieces were washed in MS medium containing 500 µg/ml cefotaxime, blotted on sterile filter paper, and transferred (underside down) onto MS plates containing 1 µM each of α-naphthaleneacetic acid (NAA) and 6-benzylamino purine (BAP), 500 µg/ml cefotaxime and 100 µg/ml kanamycin. These were kept at 27°C and transferred to fresh medium every 2 weeks. Shoots about 1cm in length were transferred onto MS plates containing 100 µg/ml kanamycin. PCR reactions were used to screen for the transgene using MangoTaq™ DNA polymerase (Bioline), according to the manufacturer's instructions, with the 35SF and OCS rev primers that were designed to the promoter and terminator sequences within the pART27 vector (Appendix F). Once shoots had rooted and grown to approximately 15-20 cm in length they were transferred to soil (20 L composted pine bark, 10 L river sand, 30 g $FeSO_4$, 60 g pH amendment, 140 g longlife osmocote) and hardened off in the glasshouse. They were grown in ambient light, with a night break during the spring season in South Australia. Day and night temperatures were about 27°C and 22°C respectively.

4.2.8 Analysis of anthocyanins in transgenic tobacco flowers

Anthocyanins were extracted from 100 mg powder from ground frozen tobacco flowers with 300 µl of 0.3% formic acid in 70 % methanol. Samples were then sonicated for 20 mins in an

ice bath and centrifuged in a microfuge for 5 mins to pellet debris. Anthocyanins were separated as described in section 3.2.4.2. Anthocyanin concentrations in tobacco extracts were determined by comparison to a standard curve of known cyanidin-3-*O*-rutinoside concentrations. Anthocyanin peaks were identified by their MS/MS parent and major daughter ions as determined using the HPLC method as described above coupled to a 6410 triple quad mass spectrometer (Agilent, Santa Clara, CA) using parameters described by Downey and Rochfort (2008). Table S2 in Appendix N summarises the MS parental ions and MS/MS major daughter ion detected for each compound, which were compared to previously reported values (Luo et al., 2007; Downey and Rochfort, 2008).

4.2.9 Bioinformatics

Sequence chromatograms were all viewed using Chromas Lite 2.1.1 software (www.technelysium.com.au). All sequence alignments were carried out using AlignX (a component of Vector NTI Advance 11.0, Invitrogen) and viewed in GeneDoc version 2.7 (Nicholas and Nicholas Jr, 1997). All BLAST searches were carried out in NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi). N-terminal signal peptide prediction was determined using the SignalP 4.1 prediction server (Petersen et al., 2011).

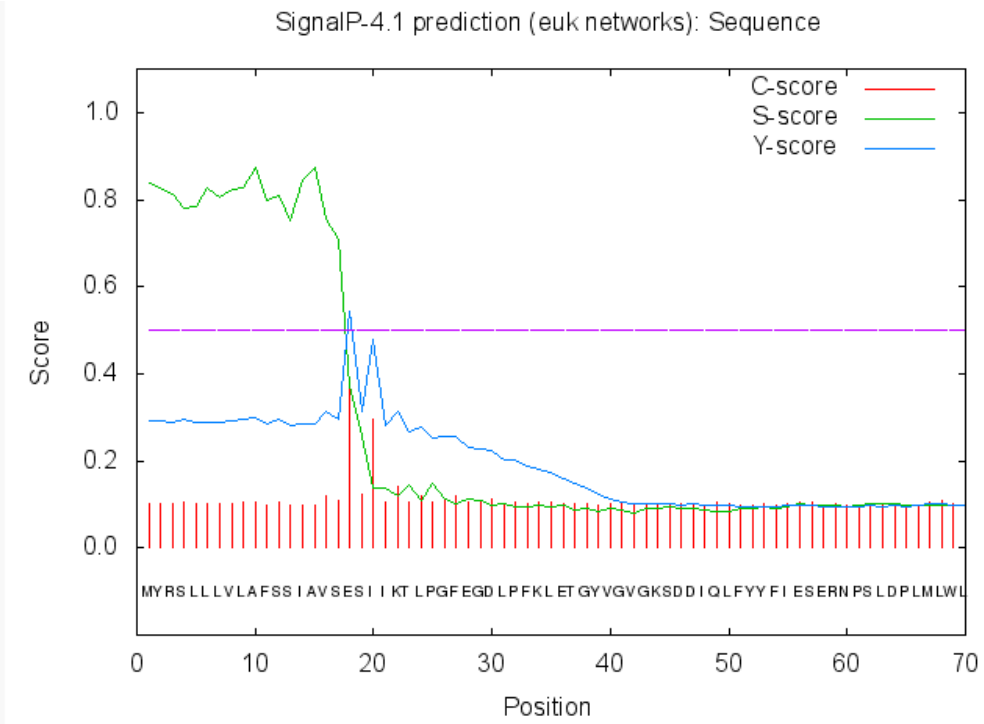
4.3 Results

4.3.1 *VvSCPL1* belongs to the serine carboxypeptidase-like gene family

The predicted cDNA sequence of the *VvSCPL1* gene (Microarray ID VIT_03s0091g01240) was obtained from the 12X grapevine genome sequence (V1 gene prediction version) (http://genomes.cribi.unipd.it/grape/). A nucleotide BLASTx (search of protein database using translated nucleotide query) was performed using this sequence in NCBI. This revealed that the VvSCPL1 protein showed homology to a number of putative and characterised SCPL proteins from *V. vinifera* and other species (data not shown). Those that had been functionally characterised were a number of glucose acyltransferases from *Solanum berthaultii* (accession numbers, AAD01263.1, AAD01264.1 and AAD01265.1) (Li and Steffens, 2000), and *Solanum pennelli* (AAF64227.1) (Li et al., 1999) that showed 49 – 51% sequence identity over 80 – 83 % coverage of the VvSCPL1 sequence, and two 1-*O*-acylglucose:anthocyanin-*O*-acyltransferases from *Clitoria tenatea* (accession numbers BAF99695.1 and BAF99694.1)

(Noda et al., 2006), that had 50 % sequence identify to 81 % of the VvSCPL1 sequence. An alignment of these protein sequences with the VvSCPL1 sequence can be found in Appendix K. Three putative proteins from *Genitana triflora,* annotated as 1-*O*-acylglucose:anthocyanin-*O*-acyltransferase-like proteins (accession numbers BAF99696.1, BAF99698.1 and BAF99697.1) showed 48 – 51% sequence identify over 81% coverage of the VvSCPL1 protein. Most other BLAST hits were annotated as putative or predicted SCPL proteins (data not shown).

The SignalP 4.1 prediction server (Petersen et al., 2011) predicted that the VvSCPL1 protein sequence likely contained an N-terminal signal peptide with a cleavage site between position 17 and 18 of the amino acid sequence (Figure 4.1).




```
# Measure  Position  Value    Cutoff   signal peptide?
  max. C    18        0.363
  max. Y    18        0.544
  max. S    15        0.874
  mean S     1-17     0.808
       D     1-17     0.687   0.450    YES
Name=Sequence   SP='YES' Cleavage site between pos. 17 and 18: AVS-ES D=0.687 D-cutoff=0.450 Networks=SignalP-noTM
```

Figure 4.1: Signal P 4.1 prediction result obtained using the VvSCPL protein sequence

Screen print of the output from SignalP 4.1 prediction server indicating that the first 17 amino acids of the VvSCPL1 protein (obtained from the 12X grapevine genome V1 annotation) are likely a signal peptide. Instructions on interpreting this output can be found in Appendix L.

4.3.2 Sequencing *VvSCPL1* coding region and whole gene in Cabernet Sauvignon and Pinot Noir

To determine if there were any differences in the sequence of *VvSCPL1* between Cabernet Sauvignon and Pinot Noir, the cDNA and gDNA sequences of the gene from both cultivars were analysed. From start to stop codons *VvSCPL1* is 3352 bp in length and contains 13 exons (based on the 12X grapevine genome sequence annotation) that code for a protein 464 aa in length. Cabernet Sauvignon is heterozygous for this gene, with alleles differing by one nucleotide, an A/G polymorphism at position 2106 of the gDNA sequence (residing in exon 7), which is a silent mutation. Pinot Noir is homozygous for the allele containing the A nucleotide at position 2106 and has no differences to the Cabernet Sauvignon version of this allele. Hence the Cabernet Sauvignon and Pinot Noir VvSCPL1 protein sequences, coded for by two alleles, are identical.

4.3.3 Determining the correct gene structure (exon/intron structure) and start codon position of the *VvSCPL1* gene

As mentioned previously, the *VvSCPL1* gene sequence (microarray ID VIT_03s0091g01240) and its putative intron/exon annotation was first obtained from the 12X grapevine genome sequence database. A nucleotide BLAST (blastn) with this sequence, in the NCBI nucleotide collection database, matched one *V. vinifera* contig (accession number AM462732) with 100% identity; however in this database the intron/exon prediction of this gene was different to that in the 12X genome database. The NCBI annotation of the gene identified another ATG as the start codon which was further upstream to the one suggested in the 12X genome database. Figure 4.2 illustrates the differences between these two annotations of the *VvSCPL1* gene. For the sake of simplicity these two annotated versions of the *VvSCPL1* gene will be referred to as *VvSCPL1*_12X and*VvSCPL1*_NCBI.

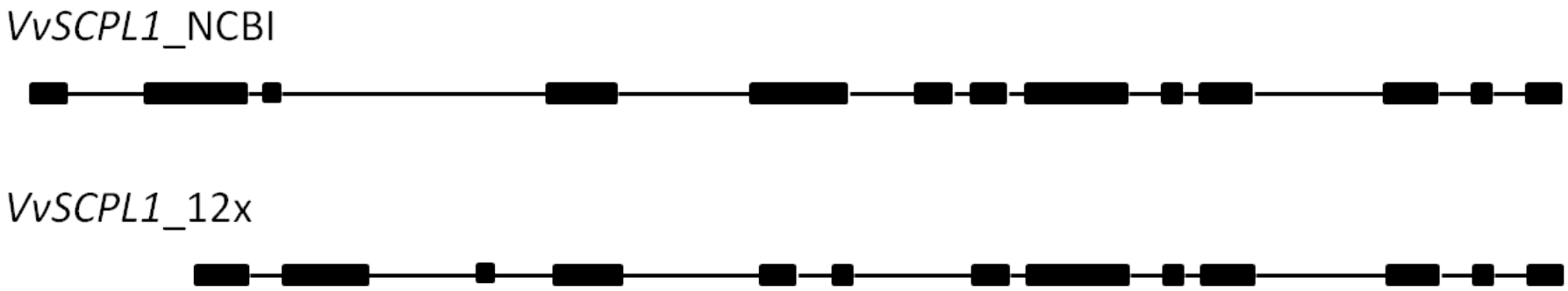


Figure 4.2: Schematic comparing exon/intron structure of two annotations of the *VvSCPL1* gene sequence

Two annotations of the *VvSCPL1* gene were discovered. The *VvSCPL1*_NCBI annotation was found within a contig in the NCBI database (accession number AM462732). The *VvSCPL1*_12X annotation was found in the 12X grapevine genome sequence database, V1 gene prediction version (microarray ID VIT_03s0091g01240). Black boxes represent exons and black lines are indicative of introns.

In order to study the function of the *VvSCPL1* gene, it was imperative to determine where the start codon resided. The coding region of *VvSCPL1* was amplified from cDNA of Cabernet Sauvignon berries 2wpv and the PCR product was sequenced (see section 4.3.2). An alignment of this sequence with the predicted cDNA sequences from the *VvSCPL1*_12X and *VvSCPL1*_NCBI gene annotations showed that it was 100 % identical to the *VvSCPL1*_12X sequence (Appendix M). While this suggested that the VvSCPL1_12X exon/intron annotation was correct, it did not rule out the possibility that the start codon was further upstream, as suggested by the *VvSCPL1*_NCBI gene annotation. To investigate this possibility further a number of bioinformatic and molecular analyses were carried out and these are presented below.

A nucleotide BLAST search in the expressed tag sequence (EST) database of NCBI was performed. It was hoped that ESTs of this gene would be available that aligned to the 5' region of the *VvSCPL1* gene where the two annotated versions differed. The only ESTs that matched the *VvSCPL1* sequence aligned to the 3' end of the gene and so no further information from this search was gained (data not shown).

To find the location of start codons in SCPL genes from other species and how they compared to the two *VvSCPL1* gene annotations, a tblastn search (translated nucleotide database BLAST search using a protein query) using both the VvSCPL1_NCBI protein sequence and the VvSCPL1_12X sequence was performed (data not shown). This showed that homologous SCPL proteins had high identity to the coding regions of both annotations of this gene. Many of these homologues started where the *VvSCPL1*_12X sequence started or after this, but there were some sequences that had homology further upstream to this. No protein showed homology to the entire 5' region of *VvSCPL1*_NCBI. These results suggested that the *VvSCPL1*_12X gene annotation, in regards to the location of the start codon, is more likely to be correct.

As the bioinformatic analyses described above did not provide definite conclusions as to the location of the start codon of the *VvSCPL1* gene annotation, 5' RACE (rapid amplification of cDNA ends) was used to sequence the 5' regions of *VvSCPL1* transcripts. To determine if there were any differences between the two varieties, RNA from Cabernet Sauvignon and Pinot Noir berries 2 wpv (12 wpf) were used. When the cDNA fragments, obtained from 5' RACE, were separated by agarose gel electrophoresis there was a single DNA band detected for Cabernet Sauvignon, but 2 bands (both at different sizes to the Cabernet Sauvignon band)

detected for Pinot Noir (Figure 4.3). Sequencing of the Cabernet Sauvignon fragment revealed that it was 176 bp in length (including the GeneRacer™ RNA Oligo that was added to it) and that Cabernet Sauvignon was homozygous in this 5' region of *VvSCPL1*. Sequencing of DNA extracted from both of the Pinot Noir DNA bands revealed that band 1 (Figure 4.3) contained two DNA fragments within it, differing in size by 18 bp (231 bp and 212 bp). DNA band 2 from Pinot Noir (Figure 4.3) was 83 bp in length.

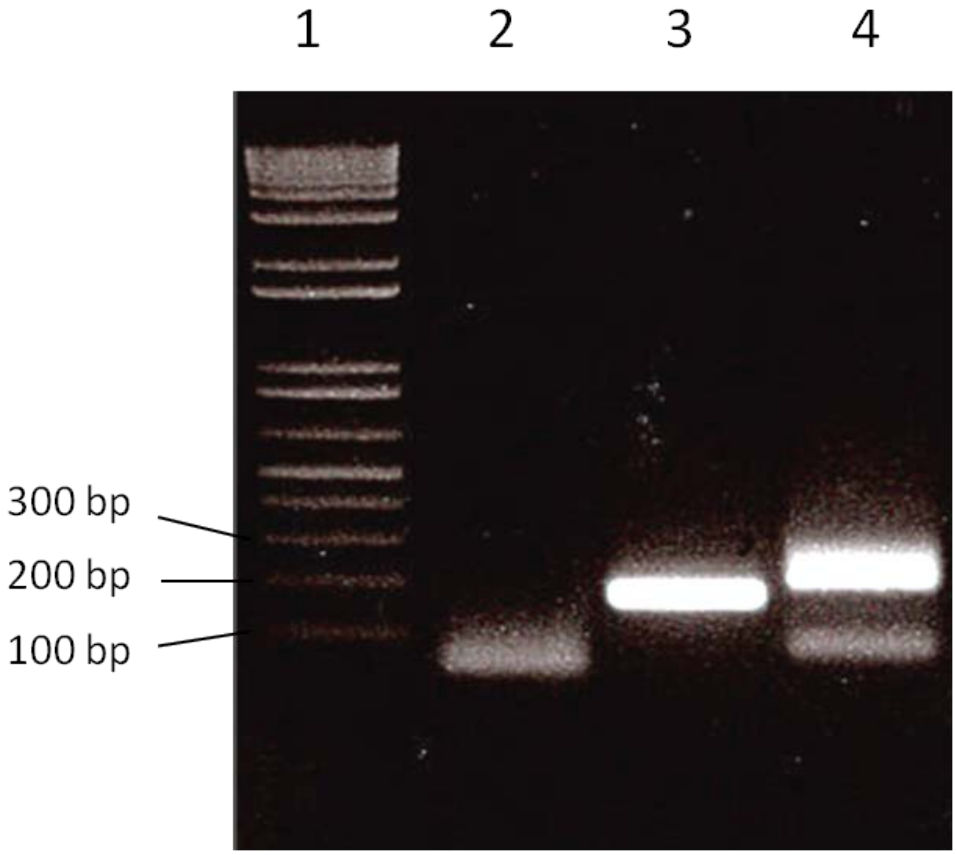


Figure 4.3: Agarose gel of 5' RACE fragments obtained from Cabernet Sauvignon and Pinot Noir *VvSCPL1* transcripts in berries

DNA fragments amplified from the 5' region of *VvSCPL1* transcripts by 5' RACE from Cabernet Sauvignon and Pinot Noir whole berries 2 weeks post veraison separated on a 2 % (w/v) agarose gel. Lane 1: 1Kb Plus DNA ladder (Invitrogen), lane 2: water control, lane 3: 5' RACE products obtained using Cabernet Sauvignon RNA, lane 4: 5' RACE products using Pinot Noir RNA. The same volume of PCR reactions was loaded into lanes 2, 3 and 4.

In Cabernet Sauvignon, the first ATG in the *VvSCPL1* transcript (CS5'RACE, Figure 4.4) was the same as the one that was annotated as the start codon in the *VvSCPL1*_12X annotation. If this was the start codon then the transcript would contain no 5' untranslated region (UTR). The largest 5' region of the Pinot Noir *VvSCPL1* transcripts (PN5'RACE-1, Figure 4.4) contained an additional 49 nucleotides further upstream to where the Cabernet Sauvignon transcript began. This extra 5' region also contained an earlier ATG 12 bp from its start. When the sequence of the PN5'RACE-1 transcript was translated using this first ATG as the start codon, it was out of frame to the second ATG, which is the first ATG in the Cabernet Sauvignon transcript and the start codon in the *VvSCPL1*_12X gene annotation (data not shown). The slightly smaller Pinot Noir transcript (PN5'RACE-2, Figure 4.4) did not contain this additional ATG, but instead contained a 30 bp region 5' to its first ATG, which was the same as that of the *VvSCPL1*_12X gene annotation. The smallest Pinot Noir 5' region (PN5'RACE-3, Figure 4.4) began 100 bp downstream of where the Cabernet Sauvignon transcript began. Its first ATG was 17 bp from its start and is out of frame to the *VvSCPL1*_12X gene annotation (data not shown). A nucleotide BLAST of these sequences against the *V. vinifera* sequence database in NCBI showed that the only gene which they had sufficient homology to have been able to be amplified by the VvSCPL_R2 primer was the *VvSCPL1* gene (data not shown). There were no transcripts identified from Cabernet Sauvignon or Pinot Noir that contained the start codon of the *VvSCPL1*_NCBI gene annotation.

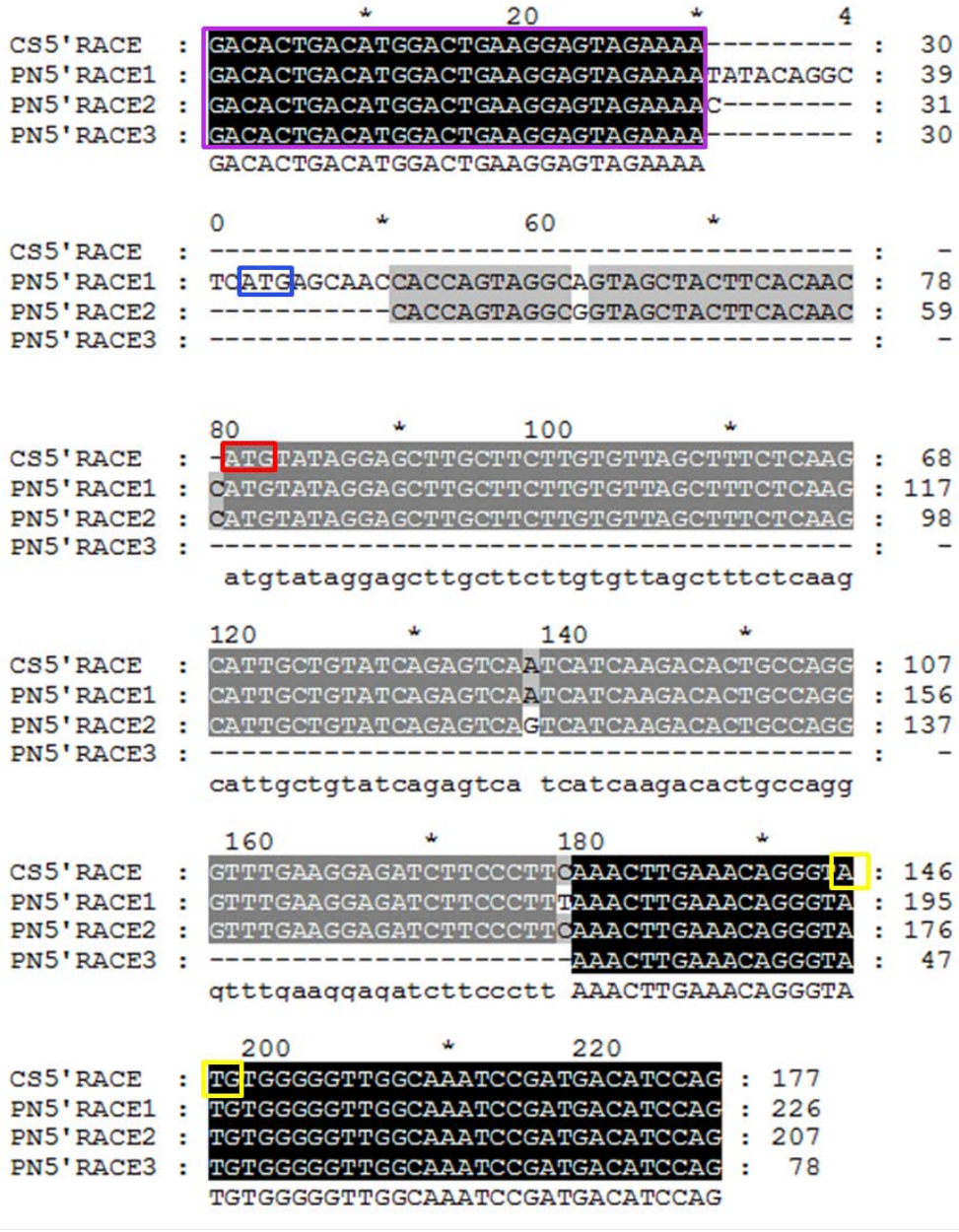


Figure 4.4: Sequence alignment of *VvSCPL1* transcript 5' ends from 5' RACE of Cabernet Sauvignon and Pinot Noir mRNA

Sequences of fragments obtained from 5' RACE of Cabernet Sauvignon (CS) and Pinot Noir (PN) mRNA from whole berries 12 weeks post flowering using a *VvSCPL1* specific primer. 5'Generacer RNA oligo added to ends of mRNA transcripts are boxed in purple. The start codon as annotated in the *VvSCPL1*_12X gene annotation is boxed in red. An earlier start codon is present in PN5'RACE1 and is boxed in blue. The second ATG in the CS5'RACE sequence, which is the first ATG in the PN5'RACE3 sequence, is boxed in yellow.

4.3.4 Pattern of gene expression of *VvSCPL1* over berry development

4.3.4.1 Gene expression of VvSCPL1 in Cabernet Sauvignon, Malian, and Shalistin

Transcript levels of *VvSCPL1* were analysed over berry development (whole berries) and berry ripening (skins only) of Cabernet Sauvignon, Malian and Shalistin varieties (Figure 4.5). *VvUFGT* transcription was also measured as an indicator of anthocyanin biosynthesis. In whole berries *VvSCPL1* transcripts were highest at 2 wpf then gradually decreased till 9 wpf with no significant differences between the three varieties (Figure 4.5a). In contrast, *VvUFGT* transcripts were not detected in whole berries between 2 and 8 wpf. At 9 and 10 wpf *VvUFGT* transcripts were highest in Cabernet Sauvignon, lower in Malian and barely detectable in Shalistin whole berries (Figure 4.5b). In berry skins, *VvSCPL1* transcript levels increased after veraison in Cabernet Sauvignon, peaking at 11 wpf and then decreasing till 15 wpf where they remained the same till 18 wpf. *VvSCPL1* transcript levels only slightly increased in Malian berry skins, and did not significantly differ in Shalistin skins after veraison, and were much lower than that of Cabernet Sauvignon (Figure 4.5c). *VvUFGT* transcript levels were highest at 11 wpf in both Cabernet Sauvignon and Malian berry skins, but transcript levels were lower in Malian. No *VvUFGT* transcripts were detected in Shalistin berry skins (Figure 4.5d).

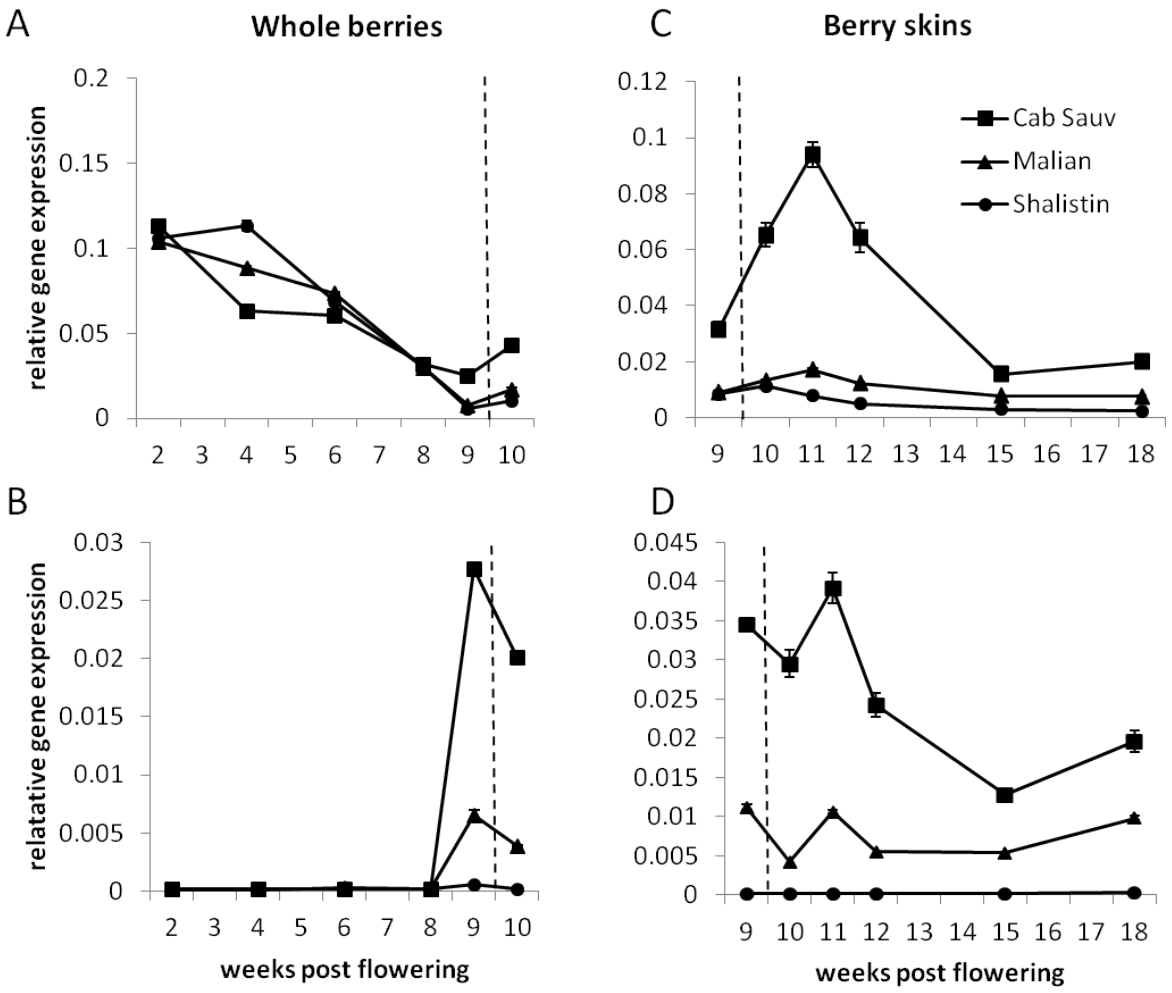


Figure 4.5: Transcript levels of *VvSCPL1* over the development of Cabernet Sauvignon, Malian and Shalistin berries

Transcript levels of **A and C)** *VvSCPL1* and **B and D)** *VvUFGT* throughout berry development of Cabernet Sauvignon (Cab sauv), Malian and Shalistin. **A and B)** Transcript levels were measured in early berry development from RNA that was extracted from whole berries 2 – 10 weeks post flowering (wpf). **C and D)** Transcript levels were measured in the skins of berries from 9 - 18 wpf. Vertical dashed line represents veraison. Gene expression was determined by qPCR and is shown relative to the the average expression levels of three housekeeping genes *VvUbiquitin*, *VvActin2*, and *VvEF1α-2*. All data is presented as a mean of three technical replicates with standard error bars.

4.3.4.2 Gene expression of *VvSCPL1* in Cabernet Sauvignon and Pinot Noir berry development

Pinot Noir does not synthesise acylated anthocyanins (Van Buren et al., 1968) and represents a mutant that can be compared to wild type cultivars. The transcript levels of *VvSCPL1* and *VvUFGT* were analysed using qPCR over the development of whole berries from Cabernet Sauvignon and Pinot Noir cultivars starting from 2 wpf through to harvest at 14 wpf (Figure 4.6). Pre-veraison, *VvSCPL1* transcripts in Cabernet Sauvignon and Pinot Noir were similar, being high in 2 wpf berries and then decreasing till 8 wpf. Post-veraison transcript levels were higher in Cabernet Sauvignon, peaking at 10 wpf, when they were 2.3 fold higher than in Pinot Noir (Figure 4.6a). *VvUFGT* transcription was not activated till veraison, after which there was little difference between Cabernet Sauvignon and Pinot Noir, except that in Cabernet Sauvignon transcript levels at 14 wpf were lower than at 10 wpf (when they peaked) whereas in Pinot Noir they were approximately the same at these two time points (Figure 4.6b).

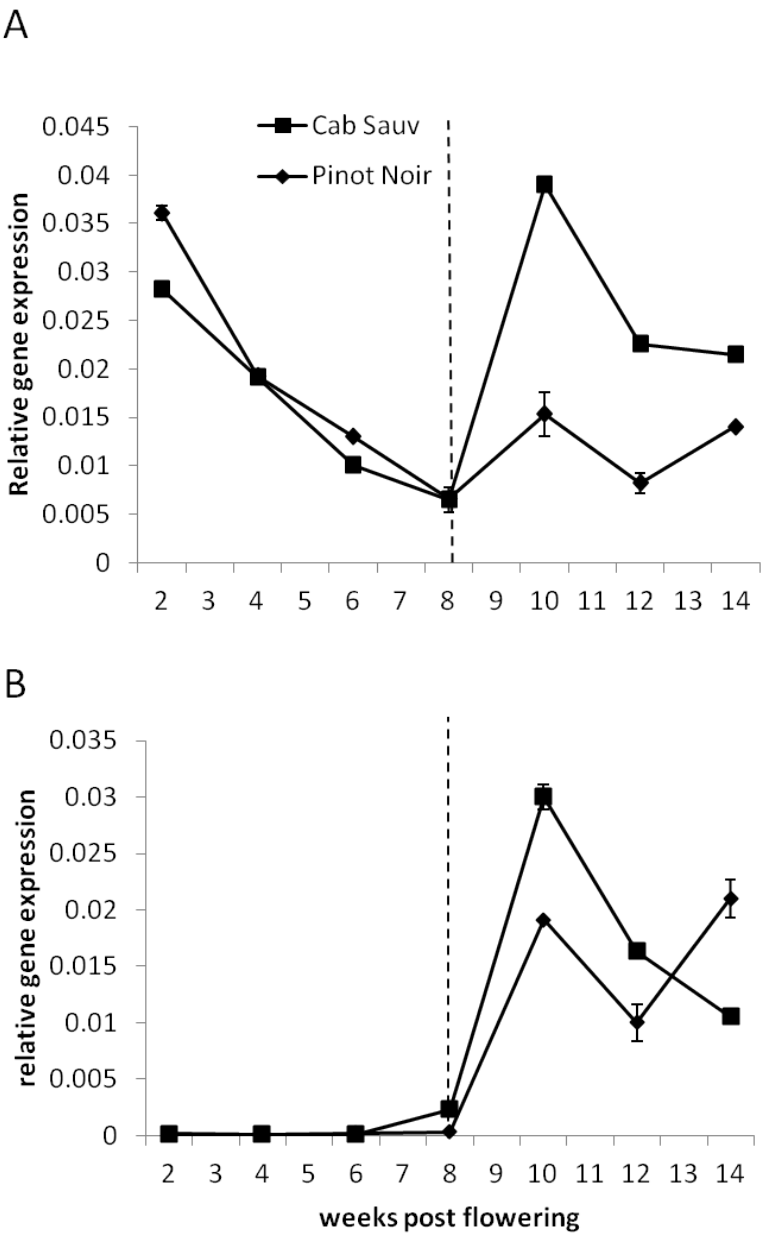


Figure 4.6: Transcript levels of *VvSCPL1* over the development of Cabernet Sauvignon and Pinot Noir berries

Transcript levels in whole berries of **A)** *VvSCPL1* and **B)** *VvUFGT* throughout berry development of Cabernet Sauvignon (Cab Sauv) and Pinot Noir. Vertical dashed line represents the time where veraison occurred. RNA was extracted from deseeded whole berries collected every 2 weeks post flowering. Transcript levels were determined by qPCR and is shown relative to the the average levels of three housekeeping genes *VvUbiquitin*, *VvActin2* and *VvEF1α-2*. All data is presented as a mean of three technical replicates with standard error bars.

4.3.5 VvMYBA does not activate the promoter of *VvSCPL1*

Transient promoter-binding luciferase activity assays were performed to determine if VvMYBA1 can activate transcription using the *VvSCPL1* promoter. Genomic DNA 1148 bp upstream of the putative protein coding start site of the *VvSCPL1* gene (*VvSCPL1*_12X annotation) was isolated from Cabernet Sauvignon and Pinot Noir. There were no sequence differences in these promoter regions between the two cultivars (data not shown), so only the Cabernet Sauvignon promoter was used in further experiments. *VvUFGT* and *VvSCPL1* promoters upstream of a luciferase reporter gene (Horstmann et al., 2004) were co-bombarded into grapevine cell suspension cultures with constructs expressing VvMYBA1 and bHLH transcription factors. Luciferase activity in cells containing the *VvUFGT* promoter construct and VvMYBA1 was 240-fold higher than the activity in control cells (no *35S:VvMYBA1* construct present). There was no significant difference in the luciferase activity in the grape cells when the *VvSCPL1* promoter was used compared to background controls (Figure 6). This indicates that VvMYBA1 does not activate the transcription of *VvSCPL1*

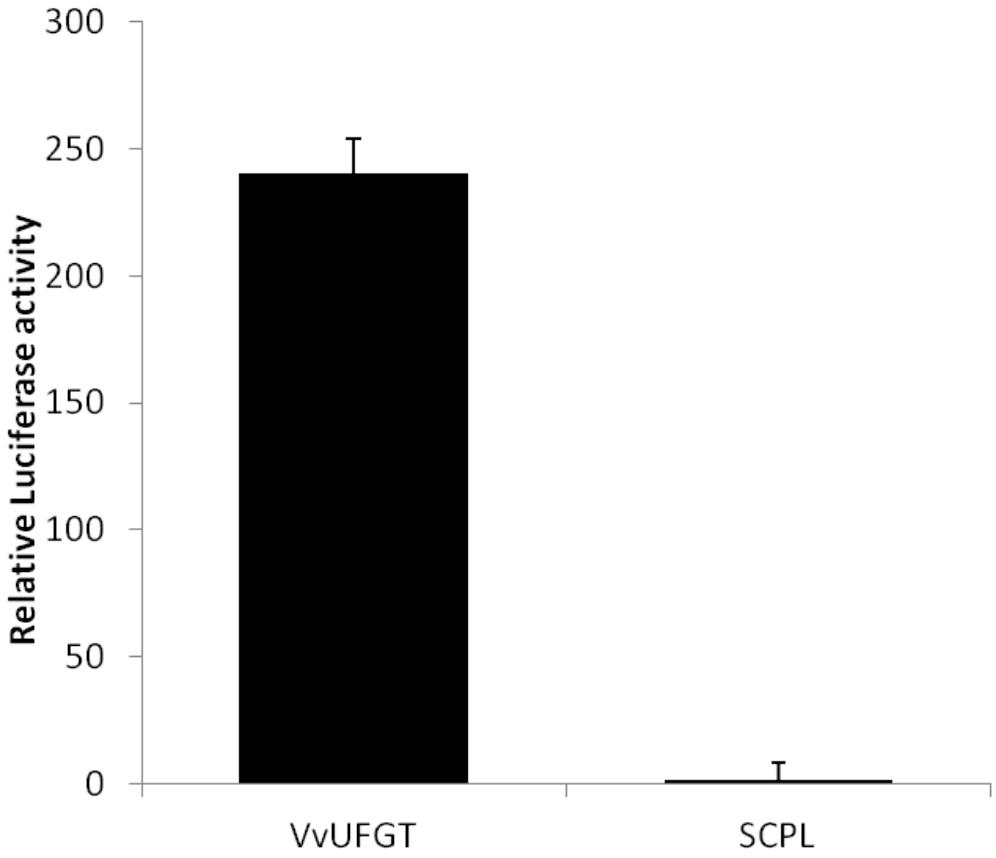


Figure 4.7: VvMYBA1 trascriptional activation assays of *VvUFGT* and *VvSCPL1* gene promoters

Promoters were ligated to the pLUC vector so they were preceding the firefly luciferase gene. promoter:*LUC* constructs along with *35S:VvMYBA1*, *pFF19:EGL3* and *pRluc* expression vectors were delivered to grape suspension culture cells through biolistic transfection. Luciferase activity was measured after 48 hours, divided by the background luciferase activity of negative controls lacking the *35S:VvMYBA1* construct, and reported relative to renilla activity.

4.3.6 Analysis of putative acyltransferase activity of VvSCPL1 *in planta*

Tobacco (*N. tabacum* var. Samsun) was chosen as an appropriate *in planta* model to determine if *VvSCPL1* encoded an anthocyanin acyltransferase. Tobacco produces anthocyanins in flowers, but they are not acylated (Luo et al., 2007); it is a relatively easy plant to transform, with culturing and glasshouse growth periods much shorter than for grapevine. *VvSCPL1* was constitutively expressed in tobacco under the control of the cauliflower mosaic virus 35S promoter (Gleave, 1992) through *Agrobacterium*-mediated stable transformation. Transcript levels of *VvSCPL1* were determined by qPCR in the transgenic tobacco flowers and WT controls. Eight transgenic tobacco plants (all derived from independent transformation events) expressed the *VvSCPL1* gene while the three WT plants tested did not (data not shown). There were no visual differences between the flower pigmentation of the transgenic flowers and WT controls (Figure 4.8). Anthocyanins were extracted from flowers and separated using high-performance liquid chromatography (HPLC) and peaks were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Two anthocyanin species could be detected in the WT tobacco flowers. The most abundant species was cyanidin-3-*O*-rutinoside (peak 1), with lesser amounts of pelargonidin-3-*O*-rutinoside also present (peak 2; Figure 4.8). Compared to WT there were no alterations to the anthocyanin profiles within the transgenic tobacco flowers expressing the *VvSCPL1* gene (Figure 4.8).

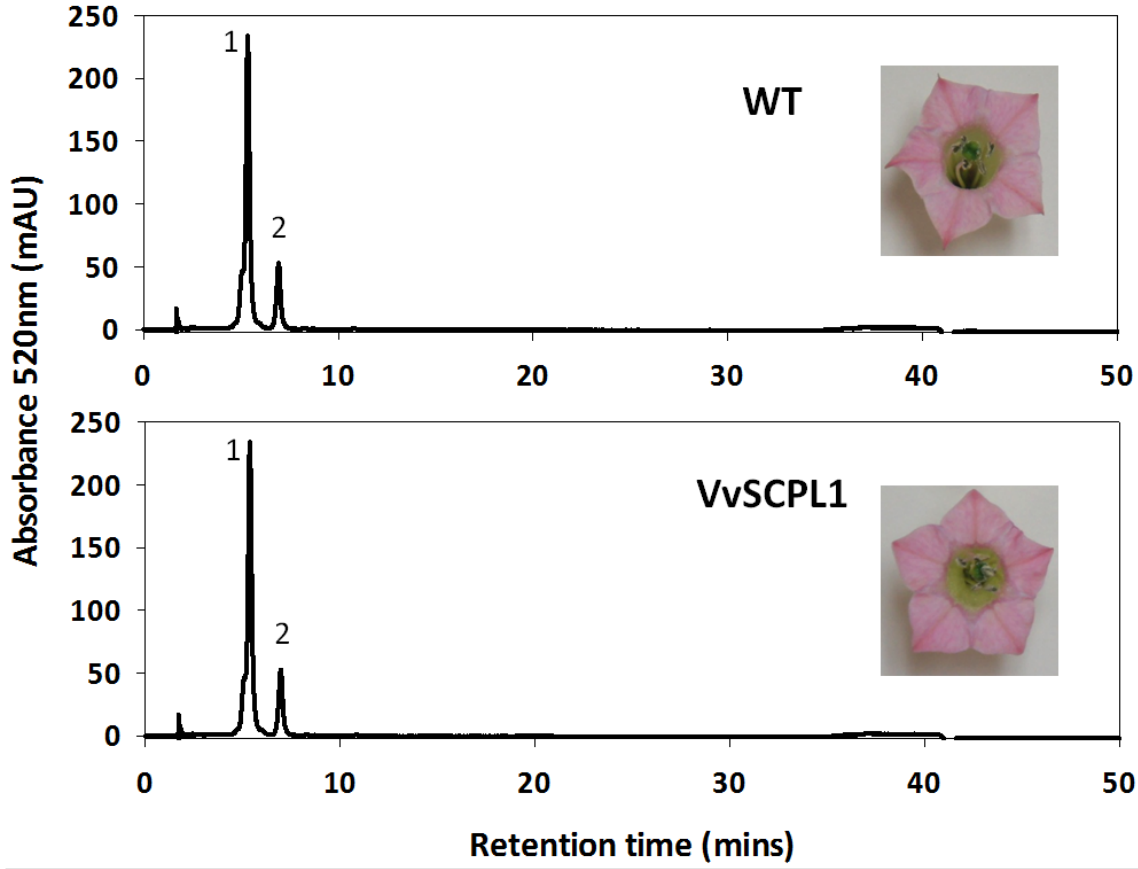


Figure 4.8: Chromatograms of anthocyanins in wildtype and transgenic tobacco expressing *VvSCPL1*

Chromatograms of anthocyanins in wild type (WT) and transgenic *Nicotiana tabacum* (var. Samsun) flowers expressing the *VvSCPL1* gene under the 35S promoter (VvSCPL1) are presented. Photos of WT and transgenic VvSCPL1 tobacco flowers are inset in the chromatograms. Peak identities were determined using LC/MS/MS: peak 1 = cyanidin-3-*O*-rutinoside, peak 2 = pelargonidin-3-*O*-rutinoside.

4.4 Discussion

4.4.1 Bioinformatic analysis of *VvSCPL1* suggests an anthocyanin acyltransferase function

In Chapter 2, an uncharacterised gene annotated as belonging to the SCPL acyltransferase family, which was up-regulated in berries expressing VvMYBA, was identified. It was hypothesised that this gene, named *VvSCPL1*, may function as an anthocyanin acyltransferase, and in this chapter experimental work aiming to test this hypothesis has been presented.

A BLAST search of the conceptual translation of *VvSCPL1* confirmed that this protein was homologous to a number of putative and characterised SCPL proteins. A sequence alignment of VvSCPL1 with the characterised homologues identified in this BLAST search and with AtSMT, of which the structure has been modelled *in silico* (Stehle et al., 2006), showed that the Ser-Asp-His catalytic triad and many of the residues involved in the hydrogen bonding network are conserved in the VvSCPL1 sequence (Appendix K). This provides further evidence that VvSCPL1 belongs to the SCPL family of acyltransferases and could be a functional protein as it contains structural sequence elements essential for the activity of these enzymes (Stehle et al., 2006). As VvSCPL1 has homology to anthocyanin acyltransferases, this provides further support to the hypothesis that it too may have this function.

4.4.2 VvSCPL1 may be located in the vacuole

The VvSCPL1 protein sequence was predicted to contain an N-terminal signal peptide, 17 aa in length, using the SignalP 4.1 prediction server (Figure 4.1). Also using SignalP, Fraser et al. (2005) found that 49 of the 51 SCPL proteins from *Arabidopsis* possessed N-terminal signal peptides which are likely to target them to the endoplasmic reticulum (ER). These genes also posses many conserved NX(S/T) sequences which are potential sites for N-linked glycosylation (Marshall, 1972). *N*-glycosylation post-translational modifications occur to pre-proteins in the ER and Golgi apparatus prior to their transport to subcellular locations (Rayon et al., 1998). SMTs from *Arabidopsis* (Hause et al., 2002) and *Raphanus sativus* (Sharma and Strack, 1985) have been shown to be located in the vacuole. Furthermore, the sequence of the first 17 N-terminal aa of the mature SMT protein extracted from *Brassica napus* match those

following the predicted cleavage site of AtSMT, suggesting that the peptide prediction by SignalP is correct (Gräwe et al., 1992; Lehfeldt et al., 2000). Analysis of the BnSMT sequence using the PSORT server (psort.nibb.ac.jp/) predicted six putative glycosylation sites and indicated that the protein is most likely localized in the vacuole (Lehfeldt et al., 2000). From these studies it has been proposed that SCPL acyltransferases are synthesised as pre-proteins, glycosylated in the ER and Golgi apparatus and then transported to the vacuole (Milkowski and Strack, 2004). This seems to be also likely for VvSCPL1. Future experiments to investigate this could include transient or stable expression of VvSCPL1 fused to a fluorescent protein to visualise cell localisation in a model plant such as tobacco.

Anthocyanin synthesis is known to occur in the cytoplasm, from where they are then transported into the vacuole (Zhao and Dixon, 2010). Recently an ABCC-transporter , VvABCC1, was identified from grapevine and shown to preferentially transport anthocyanin-3-*O*-glucosides, in particular malvidin-3-*O*-glucoside, into the vacuole (Francisco et al., 2013). Two other anthocyanin transporters, anthoMATE1 and anthoMATE3 have been characterised and were shown to transport only acylated anthocyanins across the tonoplast (Gomez et al., 2009). This means that at least some of the anthocyanins in grapes must be acylated in the cytoplasm before they are transported. If VvSCPL1 is a vacuolar protein, as has been predicted, then it could not be responsible for the anthocyanin acyltransferase reactions occurring in the cytoplasm. This could suggest that either VvSCPL1 does not function as an anthocyanin acyltransferase, or that it does but only in the vacuole. If this protein did function as an anthocyanin acyltransferase then it could only be responsible for a portion of the anthocyanin acylation reactions in grapevine. A cytoplasmic anthocyanin acyltransferase, such as one belonging to the BAHD protein family (D'Auria, 2006), could be responsible for anthocyanin acylation in the cytoplasm. In Chapter 2 work describing an uncharacterised BAHD protein that was highly up-regulated in berries expressing VvMYBA and could potentially have this function was presented. Experimental work testing this hypothesis is presented in Chapter 5.

4.4.3 *VvSCPL1* is transcribed pre- and post-veraison and is not directly regulated by VvMYBA

VvSCPL1 transcript levels were measured in Cabernet Sauvignon, and its colour sports, Malian and Shalistin, over berry development, and *VvUFGT* gene expression was used as a

marker for anthocyanin biosynthesis controlled by VvMYBA TFs (Figure 4.5). The expression pattern of *VvUFGT* was as expected in these three varieties, i.e. transcripts were present from just before veraison and throughout post-veraison, their levels were higher in Cabernet Sauvignon compared to Malian, and they were absent in Shalistin. Boss et al. (1996b) showed that *VvUFGT* transcript levels were greatly reduced in Malian and completely absent in Shalistin compared to Cabernet Sauvignon using northern blot analysis. It was later demonstrated that this was a consequence of a DNA deletion of the 'red' colour locus containing the *VvMYBA* genes in cells derived from the L2 meristem cell layer in Malian, and from both the L1 and L2 cell layers in Shalistin (Walker et al., 2006). It is expected that genes requiring VvMYBA transcriptional activation would have very similar expression patterns to *VvUFGT*. Post-veraison *VvSCPL1* transcript levels were similar to that of *VvUFGT* except that Shalistin expressed *VvSCPL1* at low levels. Yet, pre-veraison *VvSCPL1* transcripts were also detected unlike with *VvUFGT* and this was seen in berries of Cabernet Sauvignon, Malian, Shalistin and Pinot Noir (Figure 4.5 and Figure 4.6). As Shalistin does not contain functional *VvMYBA* genes, and *VvMYBA* is not expressed in pre-veraison berries (Kobayashi et al., 2002), it must be concluded that *VvSCPL1* transcription cannot be solely controlled by VvMYBA TFs. This was further supported by results from transient promoter-binding luciferase activity assays, which indicated that *VvSCPL1* promoter activation was not achieved using the VvMYBA1 TF, in contrast to high levels of *VvUFGT* promoter expression under the same conditions (Figure 4.7). Moreover, anthocyanin biosynthesis does not occur pre-veraison, when *VvSCPL1* is transcribed, suggesting that this gene may have another or additional function to the acylation of anthocyanins.

The structural flavonoid genes, *VvLDOX*, *VvANR*, *VvLAR1*, and *VvLAR2*, and the transcription factor gene *VvMYBPA1*, are expressed early in berry development leading to early synthesis of PAs in berries (Bogs et al., 2005; 2007). *VvLAR2* transcription measured in the skins of developing Shiraz berries had a similar pattern to that observed for *VvSCPL1* pre-veraison, with highest transcript levels observed just after flowering, then decreasing till veraison (Bogs et al., 2005). It is possible therefore that VvSCPL1 is involved in acyltransferase reactions in both PA and anthocyanin biosynthesis. SCPL enzymes have been previously proposed to be involved in galloylation of PAs in oak (Gross, 1983) and persimmon (*Diospyros kaki*) fruit (Ikegami et al., 2007), although these enzymatic reactions are yet to be characterised. Another SCPL gene from grapevine, *VvGAT-like*, has already been hypothesised to be involved in PA biosynthesis (Terrier et al., 2009; Grégory et al., 2013).

Grégory et al. (2013) identified *VvGAT-like* and 19 other genes using an integrative approach where results from several previous transcriptomic and QTL mapping studies aimed at discovering genes involved in PA biosynthesis in grapevine were analysed. *VvSCPL1* was not identified as a candidate gene in this study. An alignment between *VvSCPL1* and *VvGAT-like* shows they have 46% identity between their protein sequences (data not shown).

4.4.4 *VvSCPL1* sequence and expression in Cabernet Sauvignon and Pinot Noir does not match acylated anthocyanin phenotypes of these cultivars

The grapevine cultivar Pinot Noir lacks acylated anthocyanins in its berries. As such, *VvSCPL1* transcript levels were compared in Cabernet Sauvignon and Pinot Noir developing berries. Pinot Noir expressed this gene are slightly lower levels post-veraison compared to Cabernet Sauvignon but the gene was still expressed (Figure 4.6). If *VvSCPL1* functions as an anthocyanin acyltransferase, then the expression patterns of this gene cannot explain the lack of acylated anthocyanins in Pinot Noir. Sequence analysis of the *VvSCPL1* coding region in Cabernet Sauvignon and Pinot Noir revealed that these cultivars contain no differences in the amino acid sequences of the translated protein or in the non-coding regions of the gene. 5' RACE on *VvSCPL1* transcripts from both cultivars within berries 12 wpf revealed that there were differences in the sequence of their 5' ends (Figure 4.4). This may suggest that while there are no sequence differences of *VvSCPL1* between the two cultivars, there may be differences in how the genes is transcribed. Due to extended or truncated 5' ends, approximately two thirds of Pinot Noir transcripts could contain an earlier or later start codon compared to Cabernet Sauvignon, which would result in a frameshift. This could potentially mean that only a portion of the *VvSCPL1* transcripts detected in Pinot Noir berries would code for a functional protein. If this were the case one would assume that if VvSCPL1 was an anthocyanin acyltransferase, then Pinot Noir would still contain acylated anthocyanins, albeit at lower levels to Cabernet Sauvignon. Assuming that the *VvSCPL1* transcripts are translated, these results do not support our original hypothesis that VvSCPL1 functions as an anthocyanin acyltransferase. VvSCPL1 protein levels in the berries would need to be confirmed before further conclusions can be made. Differences in post-translation modifications to the protein, may also contribute to the phenotype difference between the two cultivars and cannot be ruled out from this study. Post-translational modifications have been identified on some plant SCPL proteins, such as glycosylation and endoproteolytic cleavage

(Milkowski and Strack, 2004). AtSMT is highly glycosylated (Hause et al., 2002) and many of the predicted SCPL genes from *Arabidopsis* contain potential glycosylation sites (Fraser et al., 2005).

To date there have been no reports of SCPL splicing variants; however, *VvSCPL1* gene structure is complex (12 introns and 13 exons) so it is not unreasonable to consider this. However most documented splicing errors are caused by mutations in genes that affect splicing site signals (Graveley, 2001). The fact that there are no sequence differences between Cabernet Sauvignon and Pinot Noir genes, including within their introns, therefore makes it unlikely that there would be differences in gene splicing between these two cultivars. With this in mind it was surprising that there were differences between the two cultivars in their 5' ends of *VvSCPL1* transcripts. Other examples of this could not be found in the literature. Possible explanations for this are discussed further in section 4.4.5.

4.4.5 VvSCPL1 did not function as an anthocyanin acyltransferase in tobacco

To study the function of *VvSCPL1 in planta*, this gene was constitutively expressed in tobacco. The transgenic tobacco flowers contained no acylated anthocyanins and did not differ from the wildtype flower anthocyanin profiles or in their visual pigmentation (Figure 4.8). These results indicated that this gene did not function as an acyltransferase in tobacco. Whether this is the case in grapevine cannot be determined from this experiment. VvSCPL1 may not have catalytic activity towards the tobacco anthocyanins, as tobacco contains rutinoside conjugated anthocyanins (Luo et al., 2007) while the berries of grapevine contain monoglucosides (He et al., 2010). Enzymes involved in the synthesis of the acyl-glucose substrates utilized in SCPL reactions may not be present in tobacco, although this is unlikely as other studies have successfully demonstrated SCPL enzyme activity from transgenes using *N. tobacum* and *N. benthamiana* as *in planta* models (Clauß et al., 2008; Weier et al., 2008; Lee et al., 2012).

Another possible explanation for no anthocyanin acyltransferase activity in transgenic tobacco expressing *VvSCPL1* is that the annotation of the coding sequence used in these experiments (*VvSCPL1*_12X) was incorrect. Two different annotations of this gene were found, one in the NCBI database (*VvSCPL1*_NCBI) and the other from the 12X grapevine genome database (*VvSCPL1*_12X) (section 4.3.3). Comparison of *VvSCPL1* transcript sequences obtained from

Cabernet Sauvignon berries demonstrated that the exon/intron annotation of the *VvSCPL1*_12X sequence was correct and this sequence was used in the *in planta* experiments (Appendix M). It is possible, however, that the start codon in this annotated sequence was incorrect. Sequencing of the 5'ends of the Cabernet Sauvignon transcript by 5' RACE showed that the ATG annotated as the start codon in the *VvSCPL1*_12X sequence was the first ATG of the transcript, but if this were the start codon then there would be no 5' UTR (Figure 4.4). The 5' UTR of mRNA contains sequences that are recognized by translational machinery and is known to have a role in transcriptional regulation (Wilkie et al., 2003). There are no published examples of transcripts lacking a 5' UTR suggesting that is an essential element of mRNA transcripts. This then infers that the results obtained from 5' RACE were either incorrect, or the start codon of the Cabernet Sauvignon *VvSCPL1* transcript is further downstream from where the *VvSCPL1*_12X annotation suggests. The second ATG in the Cabernet Sauvignon *VvSCPL1* transcript, 115 bp downstream from the first ATG, is out of frame and would be likely result in translation of a non-functional protein (Figure 4.4). It is possible that the 5' RACE reactions did not work as they should have so that the 5' GeneRacer^{TM} RNA oligo was not attached to the ends of the mRNA transcripts. This could possibly explain the unusual results obtained from Pinot Noir, being that it contained a range of different 5' ends, all differing from Cabernet Sauvignon, despite the fact that it was homozygous for the *VvSCPL1* gene and identical to the Cabernet Sauvignon sequence. These possibilities must be further investigated before conclusions can be made about the correct coding sequence of this gene. It is possible that the *VvSCPL1*_12X and *VvSCPL1*_NCBI annotations of *VvSCPL1* are both incorrect. Considering the large number of introns in this gene this is not unlikely. Fraser et al. (2005) reported that almost one third of the 51 *Arabidopsis* predicted SCPL genes contained misannotations in their sequences within the *Arabidopsis* genome, illustrating that misannotation of this gene family is common.

While definite conclusions cannot be made based on the present data, together the results from expression analysis in berries of Pinot Noir and Cabernet Sauvignon (and its colour mutants), the evidence that VvMYBA is not the only TF to activate *VvSCPL1* gene expression, and the fact that this gene did not acylate anthocyanins in tobacco, suggests that *VvSCPL1* does not function as an anthocyanin acyltransferase. This then raises the question of what other function this gene may possess. As discussed in section 4.4.2 and 4.4.3, VvSCPL1 is likely to be located in the vacuole and *VvSCPL1* is expressed when both PAs and anthocyanins are synthesised during grape development. *Arabidopsis* TT12 and *Medicago*

MATE1 transporters were shown to transport epicatechin 3′-*O*-glucosides, precursors for PA biosynthesis, into the vacuole (Zhao and Dixon, 2009). This indicates that PA biosynthesis occurs in the vacuole, and SCPL proteins have been hypothesized to play a role in this (Ikegami et al., 2007; Laitinen et al., 2008; Liu et al., 2012; Grégory et al., 2013). If VvSCPL1 is involved in PA biosynthesis then an analysis of PAs in the seeds from the transgenic tobacco plants constitutively expressing *VvSCPL1* would be a useful to investigate this. This hypothesis, however, would not account for the fact the *VvSCPL1* is expressed post-veraison, when PA accumulation has ceased (Downey et al., 2003a) and in a manner similar to *VvUFGT*, suggesting a link to anthocyanin biosynthesis. Perhaps VvSCPL1 does not function to acylate flavonoids directly but rather is involved in reactions required for their transport into, or storage to, the vacuole. No examples of such a function for a plant SCPL protein has been previously reported, but considering the only recent discovery of this protein family it is possible that many unknown functions are yet to be characterised. The fact that there were no sequence differences over the entire *VvSCPL1* gene and its promoter (4.5 Kb in total) between Cabernet Sauvignon and Pinot Noir is surprising. Sequence analysis in a number of different cultivars would be useful to determine the degree of sequence conservation for this gene in grapevine. A high degree of conservation may indicate an essential function of *VvSCPL1* requiring high sequence preservation. Other future work would include the production of recombinant VvSCPL1 protein to be used in *in vitro* studies into the function of this protein.

4.5 Conclusion

The experimental work presented in this chapter was aimed at characterising the function of the *VvSCPL1* gene found to be up-regulated in Shiraz and transgenic ‘red’ Chardonnay berries in microarray studies. In particular, the hypothesis that *VvSCPL1* functioned as an anthocyanin acyltransferase was tested. Gene expression analysis of this gene in developing berries showed that *VvSCPL1* had some similar characteristics to *VvUFGT* gene expression, but also some differences, such as being expressed early in berry development. The VvMYBA TF was unable to activate *VvSCPL1* promoter expression, and constitutively expressing *VvSCPL1* in tobacco did not alter flower anthocyanin profiles. Together these results suggest that VvSCPL1 does not function as an anthocyanin acyltransferase, although there is a possibility that the annotation of this gene, used in these studies, is incorrect and this

needs to be investigated further. More research is required to decipher the function of this gene and its relevance to flavonoid synthesis.

Chapter 5: Characterisation of a grapevine anthocyanin acyltransferase gene (*VvAnAT*)

Notes about this Chapter

The University of Adelaide allows students to submit PhD theses which have a combination of traditional results chapters along with manuscripts (regardless of whether they have been submitted or accepted by a peer-reviewed journal). The experimental work presented in this chapter has been drafted into a manuscript which is expected to be shortly submitted to 'The Plant Journal' and so this chapter is composed of that manuscript. The manuscript has been kept in the format that was required for 'The Plant Journal' except for the ease of the reader the references cited in this manuscript will appear in the final reference list at the end of this thesis. The figures have also been placed in the relevant sections within the text rather than at the end of the document. There is some repetiveness in the introduction and methods sections with other chapters as this manuscript must be able to stand alone. The supplementary data referred to in the manuscript can be found in Appendix N. The citation referred to as 'Rinaldo et. al., in preparation' in the manuscript refers to the work described in Chapter 2. A signed statement regarding the contribution of the authours to the work described in the manscript can be found on the following page.

Statement regarding contribution of the authors to the work described in the following manuscript

The first author of this manuscript is Amy R. Rinaldo who is also the author of this thesis. All the experimental work presented in the manscript was carried out by Amy Rinaldo except for the construction of the phylogenetic tree which was carried out by the second author of the manuscript Sarah M. A. Moss. The concepualisation of the work was by Amy Rinaldo under the supervison of the other three authors of the manuscript: her two PhD supervisors Dr. Amanda R. Walker and Assoc. Prof. Christopher M. Ford, and her external advisor Dr. Paul K. Boss. The manuscript was drafted in its entirety by Amy Rinaldo except for the method described used to build the phylogenetic tree which was writted by Sarah M.. A. Moss. All authors were involved in reading and editing drafts of the manuscript.

Declaration of all authors:

By signing this document I am agreeing to the statement above regarding the contribution of the authours to the manuscript titled '*A grapevine anthocyanin acyltransferase (VvAnAT) belonging to the BAHD protein family is regulated by VvMYBA1*'. I am also giving my permission for this manuscript to be included in Amy Rinaldo's thesis titled '*An investigation of the role of the regulator VvMYBA in colour, flavour and aroma metabolism using transgenic grapevines*'.

Signed and dated:

Amy R. Rinaldo	12/6/14
Sarah M. A. Moss	11/6/14
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Paul K. Boss	11/6/14
Amanda R. Walker	11/6/14

A grapevine anthocyanin acyltransferase gene (*VvAnAT*) belonging to the BAHD protein family is regulated by VvMYBA1

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Summary:

Anthocyanins are flavonoid compounds responsible for orange, red, purple and blue colours in the leaves, fruit and flowers of many plant species. Acylation of anthocyanins by acyltransferases has been shown to change the hue of these pigments in some fruits and flowers, and to increase their stability in products such as wine. Here the first identification and characterization of a *Vitis vinifera* anthocyanin acyltransferase gene (*VvAnAT*) is presented through gene expression studies, bioinformatics analyses and *in vitro* and *in planta* functional assays. VvAnAT belongs to the BAHD acyltransferase protein family, and recombinant enzyme kinetic studies show that it can utilise a range of CoA thioester acyl donors, with a preference towards monoglucoside anthocyanin acyl acceptor substrates. We have also shown, using promoter activation assays, that the transcription of the *VvAnAT* gene is activated through the action of the VvMYBA1 transcription factor, the colour regulator responsible for activating anthocyanin biosynthesis in grapevine berries. This is the first time that transcriptional activation of a BAHD anthocyanin acyltransferase gene has been shown through promoter binding studies. This research illustrates that VvMYBA1 co-regulates the expression of anthocyanin biosynthesis genes including *VvAnAT*.

Introduction:

Anthocyanins, a group of water-soluble flavonoid compounds, are produced by almost all vascular plants and have been shown to have a diverse range of biological functions. They are major contributors to the orange, red, purple and blue colours seen in the leaves, fruit and flowers of many plant species and hence have important roles in attracting pollinators and seed dispersers (Schaefer et al., 2004). It has been suggested that they also act as protection agents against UV (Markham, 1988) and are involved in plant stress responses (Dixon and Paiva, 1995). Anthocyanins have potent antioxidant capacity, which can explain their numerous health-promoting properties including cardiovascular disease prevention, anti-inflammatory, antimicrobial and anti-carcinogenic activities (He and Giusti, 2010).

Wine grapes (*Vitis vinifera* L.) contain both 3-*O*-monoglucoside and 3-*O*-acyl monoglucoside anthocyanins derived from 5 main anthocyanidin aglycones:- delphinidin, cyanidin, peonidin, petunidin and malvidin. The structural genes involved in their production have all been isolated and characterised (Figure 1, reviewed in He et al., 2010). The regulation of the flavonoid pathway is through the action of transcriptional complexes involving 3 transcription factor (TF) families:- a basic helix-loop-helix protein (bHLH), a tryptophan-aspartic acid repeat protein (WDR or WD40) and an R2R3-MYB protein. The MYB/bHLH/WDR complexes are thought to recognise and bind to responsive elements found in the promoters of biosynthesis genes in the pathway, usually resulting in the activation of that gene's expression (reviewed in Matus et al., 2010). The MYB TFs determine the specificity of this complex and have been shown to directly bind to the structural gene promoters (Sainz et al., 1997). Anthocyanin synthesis in grapes is specifically regulated by the VvMYBA1 and VvMYBA2 TFs in the transcription complex by activating *VvUFGT* (uridine diphosphate glucose-flavonoid 3-*O*-glucosyltransferase) transcription (Walker et al., 2007). VvUFGT catalyses the final step of anthocyanin synthesis, where anthocyanidins are glycosylated on the 3-hydroxyl group of the B ring of the flavylium molecule to produce stable anthocyanins (Ford et al., 1998). White-fruited grapevine cultivars have arisen due to a lack of a functional VvMYBA protein, caused by a retrotransposon insertion in the promoter of *VvMYBA1* (Kobayashi et al., 2004) and two non-conservative mutations in *VvMYBA2* (Walker et al., 2007).

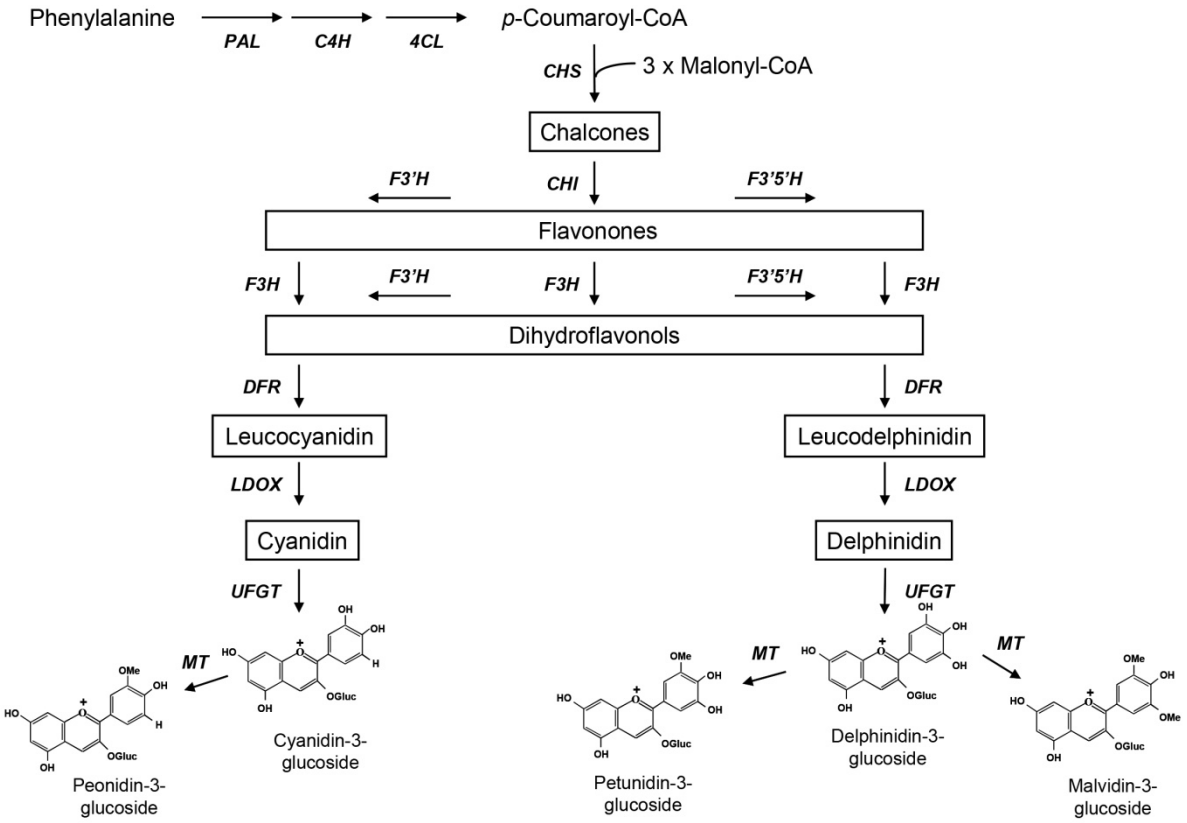


Fig 1) Schematic of the anthocyanin biosynthesis pathway in grapevine. Metabolites are boxed with genes coding for enzymes catalysing each biochemical reaction in italics. PAL = phenylalanine ammonia lyase, C4H = cinnamate 4-hydroxylase, 4CL = 4-coumaroyl CoA ligase, CHS = chalcone synthase, CHI = chalcone isomerise, F3H = flavanone-3-hydroxylase, F3'H = flavonoid 3'-hydroxylase, F3'5'H = flavonoid 3',5'-hydroxylase, DFR = dihydroflavonol 4-reductase, LDOX= leucoanthocyanidin dioxygenase, UFGT = UDP glucose-flavonoid 3-*O*-glucosyltransferase, MT = methyltransferases. (altered from Boss and Davies, 2009)

In grapevine, following glycosylation the core anthocyanins can be further modified by *O*-methyltransferases, which methylate hydroxyl groups at the 3' and 5' positions of the B-ring (Fournier-Level et al.), and acyltransferases, which produce 3-*O*-acetyl, 3-*O*-coumaroyl, and 3-*O*-caffeoyl-monoglucosides by attaching acyl groups to the C6'' position of the glucose molecule (Mazza and Francis, 1995; Nakayama et al., 2003). Acylated anthocyanins in various flowering species are more stable compared to their non-acylated counterparts, most likely due to increased intramolecular stacking (Yonekura-Sakakibara et al., 2008). Van Buren et al. (1968) showed that wines made from grapevine varieties with high proportions of acylated anthocyanins (e.g. Ives and Veeport) had greater colour stability when exposed to light compared to varieties with no acylated anthocyanins. Pinot Noir lacks acylated anthocyanins (Van Buren et al., 1968) and produces red wines with low anthocyanin content and unstable colour (Smart, 1992). Despite the importance of red colour stability to wine quality, and the association between poorly coloured red wines and cultivars containing no acylated anthocyanins, anthocyanin acyltransferases from grapevine have not yet been identified.

In a recent study (Rinaldo et al., in preparation) transgenic grapevines with altered *VvMYBA* gene expression were created. Overexpression of *VvMYBA1* in the white-berried cultivar Chardonnay resulted in pigmented fruit, while silencing the *VvMYBA1* and *VvMYBA2* genes in the pigmented Shiraz cultivar produced berries with lightly-coloured or white phenotypes. Transcriptome comparisons between these transgenic plants and their controls identified a set of genes which were up- and down-regulated in response to *VvMYBA* gene expression. Among those that were up-regulated were genes already known to be involved in the synthesis, modification and transport of anthocyanins (Rinaldo et. al., in preparation). Among the uncharacterised genes that were significantly upregulated was one with homology to the BAHD protein family. Members of the BAHD gene family, named after the first letter of the first 4 characterised proteins [BEAT, AHCT. HCBT, DAT; (St-Pierre and Luca, 2000)], are acyltransferases that utilize CoA thioesters as their donor substrates. BAHDs characterised in various plant species have been shown to acylate anthocyanins *in planta* (Fujiwara et al., 2001) and/or *in vitro* (Yonekura-Sakakibara et al., 2000; Yabuya et al., 2001; Suzuki et al., 2004b; D'Auria et al., 2007). Here we describe the functional characterisation of the BAHD gene from grapevine. Gene expression analyses, stable plant transformations and recombinant protein assays were used to demonstrate that the gene encodes an anthocyanin acyltransferase, named *Vitis vinifera anthocyanin acyltransferase* (*VvAnAT*), which can use a broad range of

acyl donor and acceptor substrates and is capable of producing all common acylated anthocyanins found in grapevine.

Results

VvAnAT belongs to the BAHD superfamily of acyltransferases within a clade distinct from most other anthocyanin acyltransferases

A full-length cDNA clone of the BAHD gene, upregulated in plants expressing *VvMYBA1* (Rinaldo et. al., in preparation) was isolated from the cultivar Cabernet Sauvignon (*VvAnAT-CS*). The Cabernet Sauvignon gene consists of a single exon 1284 bp in length. Two alleles were present in this cultivar that differ by 2 nucleotides: an A/G polymorphism at position 238 of the putative coding region, which is a silent mutation, and a C/T polymorphism at 784 bp which results in the coversion of Arg_{262} to Cys in the 428 aa protein sequence.

A comprehensive protein alignment and a phylogenetic tree were created using 72 BAHD protein sequences from 38 different plant species, including VvAnAT. Only sequences from proteins that have been genetically or biochemically characterized were included (Table S1). The tree separated the proteins into 5 major clades (Figure 2) with the VvAnAT sequence falling into clade III with an anthocyanin acyltransferase, anthocyanin 5-*O*-glucoside-4'''-*O*-malonyltransferase (Ss5MaT2) from *Salvia splendens* predicted to be its closest homologue.

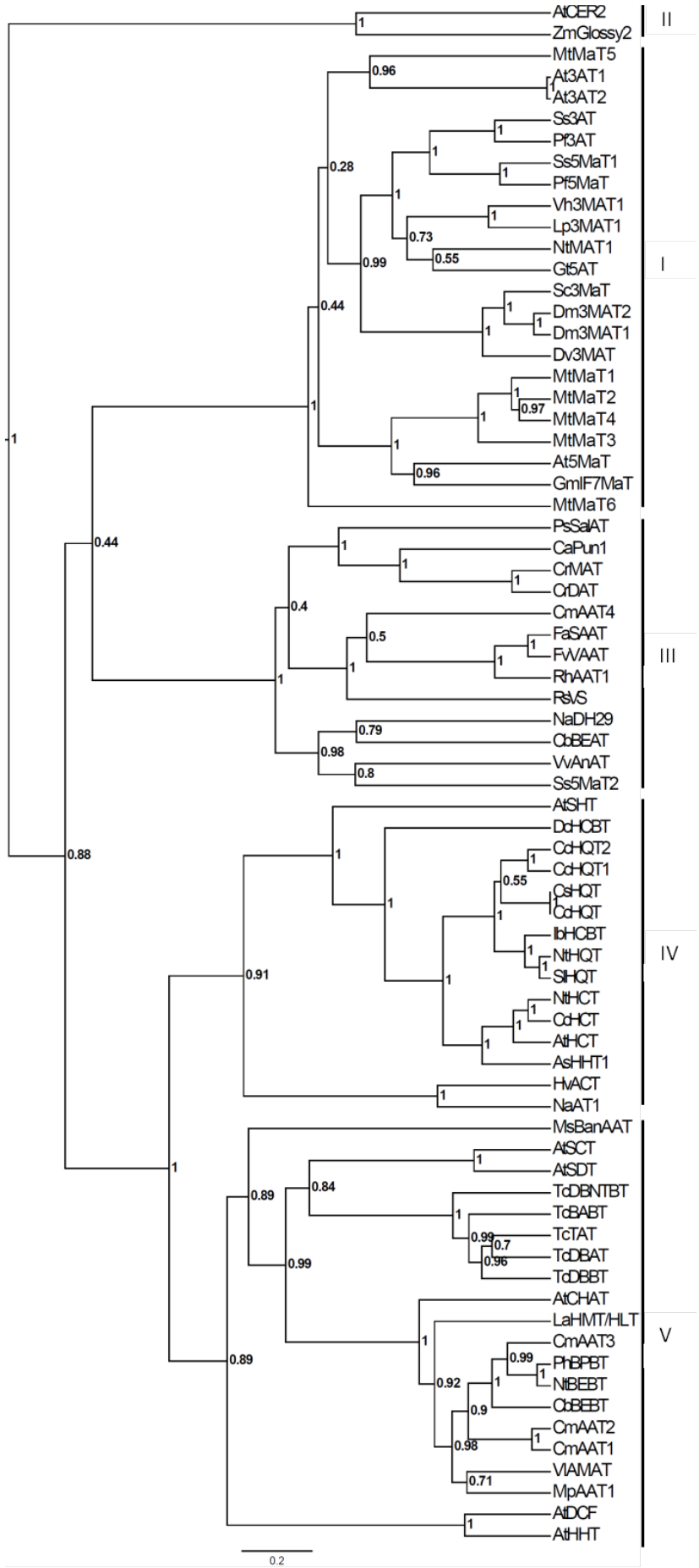

AtCER2
ZmGlossy2
II
MtMaT5
At3AT1
At3AT2
Ss3AT
Pf3AT
Ss5MaT1
Pf5MaT
Vh3MAT1
Lp3MAT1
NtMAT1
Gt5AT
I
Sc3MaT
Dm3MAT2
Dm3MAT1
Dv3MAT
MtMaT1
MtMaT2
MtMaT4
MtMaT3
At5MaT
GmIF7MaT
MtMaT6
PsSalAT
CaPun1
CrMAT
CrDAT
CmAAT4
FaSAAT
FvVAAT
RhAAT1
RsVS
III
NaDH29
CbBEAT
VvAnAT
Ss5MaT2
AtSHT
DcHCBT
CcHQT2
CcHQT1
CsHQT
CcHQT
IbHCBT
NtHQT
SlHQT
IV
NtHCT
CcHCT
AtHCT
AsHHT1
HvACT
NaAT1
MsBanAAT
AtSCT
AtSDT
TcDBNTBT
TcBABT
TcTAT
TcDBAT
TcDBBT
AtCHAT
LaHMT/HLT
CmAAT3
PhBPBT
NtBEBT
CbBEBT
CmAAT2
CmAAT1
VlAMAT
MpAAT1
AtDCF
AtHHT
V
0.2

Fig 2) A phylogenetic tree of characterised plant acyltransferases belonging to the BAHD protein family, including the putative anthocyanin acyltransferase sequence from Vitis vinifera (VvAnAT). Protein sequences were aligned using ClustalW and manually edited to remove non-conserved regions. A phylogenetic tree and the posterior probability of the nodes were generated using Bayesian Inference within BEAST v1.7.5. The clades have been named based on the groupings discussed in D'Auria (2006).

Protein sequence and gene expression patterns of *VvAnAT* explain the absence of acylated anthocyanins in Pinot Noir

Pinot Noir does not synthesise acylated anthocyanins (Van Buren et al., 1968) and represents a mutant that can be compared to wild type cultivars. Sequence analysis of cDNA clones from Pinot Noir suggested this cultivar is homozygous at the locus containing *VvAnAT* (*VvAnAT-PN)* but contained two SNPs compared to the Cabernet Sauvignon cDNA clones. The first of these is a C/T polymorphism at position 349 bp of the coding region which introduces a premature stop codon resulting in a truncated protein of 117 aa. An alignment of VvAnAT-CS and VvAnAT-PN against previously characterized BAHD proteins showed that the predicted VvAnAT-PN protein did not contain either of the functional motifs, HXXXDG and DFGWG, found in almost all members of the BAHD family (St-Pierre and Luca, 2000), most likely rendering it inactive (Figure 3).

The transcript level of *VvAnAT* was analysed using qPCR over the development of whole berries from Cabernet Sauvignon and Pinot Noir cultivars starting from 2 weeks post flowering (wpf) through to harvest at 14 wpf (Figure 4a). This was compared to the expression of *VvUFGT* as an indicator of anthocyanin biosynthesis (Figure 4b). Veraison is defined as the onset of ripening in a developing berry and it is after this point that anthocyanins begin to accumulate. *VvUFGT* expression was activated post-veraison in both Cabernet Sauvignon and Pinot Noir as expected (Boss et al., 1996a). *VvAnAT* gene expression followed a very similar pattern to *VvUFGT* in the Cabernet Sauvignon cultivar suggesting a link between the function and regulation of these two genes. In comparison, *VvAnAT* gene expression in Pinot Noir was low throughout development with only a slight increase post-veraison and was not similar to *VvUFGT* (Figure 4a).

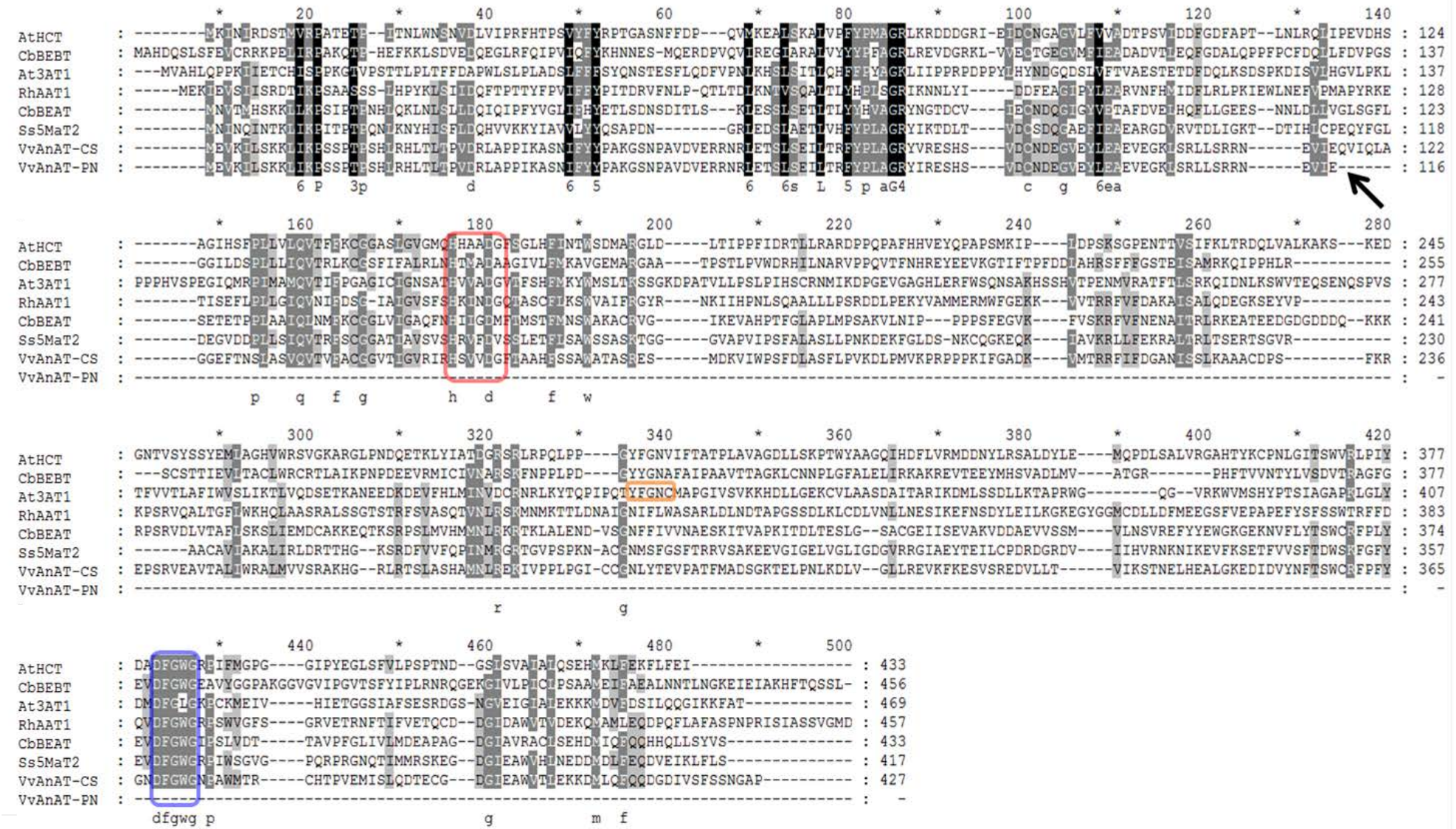

AtHCT
CbBEBT
At3AT1
RhAAT1
CbBEAT
Ss5MaT2
VvAnAT-CS
VvAnAT-PN

Fig 3) Amino acid sequence alignment of VvAnAT from Cabernet Sauvignon (VvAnAT-CS) and Pinot Noir (VvAnAT-PN) with previously characterised BAHD proteins. Black arrow indicates a nonsense mutation in VvAnAT-PN resulting in a truncated protein that does not contain either of the BAHD family functional motifs HXXXDG (boxed in red) and DFGWG (boxed in blue). The YFGNC motif common to most anthocyanin acyltransferases in clade I is boxed in orange. Other genes, species and accession numbers are as follows: *Arabidopsis thaliana* hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (AtHCT, NP_199704), *Clarkia breweri* benzoyl coenzyme A: benzyl alcohol benzoyl transferase (CbBEBT, AAN09796), *Arabidopsis thaliana* coumaroyl-CoA:anthocyanidin 3-*O*-glucoside-6"-*O*-coumaroyltransferase 1 (At3AT1, NP_171890), *Rosa hybrid cultivar* acetyl CoA geraniol/citronellol acetyltransferase (RhAAT1, AAW31948), *Clarkia breweri* acetyl CoA: benzylalcohol acetyltransferase (CbBEAT, AAN09796), and *Salvia splendens* malonyl CoA:anthocyanin 5-*O*-glucoside-6"-*O*-malonyltransferase (Ss5MaT2, AAR26385)

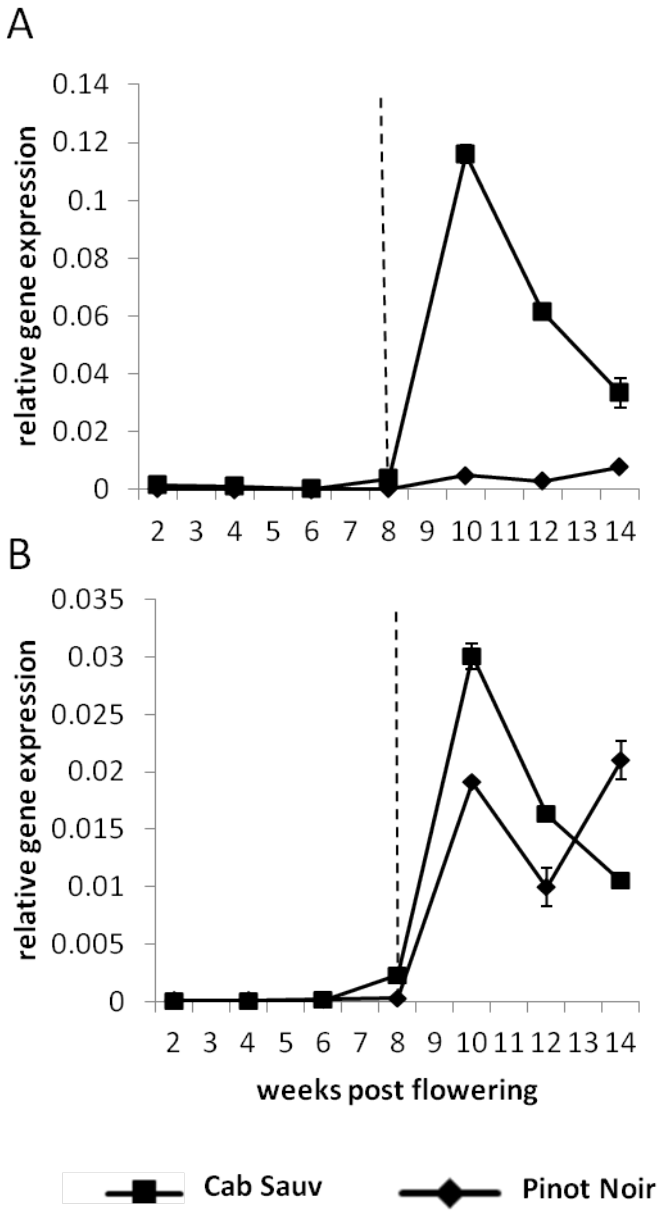


Fig 4) Relative gene expression of **A)** *VvAnAT* and **B)** *VvUFGT* genes throughout grape berry development in Cabernet Sauvignon (Cab Sauv) and Pinot Noir. The vertical dashed line represents the onset of ripening (veraison). Gene expression was determined by quantitative PCR and is shown relative to the the average expression levels of three housekeeping genes *VvUbiquitin, VvActin2* and *VvEF1α-2*. All data is presented as a mean of three technical replicates with standard error bars.

The grapevine colour regulator VvMYBA1 activates the expression of *VvAnAT* in Chardonnay suspension cells

Two colour sports of Cabernet Sauvignon (Malian and Shalistin) have arisen due to a deletion of the berry colour locus carrying the *VvMYBA* genes (Walker et al., 2006). In Malian this deletion has occurred in the L2 cell layer resulting in bronze/rose coloured berries, while in Shalistin the deletion has extended to the L1 cell layer resulting in white berries. To further investigate the link between *VvAnAT* gene expression and VvMYBA transcription factors, transcript levels of *VvAnAT* and *VvUFGT* were analysed over early (whole berries) and late (skins only) berry development of Cabernet Sauvignon, Malian and Shalistin varieties (Figure 5). The expression pattern of *VvAnAT* was very similar to that of *VvUFGT*. In both cases gene expression was activated after veraison in Cabernet Sauvignon and Malian, but transcript levels were lower in Malian. No expression of either *VvAnAT* or *VvUFGT* was detected in Shalistin. It has been previously shown that *VvUFGT* transcription is activated by the VvMYBA1 transcription factor (Walker et al., 2007). This would explain the expression pattern of *VvUFGT* seen here, as Malian only expresses *VvMYBA* in the L1 cell layers of the berry skin and Shalistin does not express it at all (Walker et al., 2006). These results demonstrate a strong link between the presence of the VvMYBA transcription factors and *VvAnAT* gene expression.

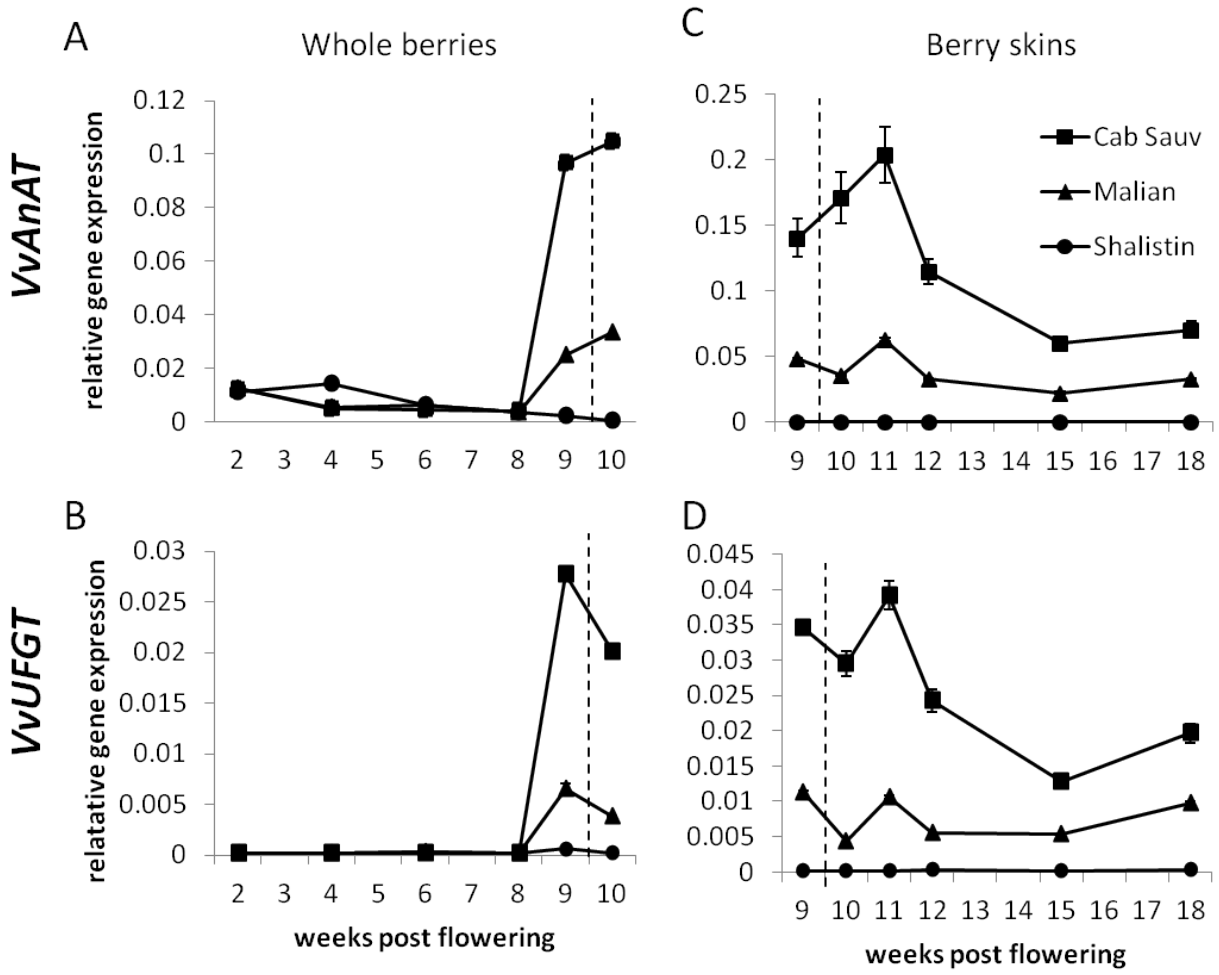


Fig 5) Relative gene expression of *VvAnAT* (**A and C)** and *VvUFGT* (**B and D)** genes throughout the development of Cabernet Sauvignon (Cab Sauv), Malian and Shalistin berries. Whole berries were used between 2 – 10 weeks post flowering (**A and B)** and skin samples were assayed from 9 - 18 weeks post flowering (**C and D)** Veraison occurred between 9 and 10 wpf (dashed line). Gene expression was determined by quantitative PCR and is shown relative to the the average expression levels of three housekeeping genes *VvUbiquitin*, *VvActin2* and *VvEF1α-2*. All data is presented as a mean of three technical replicates with standard error bars.

Transient, promoter-binding luciferase activity assays were performed to determine if VvMYBA1 can activate expression from the *VvAnAT* promoter. Genomic DNA 711 bp upstream of the putative protein coding start site of the *VvAnAT* gene was cloned from Cabernet Sauvignon and Pinot Noir. These promoter regions differed by 6 point mutations which were all located more than 400 bp upstream of the predicted start codon. *VvUFGT*, *VvAnAT-CS* and *VvAnAT-PN* promoters upstream of a luciferase reporter gene (Horstmann et al., 2004) were co-bombarded into grapevine cell suspension cultures with constructs expressing the VvMYBA1 and bHLH transcription factors. Luciferase activity in cells bombarded with the *VvUFGT* promoter construct and VvMYBA1 was 200-fold higher than the activity in control cells (no VvMYBA1 construct present). The luciferase activity in the grape cells when the promoters of *VvAnAT-CS* and *VvAnAT-PN* were used was 50-fold higher than background controls (Figure 6). This indicates that VvMYBA1 is capable of activating expression from both the Cabernet Sauvignon and Pinot Noir promoters of *VvAnAT* and suggests that it can activate the expression of this gene *in planta*.

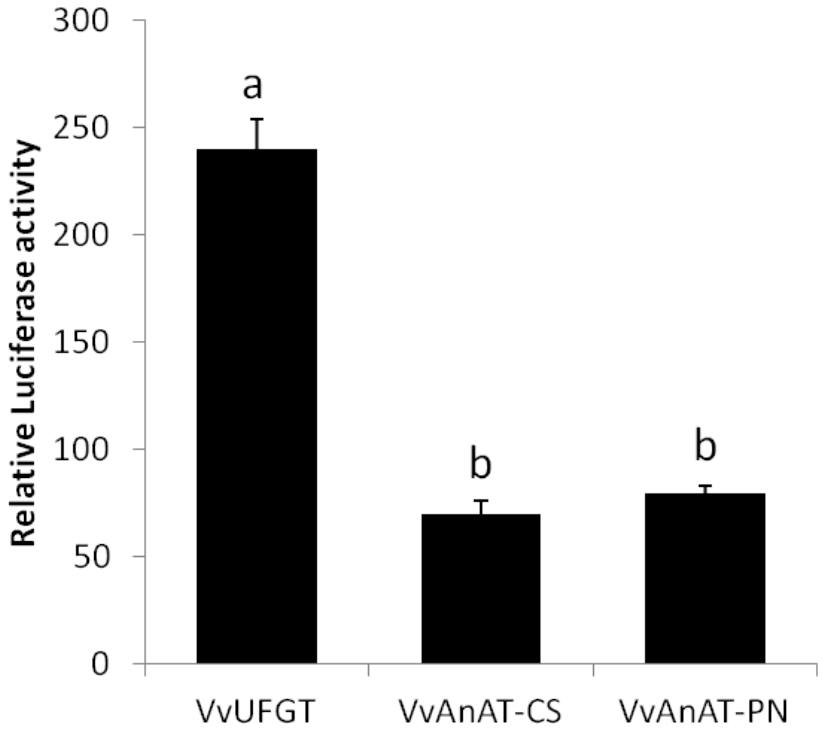


Fig 6) Transcriptional activation of *VvUFGT, VvAnAT-CS* and *VvAnAT-PN* gene promoters by VvMYBA1. Luciferase activity was measured 48 hours after bombardment of grape suspension culture cells with promoter:pLUC constructs along with 35S:VvMYBA1 in pART7, pFF19:EGL3 and pRluc expression vectors. Activity was divided by background luciferase activity of negative controls lacking the 35S:VvMYBA1 construct and is reported relative to renilla activity. The letters a and b indicate where a difference is statistically significant as determined by a t-test ($p < 0.05$).

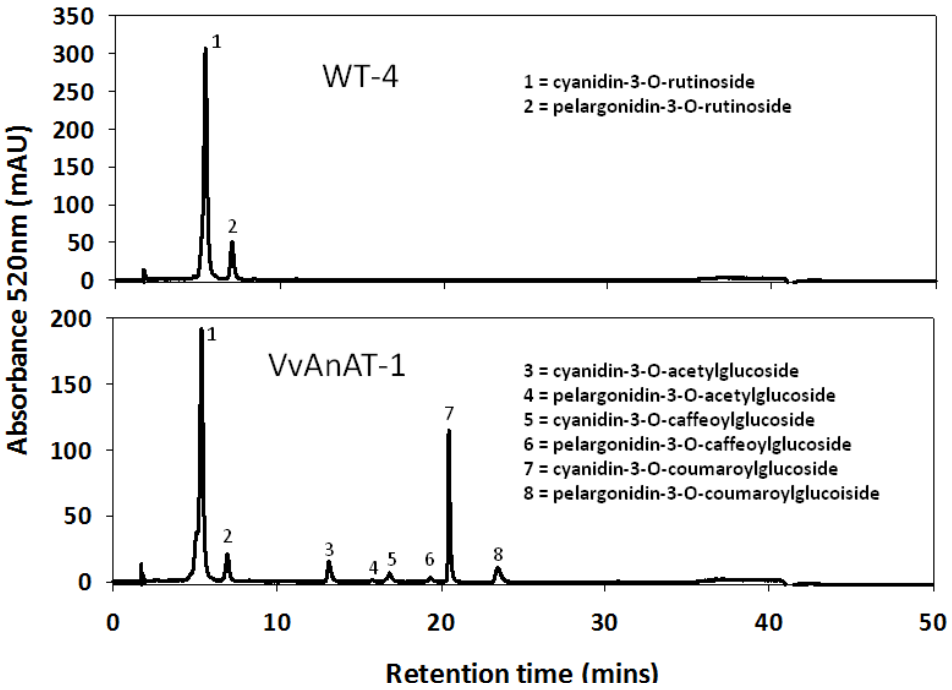
Constitutive expression of *VvAnAT* in tobacco results in the production of acylated anthocyanins

VvAnAT was constitutively expressed under the control of the cauliflower mosaic virus 35S promoter (Gleave, 1992) in *Nicotiana tabacum* var. Samsun through *Agrobacterium*-mediated stable transformation. Some flowers from certain transgenic lines expressing *VvAnAT* displayed a slightly more blue/purple hue compared to WT controls (Figure 7A), but this was not consistent in all transgenic lines, some of which displayed no significant difference to controls. Anthocyanins were extracted from flowers and separated using high-performance liquid chromatography (HPLC) and peaks were identified using liquid chromatography-tandem mass spectrometry (LC-MS/MS). Two anthocyanin species could be detected in the WT tobacco flowers. The most abundant species was cyanidin-3-*O*-rutinoside (peak 1) with lesser amounts of pelargonidin-3-*O*-rutinoside also present (peak 2; Figure 7B and Table S2). Anthocyanin extracts of tobacco flowers constitutively expressing *VvAnAT* contained 6 extra peaks not present in the WT samples. These peaks were identified as cyanidin-3-*O*-acetylglucoside (peak 3), pelargonidin-3-*O*-acetylglucoside (peak 4), cyanidin-3-*O*-caffeoylglucoside (peak 5), pelargonidin-3-*O*-caffeoylglucoside (peak 6), cyanidin-3-*O*-coumaroylglucoside (peak 7) and pelargonidin-3-*O*-coumaroylglucoside (peak 8; Figure 7B and Table S2). Of the acylated anthocyanins, all of the transgenic tobacco lines expressing *VvAnAT* contained more coumaroylated anthocyanins than acetylated anthocyanins with even lesser amounts of caffeoylated anthocyanins (Figure 7C).

A


WT-4
VvAnAT-1

B


WT-4
1 = cyanidin-3-O-rutinoside
2 = pelargonidin-3-O-rutinoside
VvAnAT-1
3 = cyanidin-3-O-acetylglucoside
4 = pelargonidin-3-O-acetylglucoside
5 = cyanidin-3-O-caffeoylglucoside
6 = pelargonidin-3-O-caffeoylglucoside
7 = cyanidin-3-O-coumaroylglucoside
8 = pelargonidin-3-O-coumaroylglucoiside
Absorbance 520nm (mAU)
Retention time (mins)

C

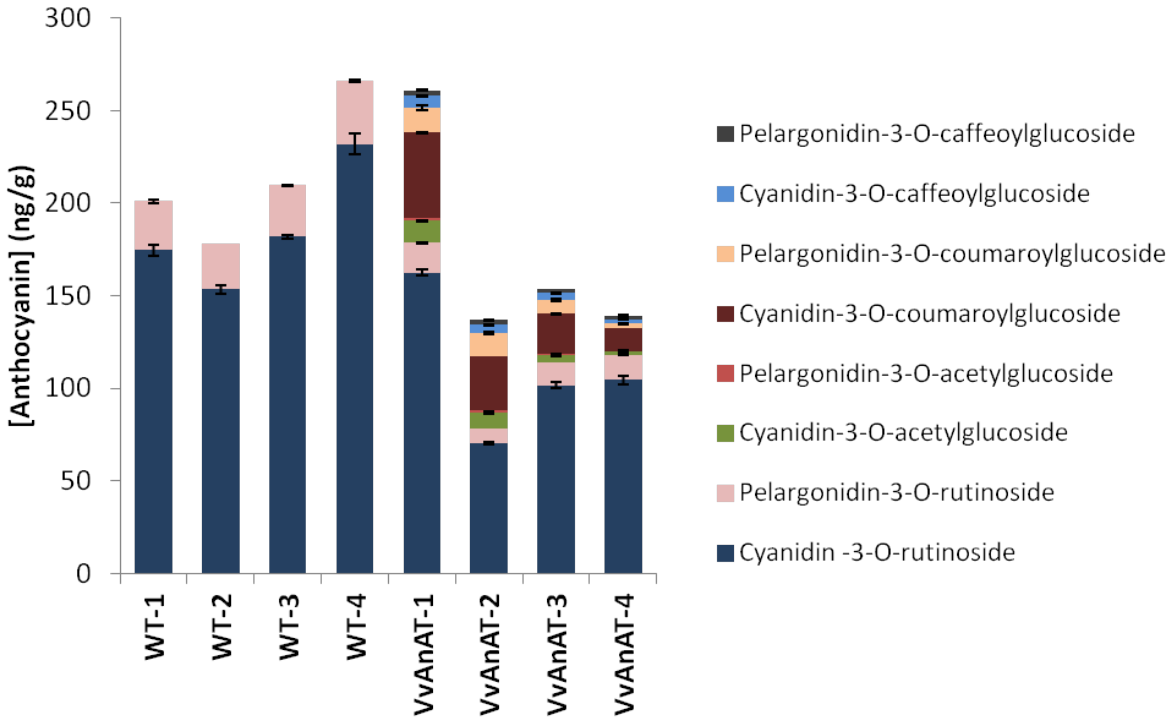

[Anthocyanin] (ng/g)
WT-1
WT-2
WT-3
WT-4
VvAnAT-1
VvAnAT-2
VvAnAT-3
VvAnAT-4
Pelargonidin-3-O-caffeoylglucoside
Cyanidin-3-O-caffeoylglucoside
Pelargonidin-3-O-coumaroylglucoside
Cyanidin-3-O-coumaroylglucoside
Pelargonidin-3-O-acetylglucoside
Cyanidin-3-O-acetylglucoside
Pelargonidin-3-O-rutinoside
Cyanidin -3-O-rutinoside

Fig 7) A) Wild type (WT) and transgenic *Nicotiana tabacum* var. Samsun flowers expressing the *VvAnAT* gene under the 35S promoter (VvAnAT) representing the range of colours found on those plants. B) Chromatogram of anthocyanins in WT and transgenic VvAnAT tobacco flowers. Peak identities were determined using LC/MS/MS. C) Total anthocyanin content (ng/g) of WT and transgenic VvAnAT tobacco lines with relative proportion of each type of anthocyanin in that sample.

Recombinant VvAnAT can acylate anthocyanins *in vitro*

In order to study the function of VvAnAT and its substrate preferences *in vitro*, recombinant His-tagged VvAnAT protein was generated and purified using affinity chromatography. The kinetic properties of this enzyme were determined using various acyl donor and acceptor substrates (Table 1). First, various acyl donors were assayed using malvidin-3-*O*-glucoside as the anthocyanin acyl acceptor, as this is the predominant anthocyanin found in the berry skins of grapevine (Mazza and Francis, 1995). As acetyl-, coumaroyl- and caffeoyl- conjugated anthocyanins are present in grapevine, VvAnAT activity was tested using acetyl-CoA, coumaroyl-CoA and caffeoyl-CoA donors. It was found that VvAnAT was capable of using all three of these acyl donors in transferase reactions. The lowest K_m was observed when coumaroyl-CoA was the acyl donor (1.4 μM), while the enzyme had the lowest affinity for acetyl-CoA with a K_m of 10.2 μM. When acetyl-CoA was the acyl donor, a k_{cat} of 1.8 sec^{-1} was observed, which was 9 fold higher than the k_{cat} when both coumaroyl- and caffeoyl-CoA were the acyl donors (0.2 sec^{-1}). The specificity constant, taking into account both the K_m and k_{cat} of the substrate, was highest when acetyl-CoA was used in the assays (0.172 μM^{-1} $sec\text{-}^{1}$) followed by coumaroyl-CoA (0.117 μM^{-1} $sec\text{-}^{1}$) then caffeoyl-CoA (0.049 μM^{-1} $sec\text{-}^{1}$). Malonyl-CoA was also tested to see if it could act as substrate for VvAnAT, as many other BAHD anthocyanin acyltransferases use this compound as an acyl donor, including Ss5MAT2, the closest homologue of VvAnAT. While VvAnAT could utilise malonyl-CoA as a substrate, the K_m was calculated at 440.9 μM, which was much larger than the other acyl donors tested, and the specificity constant was approximately 100 fold lower at 0.002 μM^{-1} $sec\text{-}^{1}$. Therefore, VvAnAT can catalyse the acylation of malvidin-3-*O*-glucoside with a range of CoA-conjugated acyl donors.

Table 1 – Kinetics of recombinant VvAnAT enzyme with various acyl donor and acceptor substrates

	K_m (µM)	**k_{cat} (sec^{-1})**	**specificity constant (μM^{-1} sec^{-1})**
Acyl donor[a]			
Acetyl-CoA	10.2	1.8	0.172
Caffeoyl-CoA	4.5	0.2	0.049
Coumaroyl-CoA	1.4	0.2	0.117
Malonyl-CoA	440.9	0.9	0.002
Acyl acceptor[b]			
Malvidin-3-*O*-glucoside	82.9	13.7	0.166
Cyanidin-3-*O*-glucoside	483.1	69.7	0.144
Delphinidin-3-*O*-glucoside	738.8	84.6	0.115
Peonidin-3-*O*-glucoside	88.3	15.7	0.178
Cyanidin-3,5-*O*-diglucoside	59.6	0.2	0.003
Cyanidin-3-*O*-rutinoside	NA[c]	NA[c]	NA[c]

[a] These reactions were carried out using malvidin 3-glucoside as an acyl acceptor.
[b] These reactions were carried out using acetyl CoA as an acyl donor.
[c] NA, no activity.

When coumaroyl-CoA and caffeoyl-CoA were used as acyl donors in concentrations above 100 μM, it was found that they could inhibit the production of the expected acylated anthocyanin. No substrate inhibition was observed when using acetyl-CoA in concentrations up to 1 mM (Figure 8). For this reason, VvAnAT enzyme kinetics using various anthocyanin acyl acceptors was conducted using acetyl-CoA as the acyl donor.

The 3-*O*-glucosides of malvidin, cyanidin, delphinidin, peonidin and petunidin are the five major anthocyanin species found in grapes (He et al., 2010). The activity of VvAnAT was assayed using all of these anthocyanins as acyl acceptors except petunidin-3-*O*-glucoside as it is not commercially available. The kinetic parameters of VvAnAT when malvidin- and peonidin-3-*O*-glucosides were used as substrates were very similar with K_ms of 82.9 μM and 88.3 μM, k_{cat} values of 13.7 sec^{-1} and 15.7 sec^{-1}, and specificity constants of 0.166 μM^{-1} sec^{-1} and 0.178 μM^{-1} sec^{-1} respectively. K_m values calculated with cyanidin-3-*O*-glucoside and delphinidin-3-0-glucoside acyl acceptors were much higher at 483.1 μM and 738.8 μM respectively, but the k_{cat} values were also higher at 69.7 sec^{-1} and 84.6 sec^{-1}, meaning the specificity constants of 0.144 μM^{-1} sec^{-1} and 0.115 μM^{-1} sec^{-1} respectively, were not that dissimilar to those of malvidin- and peonidin-3-0-glucosides. To see if VvAnAT would also acylate anthocyanins with other glycosylation patterns, activity using cyanidin-3,5-*O*-diglucoside and cyanidin-3-*O*-rutinoside as acyl acceptors was also tested. VvAnAT was capable of acylating cyanidin-3,5-*O*-diglucoside with a K_m of 59.6 μM, a k_{cat} of 0.2 sec^{-1} and a specificity constant of 0.003 μM^{-1} sec^{-1}, the latter two being much lower than those calculated when the monoglucoside anthocyanins were used as substrates. Recombinant VvAnAT showed no activity with cyanidin-3-*O*-rutinoside.

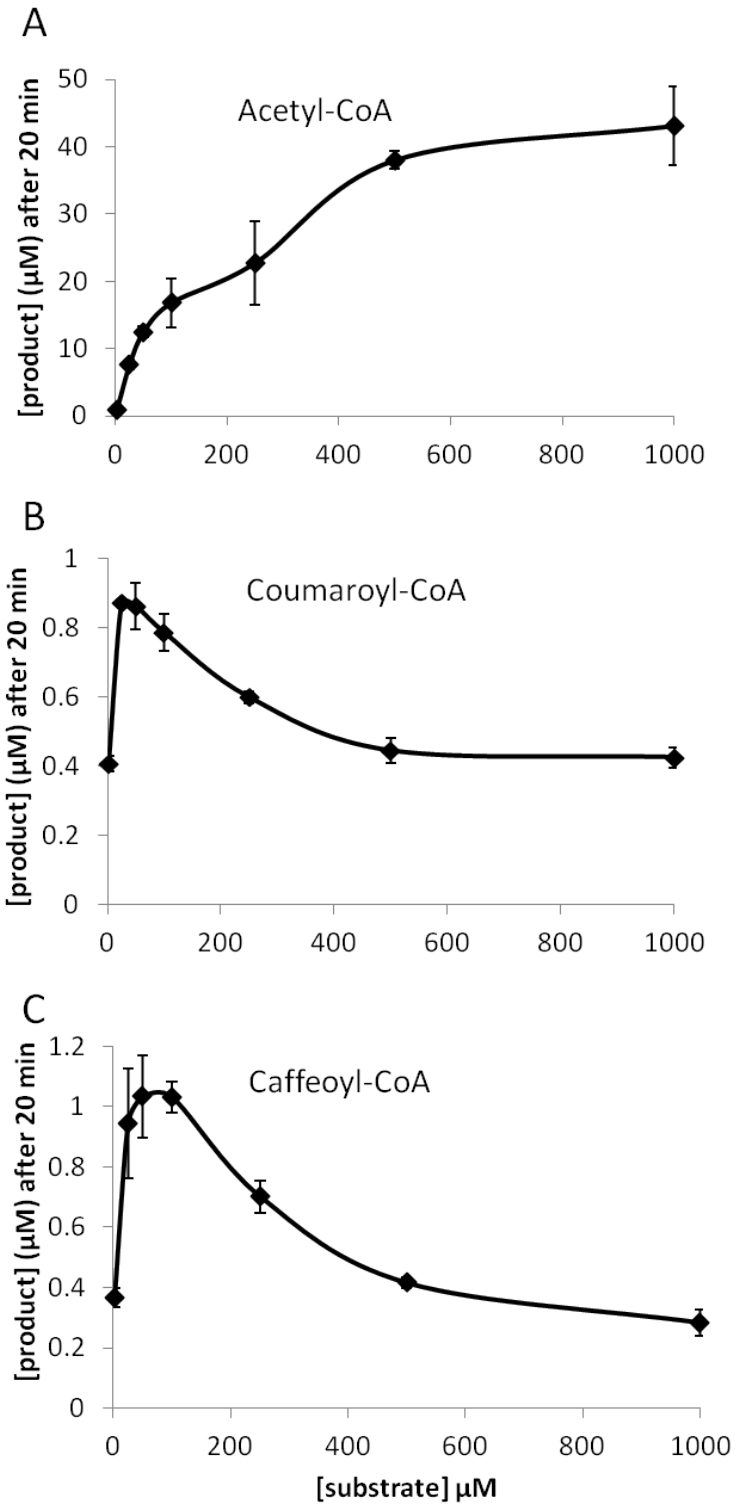


Fig 8) Activity of VvAnAT protein using malvidin-3-*O*-glucoside (200 µM) as the acyl acceptor with **A)** acetyl-CoA **B)** coumaroyl-CoA and **C)** caffeoyl-CoA as acyl donors in *in vitro* bioassays. High concentrations of coumaroyl-CoA and caffeoyl-CoA result in a reduction of product formation, which is not seen with acetyl-CoA.

Discussion

The sequence encoding a putative BAHD anthocyanin acyltransferase was identified in a screen for genes upregulated when active VvMYBA1 was present in grape berries (Rinaldo et. al., in preparation). A comprehensive phylogenetic analysis of characterised BAHD plant acyltransferases, including this protein sequence (VvAnAT), found that the proteins are grouped into five major clades which could be classified predominantly by the substrate specificities of the enzymes (Figure 3 and Table S1). In agreement with previously published phylogenetic trees (D'Auria, 2006; Yu et al., 2009), most of the anthocyanin and flavonoid acyltransferases clustered together in clade I, but VvAnAT was placed into clade III. The majority of enzymes in clade I utilize malonyl-CoA as their major acyl donor while those in clade III mostly utilize acetyl-CoA. This would suggest that the clade III enzymes are grouped based on their acyl donor preference. Malonylated anthocyanins have not been detected in grapevine, while acetylated anthocyanins are common (reviewed in He et al., 2010). However, another anthocyanin acyltransferase in this clade, from *Salvia splendens* (Ss5MAT2), has been shown to utilize malonyl-CoA to acylate anthocyanin-5-*O*-glucosides (Suzuki et al., 2004b). This enzyme lacks the characteristic YFGNC motif common to the anthocyanin transferases present in clade I which is also absent from VvAnAT (Figure 3) and possibly explains its phylogenetic classification into clade III. This suggests that VvAnAT, like Ss5MAT2, has evolved from a different branch of the BAHD family than the other anthocyanin acyltransferases.

Due to the absence of acylated anthocyanins in the grapevine cultivar Pinot Noir (Van Buren et al., 1968), we compared *VvAnAT* gene sequences and transcript levels in this variety with those of Cabernet Sauvignon, which does produce acylated anthocyanins. Very low or undetectable levels of *VvAnAT* transcript were present in Pinot Noir berries compared to fruit from Cabernet Sauvignon, which accumulated the transcript post-veraison peaking at 10 wpf (2 weeks after veraison; Figure 4). Furthermore, any transcript that was expressed in Pinot Noir would contain a premature stop codon, due to a nonsense mutation in the Pinot Noir gene sequence and this would result in a truncated protein lacking two biochemically important motifs, HXXXDG and DFGWG (St-Pierre and Luca, 2000) (Figure 2). This suggests that Pinot Noir does not possess a functional VvAnAT protein. If this is the only functional anthocyanin acyltransferase in *V. vinifera* then these results would explain why this variety does not contain acylated anthocyanins. There are other *V. vinifera* cultivars which do

not produce acylated anthocyanins such as Gamay Beujolais (Fong et al., 1971), Gaglioppo (Lovino et al., 2006), and Tintilia (Mattivi et al., 2006) as well as several grape-related species such as *V. rotundifolia* and *V. amurensis* (He et al., 2010). An analysis of the *VvAnAT* gene sequence and expression in these cultivars and species may help us determine if VvAnAT is the only functional anthocyanin acyltransferase in grapevine.

Transient promoter-binding luciferase gene expression assays using grapevine suspension cells showed that VvMYBA can activate the expression of *VvAnAT*. This result was supported by the pattern of *VvAnAT* transcription compared in berries of Cabernet Sauvignon, Malian and Shalistin that differ in *VvMYBA* gene expression (Figure 5). VvMYBA is known to activate *VvUFGT* transcription (Walker et al., 2007) and the expression of this gene followed almost identical patterns to that of *VvAnAT* in the three varieties. There has been relatively little work done on the regulation of BAHD gene transcription as studies have mostly focused on their enzymatic functions. Onkokesung et al. (2012) identified the putrescine and spermidine acyltransferases, NaAT1 and NaDH29, through microarray studies utilising *N. attenuate* plants where the *NaMYB8* gene, a transcription factor known to regulate phenolamide biosynthesis, was silenced. Strong links between the expression of *NaMYB8*, *NaAT1* and *DH29* were demonstrated suggesting that their expression was controlled by this TF. In another study, Lou et al. (2008) expressed the *AtMYB12* gene in tomato. This TF is known to activate the biosynthesis of chlorogenic acids (CGAs) in *Arabidopsis*, compounds which are derived from the phenylpropanoid pathway. The expression of a number of tomato genes involved in CGA biosynthesis was increased in *AtMYB12* expressing lines including *hydroxycinnamoyl CoA quinate transferase* and *hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase,* two characterised BAHD acyltransferases. Both of these studies suggested links between the expression of BAHD enzymes and MYB TFs, but this is the first study to show transcriptional activation of a BAHD gene through promoter binding assays (Figure 6). Whether VvMYBA directly binds to the promoter of *VvAnAT* to achieve this transcriptional activation, or whether it activates the expression of other TFs that do this, cannot be determined from these assays. Methods that can detect protein-DNA interactions such as DNA Electrophoretic Mobility Shift Assay (EMSA) (Hellman and Fried, 2007) and DNA Pull-down assays (Wu, 2006) could be used to further investigate this.

The expression of both the Cabernet Sauvignon and Pinot Noir *VvAnAT* gene promoters were activated to similar levels by VvMYBA, even though very low gene expression was detected

in Pinot Noir berries post-veraison compared to Cabernet Sauvignon. Premature stop codons in mRNA transcripts can lead to the activation of the nonsense-mediated mRNA decay pathway resulting in the rapid degradation of nonsense transcripts (reviewed in van Hoof and Green, 2006). There is evidence that nonsense-mediated mRNA decay acts on many plant gene mutants including the flavonoid biosynthetic pathway gene *CHS* in petunia (Que et al., 1997). It is possible that the premature stop codon in *VvAnAT-PN* is detected by this pathway resulting in rapid degradation of the transcript, suggesting why only very low amounts of *VvAnAT* mRNA were detected post-veraison in Pinot Noir berries.

The *in planta* function of VvAnAT was tested by constitutive expression in tobacco. A visible comparison of the colour of our WT and transgenic flowers did not reveal any consistent significant differences in the hue of their pigmentation. Biochemical analyses showed that WT flowers contained cyanidin and pelargonidin-3-*O*-rutinosides, while the flowers expressing *VvAnAT* also contained cyanidin- and pelargonidin-3-O coumaroyl- caffeoyl- and acetyl- monoglucosides (Figure 7). No acylated rutinoside anthocyanin conjugates were detected in the transgenic tobacco flowers, suggesting that *VvAnAT* is not able to acylate such anthocyanins. Rutinose is a disaccharide made up of rhamnose and glucose. It has been shown in various plant species that these two sugar molecules are conjugated to anthocyanins by two different enzymes; a glucosyltransferase which adds a glucose molecule in the first step, and a rhamnosyltransferase which then adds the rhamnose sugar (Aharoni et al., 2001). The results suggest that VvAnAT has affinity to monoglucoside anthocyanin molecules and hence competes with the rhamnosyltransferase for these substrates. This preference was also reported by Luo et al. (2007) who expressed the *Arabidopsis coumaroyl-CoA:anthocyanidin 3-O-glucoside-6''-O-coumaroyltransferase 1* and *2* (*At3AT1* and *At3AT2*) genes in tobacco. They also found that there was no visible significant difference in transgenic flower colour compared to their controls (Luo et al., 2007).

Substrate preference and VvAnAT enzyme kinetics were studied using *in vitro* bioassays with His-tagged recombinant VvAnAT protein. The activity of this enzyme was tested with four anthocyanin acyl acceptor molecules found in grapevine and the three most common acyl donors (acetyl-CoA, coumaroyl-CoA and caffeoyl-CoA). VvAnAT could catalyse acylation with all of these substrates and specificity constants were similar for all, falling between 0.115 and- 0.178 μM^{-1} sec^{-1}, except for caffeoyl-CoA which had a greater than 2 fold lower specificity constant of 0.049 μM^{-1} sec^{-1}. In a recent study of 34 *V. vinifera* genotypes it was

shown that the majority of these cultivars contained much less caffeoylated anthocyanins compared to their acetylated and coumaroylated counterparts (Ferrandino et al., 2012). This agrees with the kinetic properties of the VvAnAT enzyme assayed in this study (Table 1). Ferrandino et al. (2012) found varying proportions of acetylated and coumaroylated anthocyanins in the fruit of different grapevine genotypes, but in general they were present in similar concentrations or there was a higher proportion of coumaroylated pigments. One exception to this was Cabernet Sauvignon which contained greater than four-fold more acetylated anthocyanins than coumaroylated anthocyanins. The VvAnAT clone used for the kinetic studies originated from Cabernet Sauvignon, but it was only slightly more efficient when using acetyl-CoA as the acyl donor compared to coumaroyl-CoA (specificity constants of 0.172 and 0.117 μM^{-1} sec^{-1} respectively). It had a lower affinity for acetyl-CoA than coumaroyl-CoA (Table 1). Considering this, the higher concentration of acetylated anthocyanins in Cabernet Sauvignon is probably a consequence of the availability of CoA substrates, rather than the substrate preferences of the acyltransferase. The levels of the various acyl-CoA compounds in berries have not been compared between grapevine cultivars, but as these compounds act as substrates in numerous reactions, in many metabolic pathways, slight differences in cell metabolism between the cultivars would probably affect the availability of these substrates. The aromatic acyl donors, caffeoyl- and coumaroyl-CoA, were shown to inhibit the production of the corresponding acylated anthocyanin by VvAnAT at concentrations > 100 μM, which was not seen when using the aliphatic acyl donor acetyl-CoA. It is possible that the aromatic ring present in caffeoyl- and coumaroyl-CoA could be interfering with VvAnAT enzyme function when present in high concentrations, or perhaps the acyl donors can also act as acceptors given they have free hydroxyl groups. This suggests that *in planta* substrate availability may have a two-fold effect on the types of acylated anthocyanins found in a particular cultivar, as aromatic acyl donor concentrations both too low and too high will have a negative effect on the occurrence of that type of acylation event. An association study between *VvAnAT* gene sequences and the proportions of acylated anthocyanins in different grape cultivars would determine if these differences are due to mutations in VvAnAT that alter enzyme kinetics or are a consequence of differing substrate availability.

VvAnAT activity was also tested with substrates that other BAHD protein family members utilize. Most anthocyanin acyltransferases characterised utilize malonyl-CoA as the major acyl donor (Figure 3 and Table S1). VvAnAT can use malonyl-CoA as an acyl donor, but it

had lower affinity for this substrate than the other acyl donors tested, with a K_m of 440.9 μM and specificity constant of 0.002 μM^{-1} sec^{-1} (Table 1). The higher affinity of VvAnAT for acetyl-, coumaroyl- and caffeoyl-CoA substrates compared to malonyl-CoA may explain the lack of malonylated anthocyanins in grapevine, although the availability of this acyl donor may also play a role. VvAnAT could also acylate cyanidin-3,5-*O*-diglucoside but with much less efficiency compared to the monoglucosides tested (Table 1). This preference for a particular glycosylation pattern has been reported before. For example, the *Arabidopsis* anthocyanin acyltransferases At3AT1 and At3AT2 had over 100 fold lower k_{cat} values when diglucosides were used as substrates compared to monoglucosides (Luo et al., 2007) and anthocyanin malonyltransferases from chrysanthemum (Dendranthema x morifolium; Suzuki et al., 2004a) and *Dahlia variabilis* (Suzuki et al., 2002) could not acylate pelargonidin-3,5-*O*-diglucoside. Recombinant VvAnAT could not acylate cyanidin-3-*O*-rutinoside *in vitro*, in agreement with the observations of transgenic tobacco flowers constitutively expressing *VvAnAT* (Figure 7).

We have identified and characterised the *VvAnAT* gene from *V. vinifera* and shown that it acts as an anthocyanin acyltransferase *in planta* and *in vitro*. This enzyme has a preference for monoglucoside anthocyanin molecules and can use a range of Co-A thioesters as substrates. It is capable of synthesising all acylated anthocyanins identified in grapevines. The study also shows that *VvAnAT* gene expression is activated through the action of a MYB transcription factor which adds to our current understanding of the anthocyanin biosynthesis pathway and how it is regulated. The Pinot Noir cultivar does not produce acylated anthocyanins as it contains a mutated version of this gene. This research will assist breeding programs aimed at producing varieties with good potential for stable red wine colour.

Experimental procedures:

Plant Material

Berry samples of Cabernet Sauvignon, Malian and Shalistin varieties were collected from grapevines grown in a commercial vineyard at Langhorne Creek, South Australia (35° 17’ 30” South, 139° 2’ 33” East) in the season of 2010/11. Samples were collected at 2, 4, 6, 8, 9, 10, 12, 15 and 18 wpf. Approximately 5 bunches were randomly selected from the same 20 vines

(per variety) and berries from these were pooled and frozen in liquid N_2. Veraison occurred at 9 wpf.

Cabernet Sauvignon and Pinot Noir berry samples were collected during the 2007/08 season from a commercial vineyard in Slate Creek, Willunga, South Australia (35^o 15' South, 138^o 33' East) as previously described (Dunlevy et al., 2013).

Young leaves for DNA extraction were collected from Coombe Vineyard, University of Adelaide, Urrbrae, South Australia (34^o 56' South, 138^o 36' East) as previously described (Walker et al., 2007).

Nucleic Acid extractions and cDNA synthesis

RNA extractions, using 50 – 100 mg of powder from frozen whole berries of Cabernet Sauvignon, Malian and Shalistin, and tobacco flowers, were carried out with a Spectrum™ Plant Total RNA Kit (Sigma-Aldrich, MO, USA) and On-column DNase I Digestion Kit (Sigma-Aldrich) according to the manufacturer's instructions.

RNA was extracted from deseeded berries of the Cabernet Sauvignon and Pinot Noir developmental series using a perchlorate method described in Boss et al. (2001). DNA was removed from the samples using RNAse-free DNAse (Qiagen, Nimburg, Netherlands) in conjunction with the RNeasy Mini kit (Qiagen) according to their protocols.

A NanoDrop® 1000 spectrophotometer (V3.7.1, Thermo Fisher Scientific) was used to determine RNA quantity and ensure absorbance ratios (A260/280) were between 1.8 and 2.0. RNA integrity was analysed by agarose gel electrophoresis to assess the presence of intact ribosomal bands.

The Phusion ® RT-PCR Kit (Finnzymes, Massachusetts, USA) was used to synthesize cDNA from Cabernet Sauvignon, Malian, Shalistin and tobacco flower RNA samples according to their instructions. Superscript® III Reverse Transcriptase (Invitrogen, CA, USA) and an oligo$(dT)_{20}$ primer was used to synthesise cDNA from berries of the Cabernet Sauvignon and Pinot Noir developmental series according to manufacturer's instructions.

DNA was extracted from young leaves of Cabernet Sauvignon and Pinot Noir vines as described by Walker et al. (2007) and from young transgenic tobacco leaves using the

ISOLATE Plant DNA mini kit (Bioline, London, UK) according to manufacturer's instructions.

Analysis of gene expression

Specific primers were designed to amplify 100 - 300 bp products from *VvAnAT* and reporter genes (Table S3). The specificity of each primer pair was confirmed by PCR, sequencing, and detection of a single peak of fluorescence from melt curves during qPCR. cDNA was diluted 1:40 in sterile Nanopure® water (Thermo Fisher Scientific, Massachusetts, USA) before use. qPCR experiments were conducted using a LightCycler® 480 II instrument (Roche, Penzburg, Germany). Each sample was assayed in triplicate in a reaction volume of 15 µl made up of 5 µl of diluted cDNA and 0.5 µM of each primer (Table S3) in 1x LightCycler® 480 SYBR Green I Master Mix (Roche). Thermocycling conditions were as follows: initial activation at 95^{o}C for 5 mins followed by 45 cycles of 95^{o}C for 20 sec, 58^{o}C for 20s and 72^{o}C for 20s, then final extension at 72^{o}C for 5 mins. Reactions were then heated to 95^{o}C for 5 mins, cooled to 50^{o}C for 45s then heated to 95^{o}C at a 0.11^{o}C/sec ramping rate to produce melt curves. For each gene, standard curves were produced from a linear dilution series of target DNA fragments created by PCR. Mean Cp values (cycle threshold values) were plotted against DNA concentration and this was used to determine the DNA concentration within cDNA samples. These concentrations were normalised against an average value obtained from three housekeeping genes U*biquitin*, *Actin2* and *EF1α-2* (Genbank accessions CF406001, AF369525.1 and TC38276 respectively) and are reported as relative transcript levels.

Isolation of the *VvAnAT* gene from grapevine

Primers flanking the predicted start and stop codons of the VIT_03s0017g00870 gene (Table S3) based on the 12X grapevine genome (V.1 version; http://genomes.cribi.unipd.it/grape/) were used to amplify Cabernet Sauvignon and Pinot Noir gDNA and cDNA clones using Platinum® *Taq* DNA Polymerase (Invitrogen). Cycling conditions were as follows: 95^{o}C for 5 mins followed by 35 cycles of 95^{o}C for 30 sec, 55^{o}C for 30s and 72^{o}C for 2:30 mins, then final extension at 72^{o}C for 10 mins. Fragments were purified using agarose gel electrophoresis and the QIAEX II Gel Extraction Kit (Qiagen), ligated to pDrive (Qiagen) and sequenced.

Sequence analysis and phylogenetic tree construction

Nucleotide and protein sequence alignments were carried out using AlignX (a component of Vector NTI Advance 11.0, Invitrogen) except for the alignment used in the phylogenetic analysis for which a ClustalW alignment was used. In this case, the final sequence alignment was generated by manually editing a ClustalW alignment to select for conserved positions (Larkin et al., 2007). Phylogenies were constructed using the BEAST (Bayesian Evolutionary Analysis by Sampling Trees) v1.7.5 package (Ayres et al., 2012; Drummond et al., 2012). The WAG (Whelan and Goldman) substitution model with gamma + invariant site heterogeneity was used with a strict molecular clock. A random starting tree was used with the Yule Process tree prior for determining speciation. A MCMC (Markov chain Monte Carlo) maximum chain length of 50 million was set and the tree was run until completion. Tracer v1.5 was used to ensure convergence by assessing the estimated sample size (ESS) and the likelihood of the estimated parameters. TreeAnnotator v1.7.5 was used to generate the maximum clade credibility tree with a burnin of 20,000. The tree was then annotated using FigTree (Rambaut, 2007). RAxML was used to confirm the structure of the BEAST tree, the ZmGlossy2 and AtCER2 were set as the outgroup (Stamatakis et al., 2008).

Production of genetically modified tobacco containing the VvAnAT gene

Primers were designed to the *VvAnAT* gene to include an XhoI restriction site immediately 5' of the start codon and an Asp718 site immediately 3' of the stop codon (Table S3). These were used to amplify the gene fragment by PCR, which was then purified, ligated to pDrive and sequenced. The gene was then inserted into the multiple cloning site of the pART7 cloning vector, which sat between a *cauliflower mosaic virus* 35S promoter and an octopine synthase gene (OCE) transcriptional terminator (Gleave, 1992), using the XhoI and Asp718 restriction sites. This expression cassette was excised from pART7 using a NotI restriction enzyme and ligated to the plant expression vector pART27 (Gleave, 1992) to create the 35S:VvAnAT construct.

Agrobacterium tumefaciens strain LBA4404 containing the 35S:VvAnAT construct was used to transform *N. tabacum* var. Samsun. This was grown on LB containing Bacto^{TM} Agar (Jomar Bioscience), 25 µg/ml rifampicin, 50 µg/ml spectinomycin and 200 µM acetosyringone at 28^{o}C for 4 days. Bacteria were resuspended in 30 ml of Murashige and Skoog (MS) medium (1x MS salts and 1x Gamborg's vitamins (*Phyto*Technology

Laboratories®), 30 g/L sucrose) and adjusted to an OD_{600nm} of 0.8 – 1.0. Incisions were made on the underside of tobacco leaves parallel to the midrib and submerged in the MS/*Agrobacterium* mixture for 10 mins before blotting on sterile filter paper and transferring (topside down) to MS plates (MS medium with 5 mg/ml Phytagel™ (Sigma-Aldrich)) containing 1 µM each of α-naphthaleneacetic acid (NAA) and 6-benzylamino purine (BAP) (Sigma). *Agrobacterium* was co-cultivated on leaves for 4 days at 20°C. Leaf pieces were washed in MS medium containing 500 µg/ml cefotaxime, blotted on sterile filter paper, and transferred (underside down) onto MS plates containing 1 µM each of α-naphthaleneacetic acid (NAA) and 6-benzylamino purine (BAP), 500 µg/ml cefotaxime and 100 µg/ml kanamycin. These were kept at 27°C and transferred to fresh medium every 2 weeks. Shoots about 1cm in length were transferred onto MS plates containing 100 µg/ml kanamycin. PCR reactions were used to screen for the transgene using MangoTaq™ DNA polymerase (Bioline) according to manufacturers instructions. Once shoots had rooted and grown to approximately 15-20cm in length they were transferred to soil (20 L composted pine bark, 10 L river sand, 30 g $FeSO_4$, 60 g pH amendment, 140 g longlife osmocote) and hardened off in the glasshouse. They were grown in ambient light, with a night break, during the spring season in South Australia. Day and night temperatures were about 27°C and 22°C respectively.

VvAnAT protein expression and purification

VvAnAT was amplified using primers VvBAHDNotI_F1 and VvBAHDXhoI_R1 (Table S3) and was ligated to the pET30a(+) expression vector, using the XhoI and Asp718 restriction sites, to generate a N-terminal His-VvAnAT fusion protein.

E. coli [pBL21(DE3)] cells were co-transformed with pRIL (Stratagene, La Jolla, CA) and either the pET30a:His-VvAnAT or the empty pET30a. Cultures were used to produce recombinant His-VvAnAT protein according to an auto-induction, high-density culturing method described by Studier (2005). Cells were pelleted, lysed and the lysate was clarified as previously described by Dunlevy et al. (2010). His-tagged protein was purified using His GraviTrap columns (GE Healthcare, Little Chalfont, UK) and washed with resuspension buffer (20 mM sodium phosphate pH 7.4, 500 mM sodium chloride) containing increasing concentrations of imidazole (20, 50, 70, 100, 150, and 200 mM). Recombinant His-VvAnAT protein eluted in the 100 and 150mM imidazole fractions which were pooled then concentrated and buffer was exchanged with 0.1M sodium phosphate buffer pH 6.5 using

Ultracel® - 30K Amicon® centrifugal filters (Millipore). Glycerol was added to the samples to a final concentration of 10%. Samples were then frozen in liquid nitrogen and stored at -80^{o}C. Western blot analysis was carried out as described in Böttcher et al. (2010). The concentration of recombinant protein was determined using a His-Tag Protein ELISA Kit (Cell Biolabs, CA, USA) according to the manufacturer's instructions.

Enzyme assays

Recombinant enzyme assays were conducted in a total volume of 50 µl using 0.1 M sodium phosphate buffer pH 6.5 and containing 0.5 µl of concentrated protein fraction, 5 µl of CoA-conjugated acyl donor dissolved in 0.1 M sodium phosphate buffer and 1 µl of anthocyanin acyl acceptor dissolved in 100 % methanol. When determining K_m, acyl donor and acceptors were maintained at 200 µM while the concentration of the other substrate was varied. Reactions were carried out at 30^{o}C for 20 mins and stopped by the addition of 50 µl of 100 % methanol.

Extraction and detection of anthocyanins

Anthocyanins were extracted from 100 mg aliquots of ground, frozen tobacco flowers with 300 µl of 0.3% formic acid in 70% methanol, sonicated for 20 mins in an ice bath and centrifuged to pellet debris.

Anthocyanins were separated and quantified using a Hewlett Packard 1100 HPLC system with a Wakosil C18 analytical column (3µm, 150mm x 4.6mm, SGE, USA) protected by an C18 guard column (SGE), following the method described by Downey and Rochfort (2008). Anthocyanin concentrations in tobacco extracts were determined by comparison to a standard curve of known cyanidin-3-*O*-rutinoside concentrations. Acylated anthocyanin product concentrations within the recombinant enzyme assays were determined by comparing peak areas to standard curve of known malvidin-3-*O*-glucoside concentrations. Anthocyanin peaks were identified by their MS/MS parent and major daughter ions as determined using the HPLC method as described above coupled to a 6410 triple quad mass spectrometer (Agilent, Santa Clara, CA) using parameters described by Downey and Rochfort (2008). Table S2 summarises the MS parental ions and MS/MS major daughter ion detected for each compound, which were compared to previously reported values (Luo et al., 2007; Downey and Rochfort, 2008).

Luciferase binding assays

Promoter regions consisting of 711 bp upsteam of the putative start codon were amplified from Cabernet Sauvignon and Pinot Noir gDNA using primers VvBAHDPrF2_SacI and VvBAHDPrR1_BglII (Table S3). Products were ligated into the firefly (*Photinus pyralis*) luciferase (*LUC*) plasmid pLUC (Horstmann et al., 2004) using the SacI and BglII restriction sites. Transient transfection of Chardonnay suspension cultures and luciferase assays were carried out as described by Harris et al. (2013). All transfections were done in triplicate.

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Chapter 6: Conclusions and future perspectives

6. Conclusions and Future Perspectives

Anthocyanins are important secondary metabolites found in plants, which posses a number of biological functions *in planta* (see section 1.4.1). They have been shown to have numerous benefits to human health, and have commercial importance to food and beverage industries due to their pigmentation properties (He and Giusti, 2010). There has been a large body of research aimed to understand how these compounds are synthesised. While much is now understood about the genes coding the enzymes involved in the anthocyanin biosynthetic pathway many questions regarding the regulation of the pathway are still unanswered. In grapevine the VvMYBA TFs are known to activate anthocyanin biosynthesis in red berries. One aim of the research presented in this thesis was to further investigate the role of VvMYBA TFs in anthocyanin biosynthesis in grapevine. Studies of MYB TFs homologous to VvMYBA (in other plant species) have previously demonstrated correlations between their expression and the production of aromas (see section 1.5). We proposed that VvMYBA may have a role in regulating volatile compound production, and that the resulting differences in flavour and aroma precursors present in red and white grapes would potentially contribute to the flavour differences of red and white wines. Testing this hypothesis was a second aim of this research. To meet these aims natural mutant and transgenic grapevines with altered *VvMYBA* gene expression, and hence colour, were used in an integrative approach where transcriptomes of colour mutant berries and volatile compounds present in wines made from these berries were analysed. Molecular characterisation of novel genes potentially involved in anthocyanin modification was carried out and a negative correlation between anthocyanin accumulation and the presence of important wine flavour compounds was investigated.

6.1 VvMYBA regulates the later stages of anthocyanin biosynthesis, modification and transport

From an analysis of the transcriptomes of transgenic berries (compared to non-transgenic controls), it was found that altering *VvMYBA* gene expression resulted in a large number of transcriptomic changes involving many primary and secondary metabolism pathway genes as well as genes involved in hormone signalling, stress, and defence (see section 2.3.1). This suggests that flux through the anthocyanin biosynthesis pathway can affect global metabolism in plant cells and perhaps illustrates the cross-talk that may occurs between primary and

secondary metabolism pathways. This finding was in contrast to a similar study by Tohge et al. (2005) where *AtPAP1* was overexpressed in *Arabidopsis* plants and their transcriptome and metabolome was analysed. In this case no significant effects on metabolic pathways besides the flavonoid biosynthetic pathway were observed in the overexpressing plants. Yet, in another study, transcriptome analysis of the *Arabidopsis* mutant *pho3* (a mutant that accumulates high levels of sugar and other carbohydrates) revealed that the transcripts of anthocyanin biosynthesis genes were highly upregulated (Lloyd and Zakhleniuk, 2004). This demonstrated a link between primary and secondary metabolism in plants and is in agreement to the findings presented in this thesis.

When the microarray datasets obtained from the individual grapevine cultivars (i.e. Chardonnay and Shiraz) were compared in our study, a more conserved function of VvMYBA was identified. Specifically, VvMYBA regulates the final step of anthocyanin biosynthesis (*VvUFGT*) and the steps following this, including anthocyanin modification (*VvFAOMT*, *VvBAHD*) and transport into the vacuole (*VvGST*, *VvanthoMATE*). None of the genes involved in flavonoid or general phenylpropanoid biosynthesis were consistently down-regulated by VvMYBA in both transgenic 'white' Shiraz and wild-type Chardonnay berries. From this it is concluded that VvMYBA is a positive regulator of the later steps of anthocyanin biosynthesis, following those catalysed by *VvLDOX*, including those genes involved in anthocyanin modification and vacuolar uptake.

These data support previous research reported by Cutanda-Perez et al. (2009) who found that ectopic expression of *VlMYBA1* in grapevine hairy root cultures (obtained from a number of different red and white grapevine cultivars) increased gene expression of *VvUFGT*, *VvGST*, *VvFAOMT*, and *VvanthoMATE1*. In contrast to our study, *VvAnAT* gene expression was not found to be increased in these transgenic hairy root cultures but *VvLDOX* gene expression was. This demonstrates the importance of carrying out functional studies of grapevine genes within the tissues that they are usually expressed: *VvMYBA* is expressed in red berry skin. In previous studies it was shown that VvMYBA activates *VvUFGT* expression (Kobayashi et al., 2002) while in this study we have shown that it also activates *VvAnAT* promoter expression (see Chapter 5). Future work would include verifying if VvMYBA also activates the expression of the genes coding for the other anthocyanin modification enzymes and transporters, and thus could be the sole MYB TF required for activation of the late anthocyanin biosynthesis pathway, or if other MYB TFs are involved.

6.2 Linalool synthesis is reduced in red berries expressing *VvMYBA*

Flavour and aroma compounds were analysed in wines made from berries with altered *VvMYBA* gene expression to study this effect of the TF on flavour metabolism in berries. Wines made from red berries expressing *VvMYBA* (Shiraz, Cabernet Sauvignon and transgenic 'red' Chardonnay) contained lower levels of the monoterpene linalool compared to the corresponding white wines made from the same cultivars [i.e. transgenic 'white Shiraz, Shalistin (a colourless berry natural mutant of Cabernet Sauvignon) and Chardonnay, respectively]. Linalool was the only volatile compound detected by HS-SPME-GC/MS whose abundance was significantly altered in relation to wine colour in a consistent manner across the three cultivars (see section 3.3.1.1). The microarray data did not reveal any genes that were consistently down-regulated in red berries, at the time that they were sampled, which could explain the observed difference in linalool abundance in their wines (Table 2.1). Through gene expression studies, red berries expressing *VvMYBA* were shown to have decreased transcript levels of a linalool synthase gene, *VvCSLinNer*. This difference was greatest at 12 wpf, and by 18wpf, there was no expression of *VvCSLinNer* (Figure 3.5) when the berries were at a similar TSS levels to those used in the microarray studies. This may explain why this gene was not identified as having differential expression in the transgenic berries from the microarray experiments, and illustrates a limitation of this transcriptome study. Due to the microarray experiments being performed on berries close to 'ripeness', only gene expression changes as a consequence of the presence or absence of the VvMYBA TF at this time in berry development would have been detected. Similar transcriptome studies at several other time points over post-veraison berry development may reveal further regulatory roles of VvMYBA in cell metabolism. These results also demonstrate the usefulness of using integrative approaches to answer complex biological questions such as the one posed in this PhD project. By analysing the presence of volatile compounds within the wines we were able to identify differences in berry metabolism due to *VvMYBA* gene expression which were not detected in the microarray analysis.

It is important to note, however, that there were some limitations to the flavour and aroma analysis that was done in this study. A library of 263 previously identified wine volatile compounds was used to screen the GC/MS chromatograms obtained from the wines samples. This means that the abundances of compounds that were not present within this library were not analysed and as such there may have been differences in volatiles which were not

detected. Yet a visual scan of the chromatograms did not reveal any obvious differences in unidentified peaks between the wine colour categories.

6.3 Anthocyanins cause a shading effect in red berries that reduces linalool accumulation

A light-exclusion experiment demonstrated that when Cabernet Sauvignon, Malian and Shalistin berries were shaded during development there were no significant differences in linalool levels between the wines. Furthermore, linalool levels in wines made from the three shaded treatments were similar to levels in wines made from control unshaded Cabernet Sauvignon berries (Figure 3.6). This suggests that anthocyanins impart a shading effect on red berries and that the lower levels of linalool in red wines are most likely due to the accumulation of anthocyanins rather than a direct regulation of linalool synthesis by VvMYBA. Further supporting this hypothesis is the observation that a predicted ELIP gene was consistently down-regulated in red grapes expressing *VvMYBA* genes as determined through the microarray experiments (Table 2.1). ELIP gene expression is controlled by light stress in plants (Adamska, 2001) and has been shown to be specifically triggered by blue and UV-A light (Adamska et al., 1992; Adamska, 1995). It has been suggested that *in vivo*, anthocyanins can attenuate green/yellow and blue light (Manetas et al., 2003), meaning that blue light signalling may be down-regulated in grapes containing these pigments. It is intriguing that the shading effect imparted on red berries by anthocyanins in this study only resulted in a difference in linalool abundances in the wines made from those berries and that other flavour compounds were not also affected. Perhaps this is due to a specific regulation of linalool synthesis by blue light while other wine volatile flavour compounds may not be sensitive to this. To investigate this aspect further, future studies may include an analysis of *VvCSLinNer* gene expression over a developmental series of Cabernet Sauvignon, and its colour sports Malian and Shalistin, in shaded berries (and non-shaded controls) where specific wavelengths of light are excluded.

6.4 Identification of a putative acyltransferase belonging to the SCPL family and a grapevine anthocyanin acyltransferase

A gene belonging to the BAHD acyltransferase family, which is up-regulated in berries expressing *VvMYBA*, was characterised and shown to function as an anthocyanin acyltransferase (*VvAnAT*) (see Chapter 5). This is the first anthocyanin acyltransferase discovered from grapevine and could potentially be the only one, as *in vitro* bioassays using recombinant VvAnAT protein have shown that this enzyme is capable of producing all common acylated anthocyanin species found in berries of *V. vinifera*. Furthermore, Pinot Noir, which lacks acylated anthocyanins, contains a mutated version of this gene that codes for a truncated protein lacking structural features required for BAHD protein function.

Another gene (*VvSCPL1*) belonging to the SCPL family of acyltransferases was also up-regulated in red berries expressing *VvMYBA* (Table 2.1) and we hypothesised that it could also encode be an anthocyanin acyltransferase. From studies aimed to characterise this gene it now seems unlikely that this is its function although due to uncertainty around the correct annotation of this gene no conclusions can be made at present (see Chapter 4). It is possible that VvSCPL1 is involved in PA biosynthesis in the vacuole while VvAnAT acylates anthocyanins in the cytoplasm. Certainly a comparison of the expression changes of *VvAnAT* and *VvSCPL1* in the microarray data suggest that both of these genes are involved in flavonoid synthesis, as their FCs in the transgenic berries with altered *VvMYBA* gene expression were similar to those of other flavonoid related genes. Furthermore the expression profile of *VvSCPL1* pre-veraison matches that expected of genes involved in PA biosynthesis. Yet there are also correlations between *VvSCPL1* expression and anthocyanin biosynthesis, albeit not as clearly as in the case of *VvAnAT* (Figure 4.5 and Chapter 5). Our results strongly suggest that this gene plays some role in flavonoid synthesis or modification. However more research is required to determine the correct annotation of this gene which could then be used to make recombinant protein for *in vitro* functional experiments.

6.5 Implications of the research and future perspectives

Flavonoids are important secondary metabolites in plants, which have an array of biological functions and that have been shown to have many benefits to human health. For this reason understanding how these compounds are synthesised in plants has been a focus of much

research over the years. The research carried out in this PhD has added to this current body of knowledge. In particular it has increased our understanding of MYB TFs and their role in regulating anthocyanin biosynthesis and is the first study to show a MYB protein activating the expression of an anthocyanin acyltransferase gene. This study has also shown that altering metabolic flux through the anthocyanin biosynthesis pathway can have a large effect on other metabolic pathways and is one of the first studies to show this in plants.

The outcomes of this research also have implications to the grape and wine industry. Colour and flavour are two of the most important quality attributes of grapes, and flavonoids are important compounds in that they affect both of these attributes. Further knowledge has been provided on the regulation of flavonoid synthesis in grapevine, specifically showing how anthocyanin synthesis and subsequent modification is negatively correlated to the accumulation of an important wine flavour compound which is particularly prevalent in white wines. This is the first study to illustrate that the metabolism of colour and flavour attributes may be linked in grapes. It is possible that the regulations of other major synthetic pathways are also linked in grapes and this should be considered in future investigations and modification of their biochemical pathways. The identification of the first anthocyanin acyltransferase from grapevine will be useful in future projects aimed to study or increase red wine colour stability. This research has answered questions relating to cultivars that do not contain acylated anthocyanins and from which red wines are made with unstable colour and low concentrations of anthocyanins. Knowledge of this gene, and how it is regulated, could be used in breeding programs aimed at producing new grapevine varieties with potential to produce wines with high red wine colour stability.

Appendices

Appendix A Total soluble sugar in berries using in microarray experiments

Sample	°Brix
A	
Shiraz 1	24.2
Shiraz 2	24.7
Shiraz 3	27
Shiraz 4	19.5
Shiraz VvMYBAsi rose 1	21.9
Shiraz VvMYBAsi rose 2	23.5
Shiraz VvMYBAsi rose 3	22.8
Shiraz VvMYBAsi rose 4	24.1
Shiraz VvMYBAsi white 1	24.2
Shiraz VvMYBAsi white 2	19.2
Shiraz VvMYBAsi white 3	20.4
Shiraz VvMYBAsi white 4	23.1
B	
Chardonnay 1	23.2
Chardonnay 2	25.5
Chardonnay 3	24
Chardonnay 35S:VvMYBA1 1	22
Chardonnay 35S:VvMYBA1 2	24.7
Chardonnay 35S:VvMYBA1 3	22.1
C	
Chardonnay 1	23.2
Chardonnay 2	24.1
Chardonnay 3	24
Chardonnay 35S:VvMYBA1 1	23.1
Chardonnay 35S:VvMYBA1 2	24.2
Chardonnay 35S:VvMYBA1 3	21.7

A – Control (shiraz) and transgenic shiraz expressing a *VvMYBA* silencing construct (MYBAsi) with rose or white berry phenotypes

B and C - Control (Chardonnay) and transgenic Chardonnay expressing the *VvMYBA1* gene under the control of a 35S promoter (35S:VvMYBA1) **B** = berries used to extract RNA from skins only
C = berries used to extract RNA from whole berries

Appendix B Plant ID and transformant lines of berries used in microarray experiments

	Replicate Number	Plant ID	Line
Chardonnay skin (control)	1	#231	Chardonnay 2
	2	#232	Chardonnay 12
	3	#232	Chardonnay 12
‘red’ Chardonnay skin	1	#69	CT10MybAG3D
	2	#75	CT10MybAE1A
	3	#226	CT11MybAF1.1
Chardonnay WB* (control)	1	#98	Chardonnay 1
	2	#231	Chardonnay 2
	3	#232	Chardonnay 12
‘red’ Chardonnay WB*	1	#22	CT10MybAG3B
	2	#69	CT10MybAG3D
	3	#74	CT10MybAG3D
Shiraz (control)	1	#37	Shiraz 1
	2	#38	Shiraz 1
	3	#39	Shiraz 1
	4	#166	Shiraz 2
‘rose’ Shiraz	1	#108	ST14MybA1S1B2(A)
	2	#109	ST14MybA1S1 B2(A)
	3	#110	ST14MybA1S1 B2(A)
	4	#133	ST14MybA1S1 B2(A)
‘white’ Shiraz	1	#193	ST14MybA1S1E1(C)
	2	#194	ST14MybA1S1E1(C)
	3	#196	ST14MybA1S1E1(C)
	4	#196	ST14MybA1S1E1(C)

*WB = Whole berry

Appendix C Genes with altered expression in transgenic 'white' or 'rose' Shiraz separated into gene expression trends 9-12

Trend 9 (Rose > Red > White)		Rose Vs Red (MYBAsi / control)		White Vs Red (MYBAsi / control)		Rose Vs White (MYBAsi / MYBAsi)	
Microarray gene ID	**Annotation/BLAST hits**	**Fold change**	**significant (SAM)**	**Fold change**	**significant (SAM)**	**Fold change**	**significant (SAM)**
VIT_10s0071g00770	aspartic proteinase nepenthesin-1 precursor	1.74	*	-3.62	*	-6.29	*
VIT_14s0068g00600	alpha-expansin 19	1.91	*	-2.58	*	-4.95	*
VIT_04s0044g01230	no hit	1.19	*	-1.55	*	-1.84	*
VIT_18s0001g08550	squalene monooxygenase	1.37	*	-1.24	*	-1.70	*
VIT_16s0100g01280	cytochrome b5 domain-containing protein	1.14	*	-1.20	*	-1.37	*
VIT_05s0020g02610	hemolysin	1.14	*	-1.18	*	-1.34	*
VIT_19s0085g00600	glyceraldehyde-3-phosphate dehydrogenase, cytosolic	1.15	*	-1.11	*	-1.28	*

Trend 10 (White > Red > Rose)		Rose Vs Red (MYBAsi / control)		White Vs Red (MYBAsi / control)		Rose Vs White (MYBAsi / MYBAsi)	
Microarray gene ID	**Annotation/BLAST hits**	**Fold change**	**significant (SAM)**	**Fold change**	**significant (SAM)**	**Fold change**	**significant (SAM)**
VIT_06s0009g01670	forkhead-associated domain-containing protein	-1.99	*	2.19	*	4.37	*
VIT_08s0007g03990	cellulose synthase (CSLA09)	-3.11	*	1.32	*	4.09	*
VIT_08s0007g03990	cellulose synthase (CSLA09)	-3.11	*	1.32	*	4.09	*
VIT_04s0008g03500	ankyrin	-1.62	*	2.20	*	3.55	*
VIT_19s0014g04200	S-locus protein kinase	-1.93	*	1.42	*	2.73	*

Trend 10 (White > Red > Rose) cont...

Microarray gene ID	Annotation/BLAST hits	Rose Vs Red (MYBAsi / control)		White Vs Red (MYBAsi / control)		Rose Vs White (MYBAsi / MYBAsi)	
		Fold change	significant (SAM)	Fold change	significant (SAM)	Fold change	significant (SAM)
VIT_19s0177g00230	no hit	-2.21	*	1.20	*	2.64	*
VIT_03s0132g00070	Arabidopsis thaliana HVA22 homologue A (ATHVA22A)	-1.60	*	1.54	*	2.47	*
VIT_14s0060g00250	no hit	-1.69	*	1.33	*	2.25	*
VIT_06s0004g01130	seven in absentia (SINA2)	-1.68	*	1.14	*	1.92	*
VIT_03s0038g01650	ABA-responsive protein (HVA22a)	-1.43	*	1.28	*	1.82	*
VIT_12s0142g00570	disease resistance protein RGA4	-1.36	*	1.34	*	1.82	*
VIT_09s0002g04260	unknown protein	-1.33	*	1.32	*	1.76	*
VIT_18s0001g10690	BRI1 kinase inhibitor 1 (BKI1)	-1.22	*	1.39	*	1.70	*
VIT_16s0050g01020	protein kinase family	-1.22	*	1.28	*	1.56	*
VIT_15s0048g01810	fructosamine kinase	-1.34	*	1.15	*	1.54	*
VIT_12s0035g00410	disease resistance protein	-1.24	*	1.21	*	1.50	*
VIT_08s0007g01450	IMP dehydrogenase	-1.16	*	1.27	*	1.47	*
VIT_09s0002g08420	unknown	-1.19	*	1.23	*	1.47	*
VIT_06s0004g06100	myb divaricata	-1.27	*	1.09	*	1.38	*
VIT_08s0040g01170	hydroxymethylglutaryl-CoA lyase	-1.13	*	1.22	*	1.38	*

Trend 11 (Red > White > Rose)

Microarray gene ID	Annotation/BLAST hits	Rose Vs Red (MYBAsi / control)		White Vs Red (MYBAsi / control)		Rose Vs White (MYBAsi / MYBAsi)	
		Fold change	significant (SAM)	Fold change	significant (SAM)	Fold change	significant (SAM)
VIT_09s0002g04840	no hit	-5.75	*	-1.92	*	2.99	*

Trend 12 (Rose > White > Red)		Rose Vs Red (MYBAsi / control)		White Vs Red (MYBAsi / control)		Rose Vs White (MYBAsi / MYBAsi)	
Microarray gene ID	**Annotation/BLAST hits**	**Fold change**	**significant (SAM)**	**Fold change**	**significant (SAM)**	**Fold change**	**significant (SAM)**
VIT_16s0100g01000	stilbene synthase 4	-4.10	*	-1.95	*	2.10	*
VIT_18s0001g04120	(-)-germacrene D synthase	9.04	*	2.12	*	-4.27	*
VIT_07s0104g01340	nodulin MtN3 family	6.70	*	2.21	*	-3.03	*
VIT_02s0033g01060	anthraniloyal-CoA: methanol anthraniloyal transferase	4.49	*	1.50	*	-3.00	*
VIT_02s0033g01060	anthraniloyal-CoA: methanol anthraniloyal transferase	4.49	*	1.50	*	-3.00	*
VIT_18s0001g04720	(-)-germacrene D synthase	4.53	*	1.80	*	-2.52	*
VIT_03s0063g00980	blue (type 1) copper domain	5.29	*	2.36	*	-2.24	*
VIT_19s0014g04830	YIP1	3.87	*	1.83	*	-2.11	*
VIT_12s0134g00030	E-beta-ocimene synthase	5.60	*	2.73	*	-2.05	*
VIT_18s0001g13790	CYP83C	1.54	*	1.13	*	-1.37	*
VIT_03s0063g00970	blue (type 1) copper domain	3.27	*	1.62	*	-2.01	*

*'white' and 'rose' Shiraz contained a *VvMYBAsi* construct which completely (white) or partially (rose) silenced the expression of *VvMYBA1/2* genes. Non-transgenic red/black Shiraz berries were used as the control for these experiments. Transcript levels were determined by microarrays

^SAM = Significance Analysis of Microarray. Y indicates that the fold change ratio was significant as determined by a SAM FC = Fold change

Appendix D Sugar concentrations in berries used to make wines

Line	Total Sugar (g/L)
A	
Shiraz 1	241.48
Shiraz 2	234.84
Shiraz 3	228.21
Shiraz 4	171.16
Shiraz MYBAsi rose 1	225.56
Shiraz MYBAsi rose 2	201.01
Shiraz MYBAsi rose 3	217.60
Shiraz MYBAsi rose 4	232.85
Shiraz MYBAsi white 1	220.91
Shiraz MYBAsi white 2	218.26
Shiraz MYBAsi white 3	216.27
Shiraz MYBAsi white 4	219.59
B	
Chardonnay 1	205.65
Chardonnay 2	213.61
Chardonnay 3	211.62
Chardonnay MYBA1Pr:MYBA1 1	193.71
Chardonnay MYBA1Pr:MYBA1 2	197.69
Chardonnay MYBA1Pr:MYBA1 3	185.75
Chardonnay 35S:MYBA1 1	152.58
Chardonnay 35S:MYBA1 2	161.21
Chardonnay 35S:MYBA1 3	180.44
C	
Cabernet Sauvignon 1	204.99
Cabernet Sauvignon 2	202.34
Cabernet Sauvignon 3	204.33
Cabernet Sauvignon 4	204.33
Malian 1	209.63
Malian 2	206.39
Malian 3	216.27
Malian 4	211.62
Shalistin 1	204.99
Shalistin 2	206.32
Shalistin 3	207.64
Shalistin 4	206.32
D	
Cabernet Sauvignon 1	207.64
Cabernet Sauvignon 2	216.27
Cabernet Sauvignon 3	210.98
Cabernet Sauvignon 4	207.64
Malian 1	213.61
Malian 2	218.09
Malian 3	201.67
Malian 4	210.3
Shalistin 1	210.96
Shalistin 2	209.63
Shalistin 3	197.69
Shalistin 4	214.94

A – Control (shiraz) and transgenic shiraz expressing a *VvMYBA* silencing construct (MYBAsi) (see section 1.6) with rose or white berry phenotypes. **B** - Control (Chardonnay) and transgenic Chardonnay expressing the *VvMYBA1* gene under the control of its own promoter (MYBA1Pr:MYBA1) or a 35S promoter (35S:MYBA1) (see section 1.6). **C** – Berries used to make wines made from fermentation with skins, seeds and pomace present during fermentation. **D** - Berries used to make wines made from fermentation of free-run juice only.

Appendix E Anthocyanin concentration in wines

Sample	[Total anthocyanin] (mg/L)
A	
Shiraz 1	310.6
Shiraz 2	265.3
Shiraz 3	223.7
Shiraz 4	231.0
Shiraz MYBAsi rose 1	27.2
Shiraz MYBAsi rose 2	28.1
Shiraz MYBAsi rose 3	27.4
Shiraz MYBAsi rose 4	27.7
Shiraz MYBAsi white 1	6.0
Shiraz MYBAsi white 2	6.6
Shiraz MYBAsi white 3	5.8
Shiraz MYBAsi white 4	6.4
B	
Chardonnay 1	5.6
Chardonnay 2	5.6
Chardonnay 3	5.6
Chardonnay MYBA1Pr:MYBA1 1	46.8
Chardonnay MYBA1Pr:MYBA1 2	30.7
Chardonnay MYBA1Pr:MYBA1 3	96.4
Chardonnay 35S:MYBA1 1	627.0
Chardonnay 35S:MYBA1 2	614.5
`Chardonnay 35S:MYBA1 3	515.5
C	
Cabernet Sauvignon 1	459.8
Cabernet Sauvignon 2	464.5
Cabernet Sauvignon 3	462.8
Cabernet Sauvignon 4	395.6
Malian 1	29.5
Malian 2	29.4
Malian 3	23.5
Malian 4	26.9
Shalistin 1	6.0
Shalistin 2	5.9
Shalistin 3	5.8
Shalistin 4	5.9
D	
Cabernet Sauvignon 1	297.1
Cabernet Sauvignon 2	268.2
Cabernet Sauvignon 3	220.7
Cabernet Sauvignon 4	272.2
Malian 1	7.6
Malian 2	7.2
Malian 3	8.1
Malian 4	10.3
Shalistin 1	6.0
Shalistin 2	6.0
Shalistin 3	6.0
Shalistin 4	6.0

A – Control (shiraz) and transgenic shiraz expressing a *VvMYBA* silencing construct (MYBAsi) with rose or white berry phenotypes (see section 1.6)

B - Control (Chardonnay) and transgenic Chardonnay expressing the *VvMYBA1* gene under the control of its own promoter (MYBA1Pr:MYBA1) or a 35S promoter (35S:MYBA1) (see section 1.6)

C – Wines made with skins, seeds and pomace present during fermentation

D - Wines made from free-run juice only during fermentation

Appendix F Primers used in this study

Primer Name	Purpose	Primer sequence
35SF	PCR screen of tobacco transformants	TTCGCAAGACCCTTCCTCTA
OCS rev	PCR screen of tobacco transformants	GGCGGTAAGGATCTGAGCTA
Vv1.102F2	qPCR of putative P450 gene	TGCCCGCTGGAATGAACAAGGA
Vv1.102R2	qPCR of putative P450 gene	ACTAGGGATCCTCGCCAAAAGGCA
Vv134.3F2	qPCR of *VvbOci*	CGCTTTCAATCTTGCGCGAATTTCC
Vv134.3R2	qPCR of *VvbOci*	TATCAGTGACAGGACTCGCTGCCT
VvLis54_F2	qPCR of *VvCSLinNer*	GGAACACCGAGGCTCTTCAGTT
VvLis54_R2	qPCR of *VvCSLinNer*	GCCAGGAAGGCACTGTTGGTCA
VvCMKqpcrF1*	qPCR of *VvCMK*	CAGCCAATCAATGGTACACCCA
VvCMKqpcrR1*	qPCR of *VvCMK*	TGGCTTGGGCAGCGTTTATG
VvCMSqpcrF1*	qPCR of *VvCMS*	CTTGAGGTCACCGATGATGTGT
VvCMSqpcrR1*	qPCR of *VvCMS*	ACTTCTCTCCAGAGTCGAGATTC
VvDXRqpcrF1*	qPCR of *VvDXR*	GCTTCTCCTTGTTGATGGGAATGA
VvDXRqpcrR1*	qPCR of *VvDXR*	GAATGCGAAATGCATCTACACCTC
VvDXSqpcrF1*	qPCR of *VvDXS*	TACATCAGCTTTTCATTCTCTCAAC
VvDXSqpcrR1*	qPCR of *VvDXS*	GCACTTTCAAGCCTTATTGCTGAC
VvHDSqpcrF1*	qPCR of *VvHDS*	TTGGCAGATTTAGTTGGTTGATGG
VvHDSqpcrR1*	qPCR of *VvHDS*	TTGTCCGGGCATTTACTACATTGA
VvIDSqpcrF1*	qPCR of *VvIDS*	TCCAGGATGCTGAAAGGAGTGA
VvIDSqpcrR1*	qPCR of *VvIDS*	TGGATTTGAACCGTGTTGATGAG
VvMCSqpcrF1*	qPCR of *VvMCS*	CATTAATGTATACAACTTGCGCACAC
VvMCSqpcrR1*	qPCR of *VvMCS*	AGATCAAGCTCCATGAATGTTCTA
VvSCPLfor1	Sequencing of gDNA of VvSCPL1	ACCATGTATAGGAGCTTGCTTCTTG
VvSCPL_F2	Sequencing of gDNA of VvSCPL1	ATGCAAATCGCAGTGGAGACA
VvSCPL_F3	Sequencing of gDNA of VvSCPL1	GGCAAGCTGCAATGGTAAGT
VvSCPL_F6	Sequencing of gDNA of VvSCPL1	CCTACAGTGGTGATCATGACATG
VvSCPL_R2	Sequencing of gDNA of VvSCPL1 / 5'RACE	CAGAGCCAACTGGTGAATCC
VvSCPL_R3	Sequencing of gDNA of VvSCPL1	GGCGGATTGGTGAAAGGAGG

Primer Name	Purpose	Primer sequence
VvSCPL_R4	Sequencing of gDNA of VvSCPL1	CTAGAGCGGATAATAAGCCAAC
VvSCPL_R5	Sequencing of gDNA of VvSCPL1 / 5'RACE	CTGGATGTCATCGGATTTGCCA
VvSCPL_R6	Sequencing of gDNA of VvSCPL1	ACAGTTGCAAATGTCATACCACG
VvSCPLPr_F2SacI	Sequencing of gDNA of VvSCPL1 / promoter ligation into pLUC vector	GAGCTCGACTCCACCTAACCACATAC
VvSCPLPr_R1BglII	Sequencing of gDNA of VvSCPL1 / promoter ligation into pLUC vector	AGATCTGGTTGTGAAGTAGCTACTGC
VvSCPLncbiF1	Determine location of start codon of VvSCPL1	ATGGATGGCCAAGATGCAATC
VvSCPLncbiF2	Determine location of start codon of VvSCPL1	CCCCACTTGCGACCTGTATTTT
VvSCPLncbiF3	Determine location of start codon of VvSCPL1	CATGAGCAACCACCAGTAGGC
VvSCPLncbiR1	Determine location of start codon of VvSCPL1	CCCTGTTTCAAGTTTGAAGGGAAGA
GeneRacerTM 5'Primer	5'RACE of VvSCPL1	CGACTGGAGCACGAGGACACTGA
GeneRacerTM 5'Nested Primer	5'RACE of VvSCPL1	GGACACTGACATGGACTGAAGGAGTA
VvSCPLsplicevarF1	VvSCPL1 splice variant PCR	ATGGCAACGAAGCTGGACAAGAG
VvSCPLsplicevarR1	VvSCPL1 splice variant PCR	TGGTTTGTTGATGGCCAAGTTGCA
VvSCPLXhoIFor2	ligating into plant expression vectors	CTCGAGCAATGTATAGGAGCTTG
VvSCPLEcoRIRev	ligating into plant expression vectors	CAGAATTCCTAGAGCGGATAATAAG
VvSCPLNotIFor	ligating into protein expression vectors	GCGGCCGCATGTATAGGAGCTTG
VvSCPLPrR1	VvSCPL1 promoter ligation into pLUC vector	GGTTGTGAAGTAGCTACTGCCTAC
VvSCPLXhoIRev2	ligating into protein expression vectors	CTCGAGCTAGAGCGGATAATAAGCC
VvSCPLqPCRF1	qPCR of VvSCPL1	AGAGCACCGAGCTCATGGTA
VvSCPLqPCRR1	qPCR of VvSCPL1	TTTCCCTTTCAAGTTGTCGAG
VvBAHD17.47qPCR_F1	qPCR of *VvAnAT*	AGTGAGTCGCGAGGATGTGTTGT
VvBAHD17.47qPCR_R1	qPCR of *VvAnAT*	TCCAAGCAGGATTTCCCCAACCA
VvBAHD17_F1	Sequencing gDNA of *VvAnAT*	ATGGAGGTCAAAATACTGTCAAAG
VvBAHD17_R1	Sequencing gDNA of *VvAnAT*	TCAAGGAGCTCCATTGGAACTG

Primer Name	Purpose	Primer sequence
VvBAHDNotI_F1	*VvAnAT* ligation to pET30A expression vector	GCGGCCGCATGGAGGTCAAAATACTG
VvBAHDXhoI_R1	*VvAnAT* ligation to pET30A expression vector	CTCGAGTCAAGGAGCTCCATTGG
VvBAHDXhoI_F1	*VvAnAT* ligation to plant expression vector	CTCGAGATGGAGGTCAAAATACTG
VvBAHDAsp718_R1	*VvAnAT* ligation to plant expression vector	GGTACCTCAAGGAGCTCCATTGG
VvBAHDPrF2_SacI	*VvAnAT* promoter ligation into pLUC vector	GAGCTCGGAGTATAGAGAGTACAGG
VvBAHDPrR1_BglII	*VvAnAT* promoter ligation into pLUC vector	AGATCTTGACGCTACCAGCTTCAGG

* These primers were designed by a visiting postgraduate student Maryam Pezhmanmehr previous to this research project

Appendix G Volatile compounds with significantly altered abundances in Cabernet Sauvignon, Malian and Shalistin wines made from pressed berry ferments

Chemical Name	Flavour/Aroma*	Mean normalised peak area in wine colour categories #		
		Red	Rose	White
1-butanol	fusel, oil, sweet, balsamic	0.018[a]	0.0249[c]	0.0208[b]
1-decanol	floral, fruity, fatty, rose	0.0249[b]	0.025[b]	0.0193[a]
1-heptanol	oily, nutty, fatty, green	0.0144[a]	0.0231[b]	0.0215[b]
1-octanol	orange, rose	0.0525[ab]	0.0562[b]	0.0465[a]
2-ethyl-4-butanol		0.0449[a]	0.0808[b]	0.0758[b]
methionol	meaty, onion	0.0273[a]	0.0444[b]	0.0445[b]
1-Propanol, 3-ethoxy-		0.019[c]	0.0161[b]	0.012[a]
2-ethyl hexanol	citrus, rose, sweet	0.0164[a]	0.0151[a]	0.0489[b]
ethyl cinnamate	balsamic, berry, plum, spice	0.0025[a]	0.0039[b]	0.0045[b]
3-(Methylthio)propanoic acid ethyl ester		0.0008[a]	0.0012[b]	0.0014[c]
beta-ionone	woody, violet, increases raspberry fruity characters	0.0043[c]	0.003[b]	0.0022[a]
7-Methoxy-2,2,4,8-tetramethyltricyclo[5.3.1.0(4,11)]undecane		0.0196[b]	0.0065[a]	0.0199[b]
beta-phenylethyl acetate	floral, sweet, rose, honey	0.1912[a]	0.3423[b]	0.3795[b]
hexyl acetate	green, apple, banana, sweet	0.0335[b]	0.0306[b]	0.0217[a]
á-Phenylethyl butyrate	grape, strawberry, floral, sweet	0.0228[a]	0.04[b]	0.52[c]
ethyl benzoate	fruity, dry, musty, sweet, wintergreen	0.0021[a]	0.0027[b]	0.003[c]
ethyl-2-methylbutanoate	sweet, green, apple	0.0046[a]	0.0067[b]	0.0066[b]
decanal	citrus, orange	0.0007[a]	0.0006[a]	0.0019[b]
dodecanoic acid, 1-methylethyl ester		0.0012[a]	0.0027[b]	0.0015[a]
gamma-butyrolactone	oily, fatty, caramel	0.0063[a]	0.0081[b]	0.0085[b]
hexanal	green, fruity	0.0025[b]	0.0011[a]	0.0007[a]
hotrienol	sweet, tropical	0.0005[a]	0.0013[ab]	0.002[b]
isoamyl lactate	creamy, nutty	0.0193[b]	0.005[a]	0.0077[a]
isopentyl hexanoate	fruity, apple, banana, peach, plum	0.0335[a]	0.0429[ab]	0.0483[b]

Chemical Name	Flavour/Aroma*	Mean normalised peak area in wine colour categories [#]		
		Red	Rose	White
linalool	citrus, floral	0.0086[a]	0.0091[a]	0.0129[b]
ethyl nonanoate	fruity, rose, waxy, wine, grape	0.0211[a]	0.0269[b]	0.0165[a]
n-propyl acetate	pear, raspberry, melon, strawberry	0.0062[a]	0.01[b]	0.0149[c]
phenylethyl octanoate	sweet, waxy, green, cocoa, fruity	0.0036[a]	0.0051[ab]	0.0068[b]
methyl caprylate	green, fruity	0.0213[b]	0.0224[b]	0.0121[a]
ethyl isobutyrate	sweet, ethereal, fruity, alcoholic, fusel, rummy	0.0065[a]	0.0068[a]	0.0075[b]
ethyl propanoate	sweet, fruity, ethereal, rum-like	0.0281[a]	0.0269[a]	0.0342[b]
propyl octanoate	coconut, gin	0.0062[c]	0.0045[b]	0.0028[a]
3-hexen-1-ol (Z)	green, leafy	0.0028[a]	0.0035[b]	0.007[c]
1-nonanol	waxy, citrus, floral	0.0128[b]	0.0176[c]	0.0103[a]
2-nonanol	waxy, citrus, fruity, creamy	0.0138[a]	0.0169[b]	0.0141[a]
ethyl butyrate	fruity, pineapple, apple	0.1382[a]	0.1554[b]	0.1375[a]
ethyl isovalerate	sweet, fruity, pineapple, apple	0.0046[a]	0.0068[b]	0.0061[b]
ethyl Phenylacetate	sweet, floral, honey, rose, balsamic	0.0178[a]	0.0303[b]	0.038[c]
ethyl-trans-3-Hexenoate	sweet, fruity, pineapple, green	0.0034[a]	0.0049[b]	0.0066[c]
propyl hexanoate	pineapple, fruity, sweet, green	0.0055[b]	0.0033[a]	0.0028[a]
isobutyl acetate	sweet, fruity, ethereal, banana, tropical	0.0258[b]	0.0241[b]	0.0162[a]
methyl hexanoate	ethereal, pineapple, apricot, strawberry	0.0104[a]	0.0138[b]	0.0093[a]
isoamyl alcohol	fusel, whiskey, banana	1.7316[a]	1.9348[b]	2.0840[b]
1-butanol, 3-methyl-, acetate	sweet, fruity, banana	0.0390[a]	0.0557[b]	0.0351[a]
isoamyl propionate	sweet, fruity, banana, tropical	0.0010[a]	0.0013[b]	0.0014[b]
1-hexanol	sweet, green, apple, herbaceous	0.2008[c]	0.1654[b]	0.1284[a]
1-propanol	alcoholic, ripe, fruity	0.0133[c]	0.0094[b]	0.0069[a]
ethyl ethanoate	ethereal, fruity, sweet, grape, cherry	0.2520[b]	0.2482[b]	0.2065[a]
Eehyl hexanoate	sweet, fruity, pineapple, banana, green	0.0915[a]	0.0990[b]	0.1223[c]
ethyl octanoate	fruity (banana, apricot, pear), waxy, wine	0.0448[a]	0.0540[b]	0.0454[a]
phenylethyl alcohol	rose, honey, floral	0.2455[a]	0.3987[b]	0.4259[c]

[#] Volatile peak areas were normalised to the peak area of a D_3-hexanol standard

*These aroma descriptors were reported by The Good Scents Company (http://www.thegoodscentscompany.com/) Means followed with the same letter do not significantly differ by Duncan's test at $P \leq 0.05$ p-value determined by one way ANOVA test

Appendix H Volatile compounds with significantly altered abundances in Cabernet Sauvignon, Malian and Shalistin wines made from ferments on free-run juice

Chemical Name	Flavour/Aroma*	Mean normalised peak area in wine colour categories [#]		
		Red	Red	White
neroidol	woody, tea, peach, raspberry, floral, fruity	0.0065[a]	0.0067[a]	0.0091[b]
1-decanol	floral, fruity, fatty, rose	0.0166[a]	0.0195[b]	0.0146[a]
ethyl-2-furoate	floral, Plum, Raisin, balsamic	0.0046[a]	0.0064[ab]	0.0073[b]
3(2H)-thiophenone, dihydro-2-methyl-	Sulphur, fruity, berry	0.0456[c]	0.0393[b]	0.0332[a]
beta-ionone	woody, violet, raspberry, fruity	0.0048[a]	0.0049[a]	0.0093[b]
3-hexen-1-ol	grassy, green	0.0077[b]	0.0052[a]	0.0045[a]
á-phenylethyl butyrate	grape, strawberry, floral, sweet	0.0334[a]	0.0313[a]	0.0435[b]
benzyl alcohol	sweet, fruity	0.0126[b]	0.0079[a]	0.0070[a]
ethyl-2-methylbutanoate	sweet, green, apple	0.0031[a]	0.0027[a]	0.0040[b]
ethyl isovalerate	sweet, fruity, pineapple, apple	0.0027[a]	0.0024[a]	0.0036[b]
hotrienol	sweet, tropical	0.0004[a]	0.0011[b]	0.0012[b]
linalool	citrus, floral	0.0112[a]	0.0102[a]	0.0148[b]
nerol acetate	sweet, rose, orange, blossom	0.0023[b]	0.0016[ab]	0.0012[a]
nonanoic acid	cheesy, fatty, waxy	0.0220[a]	0.0221[a]	0.0292[b]
n-propyl acetate	pear, raspberry, melon, strawberry	0.0093[b]	0.0091[b]	0.0066[a]
2-nonanol	waxy, citrus, fruity, creamy	0.0275[a]	0.0562[b]	0.0281[a]
3-methyl-1-pentanol	?	0.0241[a]	0.0276[ab]	0.0310[c]
benzaldehyde	almond	0.0029[b]	0.0020[a]	0.0022[b]
beta-citronellol	floral	0.0335[b]	0.0287[a]	0.0262[a]
ethyl phenylacetate	sweet, floral, honey, rose, balsamic	0.0433[a]	0.0382[a]	0.0560[b]
isobutyl acetate	sweet, fruity, ethereal, banana, tropical	0.0214[b]	0.0221[b]	0.0136[a]
benzyl acetate	floral, fruity (apple banana apricot)	0.0021[b]	0.0011[a]	0.0009[a]
1-hexanol	sweet, green, apple, herbaceous	0.3966[a]	0.4164[b]	0.3551[a]
1-octanol	orange, rose	0.0018[a]	0.0025[b]	0.0017[a]
ethyl acetate	ethereal, fruity, sweet (grape, cherry)	0.2092[b]	0.2258[b]	0.1633[a]

[#] Volatile peak areas were normalised to the peak area of a D_3-hexanol standard

*These aroma descriptors were reported by The Good Scents Company (http://www.thegoodscentscompany.com/) Means followed with the same letter do not significantly differ by Duncan's test at $P \leq 0.05$ p-value determined by one way ANOVA test

Appendix I Volatile compounds with significantly altered abundances in wines of transgenic 'rose' and 'white' Shiraz with controls

Chemical Name	Flavour/Aroma*	Mean normalised peak area in wine colour categories [#]		
		Red	Rose	White
isoamyl acetate	sweet, fruity, banana	0.2081[a]	0.4148[b]	0.2233[a]
1-octen-3-ol	mushroom, earthy, fungal	0.0013[a]	0.0027[b]	0.0035[c]
2-ethyl-4-butanol	?	0.0572[a]	0.0878[b]	0.0546[a]
trimethyl pentanyl diisobutyrate	?	0.0043[ab]	0.0048[b]	0.0029[a]
nerol	sweet, citrus, green, fruity	0.002[b]	0.0019[b]	0.001[a]
ethyl-2-furoate	floral, plum, raisin, balsamic	0.0046[b]	0.0022[a]	0.0042[b]
6-tridecane	?	0.0003[a]	0.0008[b]	0.0006[ab]
ethyl butyrate	fruity, pineapple, apple	0.1045[a]	0.1637[b]	0.1222[a]
palmitic acid ethyl ester	mild, waxy, sweet	0.0195[b]	0.0089[a]	0.0115[a]
hexanoic acid	cheesy, fatty	0.5333[a]	0.7626[b]	0.4132[a]
hotrienol	sweet, tropical	0.0002[a]	0.0027[b]	0.0004[a]
linalool	citrus, floral	0.0153[a]	0.0235[b]	0.0216[b]
nerol acetate	sweet, rose, orange, blossom	0.0014[b]	0.0019[c]	0.0009[a]
o-xylene	geranium (floral)	0.0016[a]	0.0021[b]	0.0017[a]
propyl octanoate	coconut, gin	0.0017[a]	0.0028[b]	0.0016[a]
trans-(2-Chlorovinyl)dimethylethoxysilane	?	0.0109[a]	0.0325[ab]	0.0569[b]
1-nonanol	waxy, citrus, floral	0.0064[a]	0.0107[b]	0.0112[b]
propyl hexanoate	pineapple, fruity, sweet, green	0.0015[a]	0.0036[b]	0.0014[a]
ethyl-3-hydroxytridecanoate	?	0.0239[a]	0.0345[b]	0.0169[a]
ethyl Acetate	ethereal, fruity, sweet, (grape, cherry)	0.1535[a]	0.2157[b]	0.2029[b]
3-methyl cyclohexene	none	0.0017[a]	0.0028[b]	0.0038[c]

Transgenic 'rose' and 'white' Shiraz contained the *VvMYBAsi* construct (see section 1.6). Wines were made from frozen ground whole berries
Means followed with the same letter do not significantly differ by Duncan's test at $P \leq 0.05$. p-value determined by one way ANOVA test
[#] Volatile peak areas were normalised to the peak area of a D_3-hexanol standard
*These aroma descriptors were reported by The Good Scents Company (http://www.thegoodscentscompany.com/)

Appendix J Volatile compounds with significantly altered abundances in wines of transgenic Chardonnay and controls

Chemical Name	Flavour/Aroma*	Mean normalised peak area in wine colour categories #		
		Red	Rose	White
1,3-dioxolane, 2,4,5-trimethyl-	?	0.0047[b]	0.0012[a]	0.0020[a]
1-heptanol, 2,4-diethyl-	?	0.0009[a]	0.0054[b]	0.0018[a]
1-propanol, 3-ethoxy-	?	0.0216[b]	0.0107[a]	0.126[a]
2,3-butanediol, [R-(R*,R*)]-	?	0.0896[b]	0.0355[a]	0.0467[a]
2-butenoic acid, ethyl ester	?	0.0333[b]	0.0149[a]	0.0092[a]
2-furancarboxylic acid, ethyl ester	floral, Plum, Raisin, balsamic	0.0004[a]	0.0021[b]	0.0012[ab]
3(2H)-thiophenone, dihydro-2-methyl-	sulphur, fruity, berry	0.0072[b]	0.0050[a]	0.0030[a]
3-hexen-1-ol	grassy, green	0.0058[b]	0.0051[b]	0.0031[a]
diethyl ketone	?	0.1754[b]	0.1334[ab]	0.1078[a]
4-hexen-1-ol, acetate	?	0.0074[b]	0.0029[a]	0.0015[a]
6-tridecane	?	0.0004[a]	0.0003[a]	0.0012[b]
acetaldehyde	?	0.0571[b]	0.0260[a]	0.0282[a]
acetic acid	?	0.0154[b]	0.0110[ab]	0.0055[a]
octyl acetate	?	0.0018[b]	0.0009[a]	0.0007[a]
acetic acid, phenylmethyl ester	floral, fruity, (apple banana apricot)	0.0018[b]	0.0007[a]	0.0009[a]
B-cyclocitral	?	0.0003[a]	0.0007[b]	0.0004[a]
benzaldehyde, 2-methyl-	fruity, cherry	0.0038[a]	0.0091[b]	0.0097[b]
isopentanoic acid	sweet, fruity, pineapple, apple	0.0020[a]	0.0029[b]	0.0031[b]
ethyl dl-2-hydroxycaproate	?	0.0046[b]	0.0035[ab]	0.0026[a]
ethyl trans-4-decenoate	?	0.0624[a]	0.1049[b]	0.0457[a]
hexanoic acid, 2-methylpropyl ester	?	0.0062[b]	0.0027[a]	0.0027[a]
hexanoic acid, hexyl ester	?	0.0062[b]	0.0019[a]	0.0017[a]

Chemical Name	Flavour/Aroma	Mean normalised peak area in wine colour categories [#]		
		Red	Rose	White
hexanoic acid, propyl ester	?	0.0086[b]	0.0021[a]	0.0021[a]
isopentyl hexanoate	fruity, apple, banana, peach, plum	0.0675[b]	0.0367[a]	0.0299[a]
isopentyl octanoate	?	0.1162[b]	0.0674[a]	0.0501[a]
linalool	citrus, floral	0.0053[a]	0.0102[b]	0.0102[b]
nerol acetate	sweet,, rose, orange, blossom	0.0009[b]	0.0006[ab]	0.0005[a]
nonanoic acid, ethyl ester	fruity, rose, waxy, wine, grape	0.0217[b]	0.0141[a]	0.0078[a]
n-propyl acetate	pear, raspberry, melon, strawberry	0.0229[b]	0.0081[a]	0.0053[a]
pentanoic acid	?	0.0210[a]	0.0257[a]	0.0314[b]
pentanoic acid, 5-hydroxy-, 2,4-di-t-butylphenyl esters	?	0.0171[a]	0.0729[b]	0.0346[a]
propyl octanoate	coconut, gin	0.0058[b]	0.0020[a]	0.0012[a]
terpinolene	?	0.0008[a]	0.0042[b]	0.0034[ab]
tetradecanoic acid, ethyl ester	?	0.0235[b]	0.0102[ab]	0.0042[a]
lactic acid	?	0.0032[a]	0.0150[b]	0.0123[b]
1,4-dihydrothujopsene-(I1)	?	0.0000[a]	0.0004[ab]	0.0007[b]
2-cyclopenten-1-one, 4-acetyl-2,3,4,5,5-pentamethyl-	?	0.0003[a]	0.0007[b]	0.0004[a]
cis-3-hexenyl Acetate	?	0.0074[b]	0.0029[a]	0.0015[a]
3-methyl-1-cyclohxene	?	0.0004[a]	0.0006[b]	0.0006[b]
ethyl 9-decenoate	?	0.0624[a]	0.1049[b]	0.0457[a]
ethyl Butyrate	fruity, pineapple, apple	0.3170[b]	0.1751[a]	0.1619[a]
ethyl heptanoate	?	0.0165[b]	0.0107[a]	0.0108[a]
ethyl Isovalerate	sweet, fruity, pineapple, apple	0.0020[a]	0.0029[b]	0.0031[b]
ethyl Phenylacetate	sweet, floral, honey, rose, balsamic	0.0065[a]	0.0176[b]	0.0173[b]
heptyl acetate	?	0.0024[b]	0.0011[a]	0.0012[a]
isobutyl Acetate	sweet, fruity, ethereal, banana, tropical	0.0290[b]	0.0098[a]	0.0083[a]

Chemical Name	Flavour/Aroma	Mean normalised peak area in wine colour categories [#]		
		Red	Rose	White
isobutyl Acetate	sweet, fruity, ethereal, banana, tropical	0.0290[b]	0.0098[a]	0.0083[a]
isobutyl octanoate	?	0.0055[b]	0.0024[a]	0.0011[a]
limonene	?	0.0013[b]	0.0004[a]	0.0003[a]
isoamylacetate	sweet, fruity, banana	0.6319[b]	0.214[a]	0.1712[a]
hexyl acetate	?	0.0429[b]	0.0176[a]	0.0093[a]
ethyl decanoate	?	0.2269[b]	0.0606[a]	0.0400[a]
ethyl Acetate	ethereal, fruity, sweet, (grape, cherry)	0.3903[b]	0.2293[a]	0.2028[a]
ethyl butyl acetate	sweet, fruity, pineapple, banana, green	0.2420[b]	0.0857[a]	0.0768[a]
octanoic acid, ethyl ester	fruity (banana, apricot, pear), waxy, wine	0.1348[b]	0.0568[a]	0.0363[a]
phenylethyl alcohol	rose, honey, floral	0.2549[a]	0.03154[ab]	0.03708[b]
propanoic acid, ethyl ester	?	0.0074[a]	0.0100[a]	0.0139[b]

Transgenic 'red' Chardonnay contained the 35S:VvMYBA construct (see section 1.6). Transgenic 'rose' Chardonnay contained the VvMYBAPr:VvMYBA construct. Wines were made from frozen ground whole berries.

Means followed with the same letter do not significantly differ by Duncan's test at $P \leq 0.05$

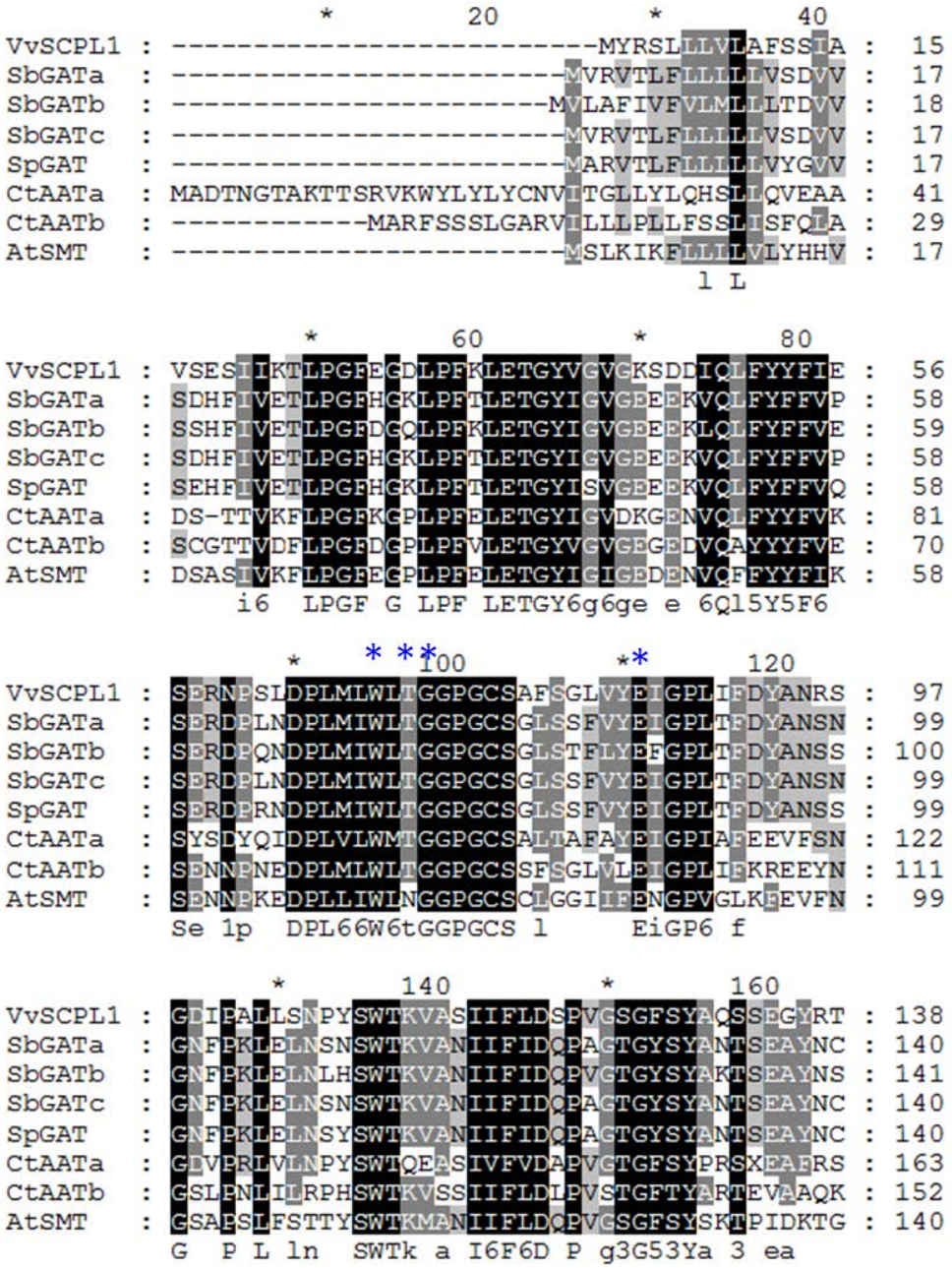
p-value determined by one way ANOVA test

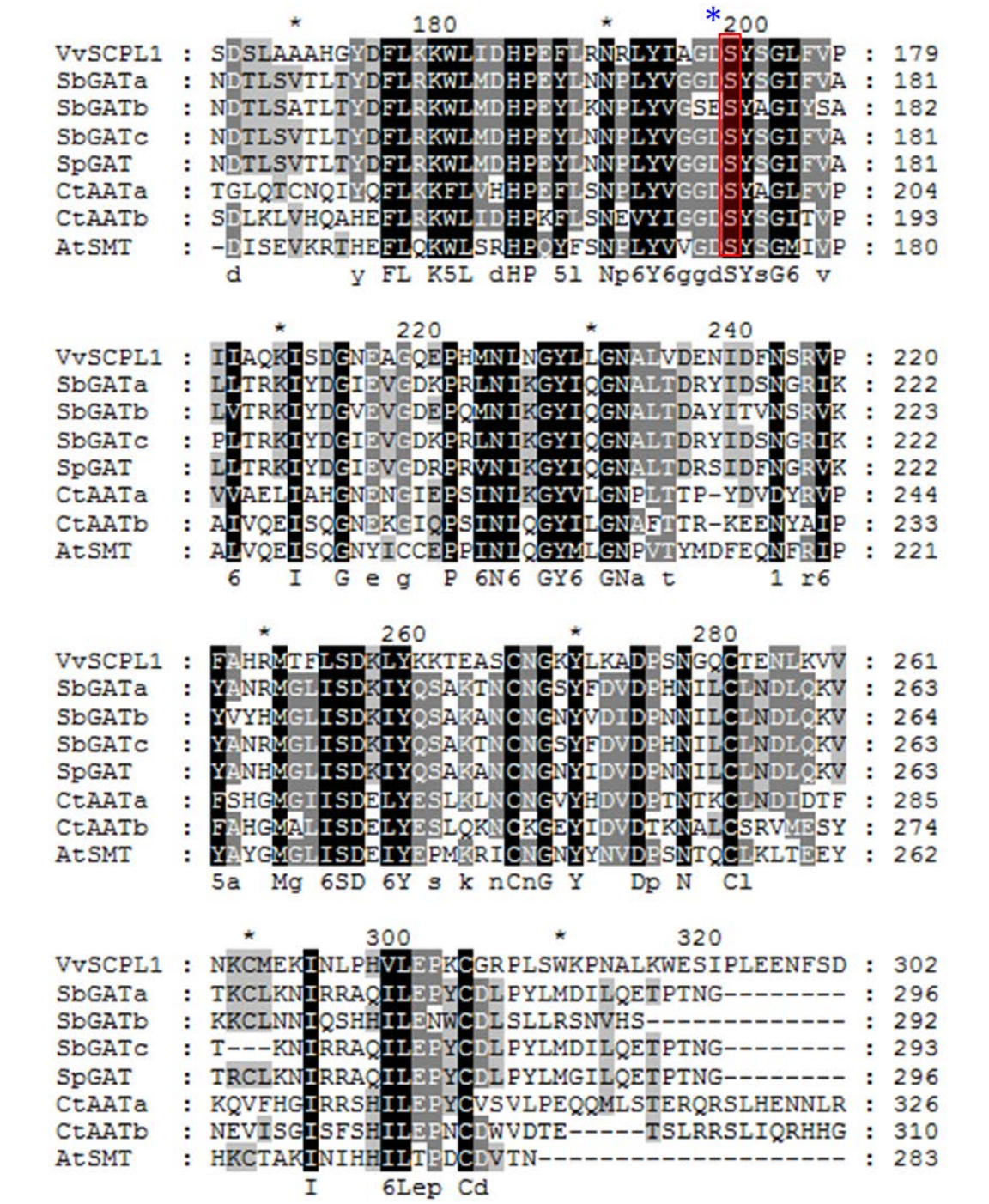
[#] Volatile peak areas were normalised to the peak area of a D_3-hexanol standard

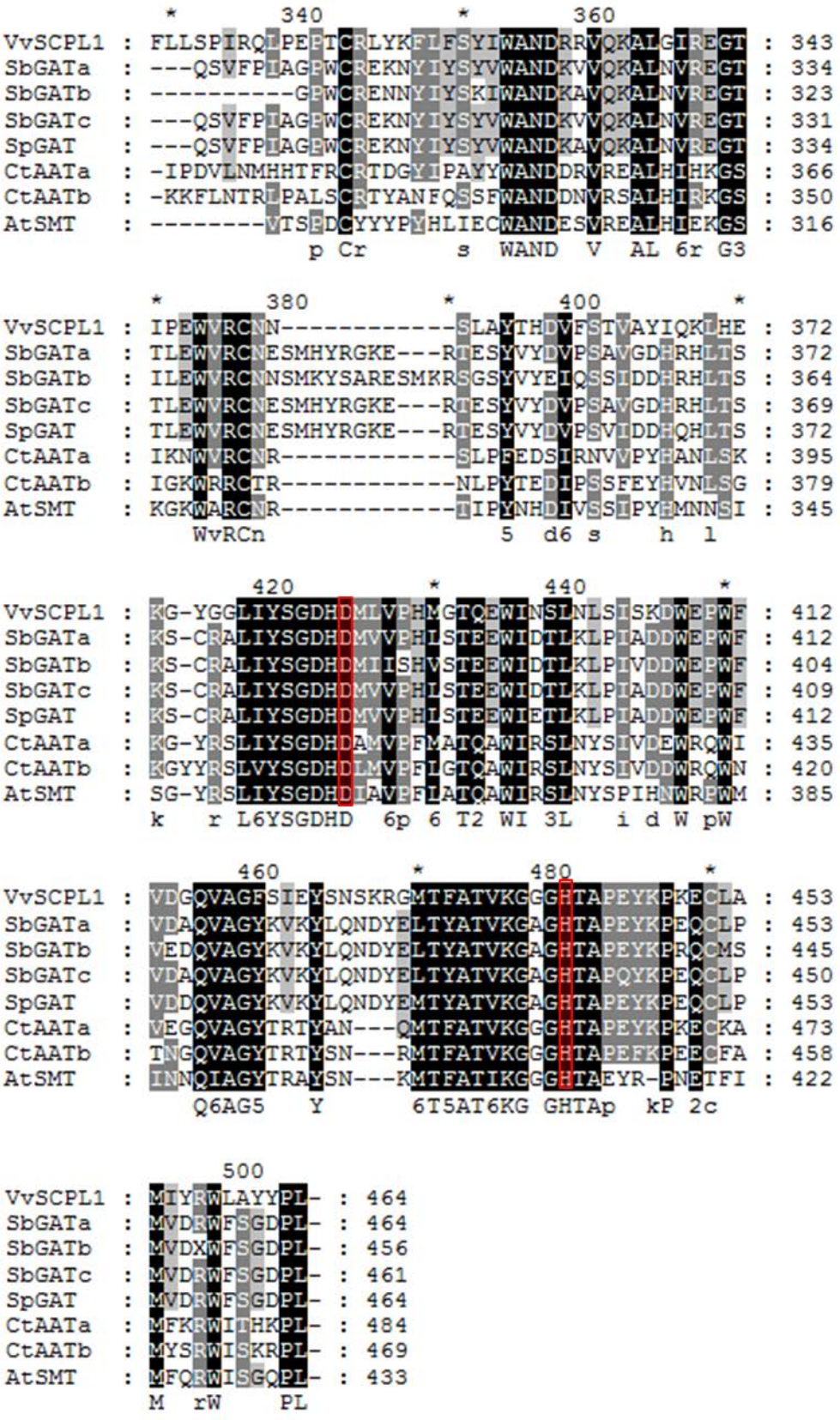
*These aroma descriptors were reported by The Good Scents Company (http://www.thegoodscentscompany.com/)

Appendix K Alignment of VvSCPL1 with homologous proteins that have been functionally characterised

Protein sequence alignment of functionally characterised homologous proteins of VvSCPL1, as determined by a BLAST search in the NCBI database. There were 5 characterised homologues: three glucose acyltransferases from *Solanum berthaultii* (SbGATa-c) that were coded for by three different alleles of the same gene, and two 1-*O*-acylglucose:anthocyanin-*O*-acyltransferases from *Clitoria tenatea* (CtAATa and CtAATb) that were coded for by two different alleles of the same gene. The 1-*O*-sinapoyl-β-glucose:l-malate sinapoyltransferase from *Arabidopsis thaliana* (AtSMT) was included to identify residues involved in the catalytic function of SCPL proteins. The Ser-Asp-His catalytic triad amino acids are boxed in red. Residues involved in the hydrogen bond network of AtSMT are indicated with a blue asterisk above the sequences.




VvSCPL1 : SDSLAAAHGYDFLKKWLIDHPEFTRNRLYIAGLSYSGLFVP : 179
SbGATa : NDTLSVTLTYDFLRKWLMDHPEYLNNPLYVGGLSYSGIFVA : 181
SbGATb : NDTLSATLTYDFLRKWLMDHPEYLKNPLYVGSESYAGIYSA : 182
SbGATc : NDTLSVTLTYDFLRKWLMDHPEYLNNPLYVGGLSYSGIFVA : 181
SpGAT : NDTLSVTLTYDFLRKWLMDHPEYLNNPLYVGGLSYSGIFVA : 181
CtAATa : TGLQTCNQIYQFLKKFLVHHPEFLSNPLYVGGLSYAGLFVP : 204
CtAATb : SDLKLVHQAHEFLRKWLIDHPKFLSNEVYIGGLSYSGITVP : 193
AtSMT : -DISEVKRTHEFLQKWLSRHPQYFSNPLYVVGLSYSGMIVP : 180
d y FL K5L dHP 5l Np6Y6ggdSYsG6 v
VvSCPL1 : IIAQKISDGNEAGQEPHMNLNGYLLGNALVDENIDFNSRVP : 220
SbGATa : LLTRKIYDGIEVGDKPRLNIKGYIQGNALTDRYIDSNGRIK : 222
SbGATb : LVTRKIYDGVEVGDEPQMNIKGYIQGNALTDAYITVNSRVK : 223
SbGATc : PLTRKIYDGIEVGDKPRLNIKGYIQGNALTDRYIDSNGRIK : 222
SpGAT : LLTRKIYDGIEVGDRPRVNIKGYIQGNALTDRSIDFNGRVK : 222
CtAATa : VVAELIAHGNENGIEPSINLKGYVLGNPLTTP-YDVDYRVP : 244
CtAATb : AIVQEISQGNEKGIQPSINLQGYILGNAFTTR-KEENYAIP : 233
AtSMT : ALVQEISQGNYICCEPPINLQGYMLGNPVTYMDFEQNFRIP : 221
6 I G e g P 6N6 GY6 GNa t 1 r6
VvSCPL1 : FAHRMTFLSDKLYKKTEASCNGKYLKADPSNGQCTENLKVV : 261
SbGATa : YANRMGLISDKIYQSAKTNCNGSYFDVDPHNILCLNDLQKV : 263
SbGATb : YVYHMGLISDKIYQSAKANCNGNYVDIDPNNILCLNDLQKV : 264
SbGATc : YANRMGLISDKIYQSAKTNCNGSYFDVDPHNILCLNDLQKV : 263
SpGAT : YANHMGLISDKIYQSAKANCNGNYIDVDPNNILCLNDLQKV : 263
CtAATa : FSHGMGIISDELYESLKLNCNGVYHDVDPTNTKCLNDIDTF : 285
CtAATb : FAHGMALISDELYESLQKNCKGEYIDVDTKNALCSRVMESY : 274
AtSMT : YAYGMGLISDEIYEPMKRICNGNYYNVDPSNTQCLKLTEEY : 262
5a Mg 6SD 6Y s k nCnG Y Dp N Cl
VvSCPL1 : NKCMEKINLPHVLEPKCGRPLSWKPNALKWESIPLEENFSD : 302
SbGATa : TKCLKNIRRAQILEPYCDLPYLMDILQETPTNG-------- : 296
SbGATb : KKCLNNIQSHHILENWCDLSLLRSNVHS------------- : 292
SbGATc : T---KNIRRAQILEPYCDLPYLMDILQETPTNG-------- : 293
SpGAT : TRCLKNIRRAQILEPYCDLPYLMGILQETPTNG-------- : 296
CtAATa : KQVFHGIRRSHILEPYCVSVLPEQQMLSTERQRSLHENNLR : 326
CtAATb : NEVISGISFSHILEPNCDWVDTE-----TSLRRSLIQRHHG : 310
AtSMT : HKCTAKINIHHILTPDCDVTN------------------- : 283
I 6Lep Cd


VvSCPL1 : FLLSPIRQLPEPTCRLYKFLFSYIWANDRRVQKALGIREGT : 343
SbGATa : ---QSVFPIAGPWCREKNYIYSYVWANDKVVQKALNVREGT : 334
SbGATb : ----------GPWCRENNYIYSKIWANDKAVQKALNVREGT : 323
SbGATc : ---QSVFPIAGPWCREKNYIYSYVWANDKVVQKALNVREGT : 331
SpGAT : ---QSVFPIAGPWCREKNYIYSYVWANDKAVQKALNVREGT : 334
CtAATa : -IPDVLNMHHTFRCRTDGYIPAYYWANDDRVREALHIHKGS : 366
CtAATb : -KKFLNTRLPALSCRTYANFQSSFWANDDNVRSALHIRKGS : 350
AtSMT : --------VTSPDCYYYPYHLIECWANDESVREALHIEKGS : 316
p Cr s WAND V AL 6r G3
VvSCPL1 : IPEWVRCNN----------SLAYTHDVFSTVAYIQKLHE : 372
SbGATa : TLEWVRCNESMHYRGKE---RTESYVYDVPSAVGDHRHLTS : 372
SbGATb : ILEWVRCNNSMKYSARESMKRSGSYVYEIQSSIDDHRHLTS : 364
SbGATc : TLEWVRCNESMHYRGKE---RTESYVYDVPSAVGDHRHLTS : 369
SpGAT : TLEWVRCNESMHYRGKE---RTESYVYDVPSVIDDHQHLTS : 372
CtAATa : IKNWVRCNR----------SLPPEDSIRNVVPYHANLSK : 395
CtAATb : IGKWRRCTR----------NLPYTEDIPSSFEYHVNLSG : 379
AtSMT : KGKWARCNR----------TIPYNHDIVSSIPYHMNNSI : 345
WvRCn 5 d6 s h l
VvSCPL1 : KG-YGGLIYSGDHDMLVPHMGTQEWINSLNLSISKDWEPWF : 412
SbGATa : KS-CRALIYSGDHDMVVPHLSTEEWIDTLKLPIADDWEPWF : 412
SbGATb : KS-CRALIYSGDHDMIISHVSTEEWIDTLKLPIVDDWEPWF : 404
SbGATc : KS-CRALIYSGDHDMVVPHLSTEEWIDTLKLPIADDWEPWF : 409
SpGAT : KS-CRALIYSGDHDMVVPHLSTEEWIETLKLPIADDWEPWF : 412
CtAATa : KG-YRSLIYSGDHDAMVPFMATQAWIRSLNYSIVDEWRQWI : 435
CtAATb : KGYYRSLVYSGDHDLMVPFLGTQAWIRSLNYSIVDDWRQWN : 420
AtSMT : SG-YRSLIYSGDHDIAVPFLATQAWIRSLNYSPIHNWREWM : 385
k r L6YSGDHD 6p 6 T2 WI 3L i d W pW
VvSCPL1 : VDGQVAGFSIEYSNSKRGMTFATVKGGGHTAPEYKPKECLA : 453
SbGATa : VDAQVAGYKVKYLQNDYELTYATVKGAGHTAPEYKPEQCLP : 453
SbGATb : VEDQVAGYKVKYLQNDYELTYATVKGAGHTAPEYKPRQCMS : 445
SbGATc : VDAQVAGYKVKYLQNDYELTYATVKGAGHTAPQYKPEQCLP : 450
SpGAT : VDDQVAGYKVKYLQNDYEMTYATVKGAGHTAPEYKPEQCLP : 453
CtAATa : VEGQVAGYTRTYAN---QMTFATVKGGGHTAPEYKPKECKA : 473
CtAATb : TNGQVAGYTRTYSN---RMTFATVKGGGHTAPEFKPEECFA : 458
AtSMT : INNQIAGYTRAYSN---KMTFATIKGGGHTAEYR-PNETFI : 422
Q6AG5 Y 6T5AT6KG GHTAp kP 2c
VvSCPL1 : MIYRWLAYYPL- : 464
SbGATa : MVDRWFSGDPL- : 464
SbGATb : MVDXWFSGDPL- : 456
SbGATc : MVDRWFSGDPL- : 461
SpGAT : MVDRWFSGDPL- : 464
CtAATa : MFKRWITHKPL- : 484
CtAATb : MYSRWISKRPL- : 469
AtSMT : MFQRWISGQPL- : 433
M rW PL

Appendix L Description of the SignalP 4.1 prediction output

Below are the descriptions of the SignalP 4.1 preduction output copied from their website: http://www.cbs.dtu.dk/services/SignalP/output.php

The neural networks in SignalP produce three output scores for each position in the input sequence:

C-score (raw cleavage site score)

The output from the CS networks, which are trained to distinguish signal peptide cleavage sites from everything else.

Note the position numbering of the cleavage site: the C-score is trained to be high at the position immediately *after* the cleavage site (the first residue in the mature protein).

S-score (signal peptide score)

The output from the SP networks, which are trained to distinguish positions within signal peptides from positions in the mature part of the proteins and from proteins without signal peptides.

Y-score (combined cleavage site score)

A combination (geometric average) of the C-score and the slope of the S-score, resulting in a better cleavage site prediction than the raw C-score alone. This is due to the fact that multiple high-peaking C-scores can be found in one sequence, where only one is the true cleavage site. The Y-score distinguishes between C-score peaks by choosing the one where the slope of the S-score is steep.

The graphical output from SignalP (see below) shows the three different scores, *C*, *S* and *Y*, for each position in the sequence.

In the summary below the plot, the maximal values of the three scores are reported. In addition, the following two scores are shown:

mean S

The average S-score of the possible signal peptide (from position 1 to the position immediately before the maximal Y-score).

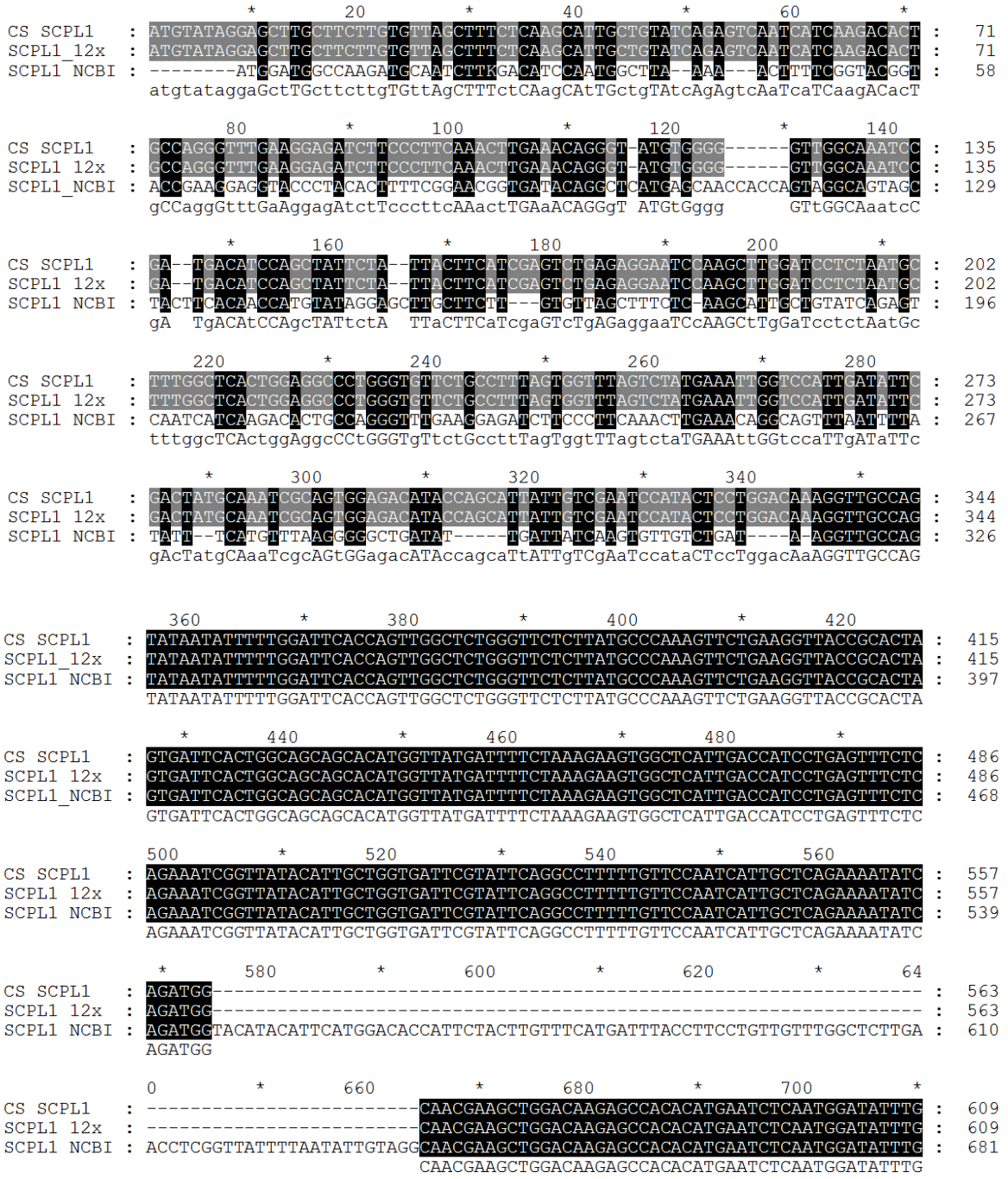
D-score (discrimination score)

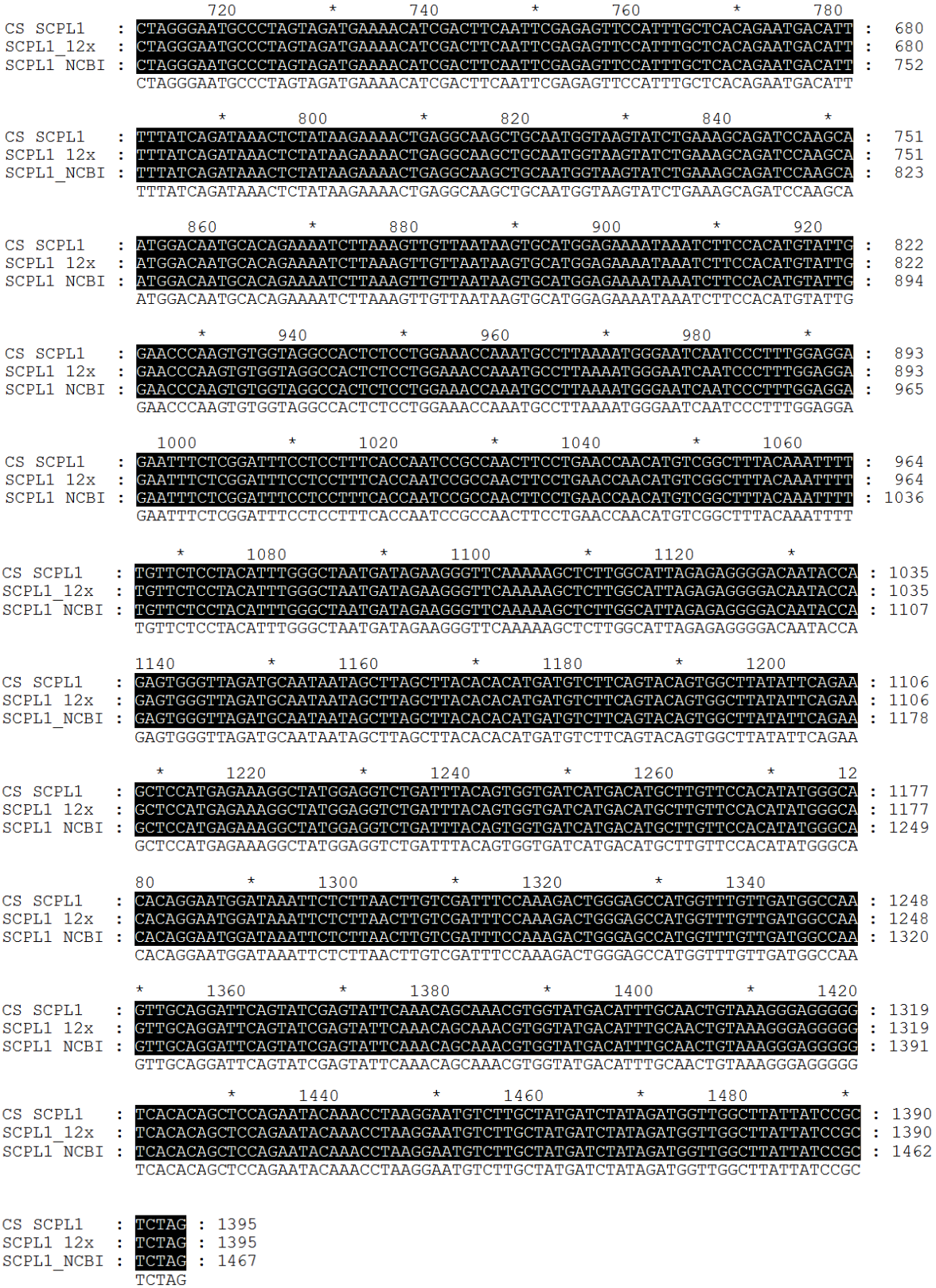
A weighted average of the mean S and the max. Y scores. This is the score that is used to discriminate signal peptides from non-signal peptides.

For non-secretory proteins all the scores represented in the SignalP output should ideally be very low (close to the negative target value of 0.1).

Appendix M Alignment of two annotation of the VvSCPL1 gene

Alignment of *VvSCPL1* cDNA from Cabernet Sauvignon (CS SCPL) with coding regions from two annotations *VvSCPL1* obtained from the NCBI database (accession number AM462732, SCPL1_NCBI) and from the 12X grapevine genome sequence database, V1 gene prediction version (microarray ID VIT_03s0091g01240, SCPL1_12X)




720 * 740 * 760 * 780
CS SCPL1 : CTAGGGAATGCCCTAGTAGATGAAAACATCGACTTCAATTCGAGAGTTCCATTTGCTCACAGAATGACATT : 680
SCPL1_12x : CTAGGGAATGCCCTAGTAGATGAAAACATCGACTTCAATTCGAGAGTTCCATTTGCTCACAGAATGACATT : 680
SCPL1 NCBI : CTAGGGAATGCCCTAGTAGATGAAAACATCGACTTCAATTCGAGAGTTCCATTTGCTCACAGAATGACATT : 752
CTAGGGAATGCCCTAGTAGATGAAAACATCGACTTCAATTCGAGAGTTCCATTTGCTCACAGAATGACATT
* 800 * 820 * 840 *
CS SCPL1 : TTTATCAGATAAACTCTATAAGAAAACTGAGGCAAGCTGCAATGGTAAGTATCTGAAAGCAGATCCAAGCA : 751
SCPL1 12x : TTTATCAGATAAACTCTATAAGAAAACTGAGGCAAGCTGCAATGGTAAGTATCTGAAAGCAGATCCAAGCA : 751
SCPL1_NCBI : TTTATCAGATAAACTCTATAAGAAAACTGAGGCAAGCTGCAATGGTAAGTATCTGAAAGCAGATCCAAGCA : 823
TTTATCAGATAAACTCTATAAGAAAACTGAGGCAAGCTGCAATGGTAAGTATCTGAAAGCAGATCCAAGCA
860 * 880 * 900 * 920
CS SCPL1 : ATGGACAATGCACAGAAAATCTTAAAGTTGTTAATAAGTGCATGGAGAAAATAAATCTTCCACATGTATTG : 822
SCPL1 12x : ATGGACAATGCACAGAAAATCTTAAAGTTGTTAATAAGTGCATGGAGAAAATAAATCTTCCACATGTATTG : 822
SCPL1 NCBI : ATGGACAATGCACAGAAAATCTTAAAGTTGTTAATAAGTGCATGGAGAAAATAAATCTTCCACATGTATTG : 894
ATGGACAATGCACAGAAAATCTTAAAGTTGTTAATAAGTGCATGGAGAAAATAAATCTTCCACATGTATTG
* 940 * 960 * 980 *
CS SCPL1 : GAACCCAAGTGTGGTAGGCCACTCTCCTGGAAACCAAATGCCTTAAAATGGGAATCAATCCCTTTGGAGGA : 893
SCPL1 12x : GAACCCAAGTGTGGTAGGCCACTCTCCTGGAAACCAAATGCCTTAAAATGGGAATCAATCCCTTTGGAGGA : 893
SCPL1 NCBI : GAACCCAAGTGTGGTAGGCCACTCTCCTGGAAACCAAATGCCTTAAAATGGGAATCAATCCCTTTGGAGGA : 965
GAACCCAAGTGTGGTAGGCCACTCTCCTGGAAACCAAATGCCTTAAAATGGGAATCAATCCCTTTGGAGGA
1000 * 1020 * 1040 * 1060
CS SCPL1 : GAATTTCTCGGATTTCCTCCTTTCACCAATCCGCCAACTTCCTGAACCAACATGTCGGCTTTACAAATTTT : 964
SCPL1 12x : GAATTTCTCGGATTTCCTCCTTTCACCAATCCGCCAACTTCCTGAACCAACATGTCGGCTTTACAAATTTT : 964
SCPL1 NCBI : GAATTTCTCGGATTTCCTCCTTTCACCAATCCGCCAACTTCCTGAACCAACATGTCGGCTTTACAAATTTT : 1036
GAATTTCTCGGATTTCCTCCTTTCACCAATCCGCCAACTTCCTGAACCAACATGTCGGCTTTACAAATTTT
* 1080 * 1100 * 1120 *
CS SCPL1 : TGTTCTCCTACATTTGGGCTAATGATAGAAGGGTTCAAAAAGCTCTTGGCATTAGAGAGGGGACAATACCA : 1035
SCPL1 12x : TGTTCTCCTACATTTGGGCTAATGATAGAAGGGTTCAAAAAGCTCTTGGCATTAGAGAGGGGACAATACCA : 1035
SCPL1 NCBI : TGTTCTCCTACATTTGGGCTAATGATAGAAGGGTTCAAAAAGCTCTTGGCATTAGAGAGGGGACAATACCA : 1107
TGTTCTCCTACATTTGGGCTAATGATAGAAGGGTTCAAAAAGCTCTTGGCATTAGAGAGGGGACAATACCA
1140 * 1160 * 1180 * 1200
CS SCPL1 : GAGTGGGTTAGATGCAATAATAGCTTAGCTTACACACATGATGTCTTCAGTACAGTGGCTTATATTCAGAA : 1106
SCPL1 12x : GAGTGGGTTAGATGCAATAATAGCTTAGCTTACACACATGATGTCTTCAGTACAGTGGCTTATATTCAGAA : 1106
SCPL1_NCBI : GAGTGGGTTAGATGCAATAATAGCTTAGCTTACACACATGATGTCTTCAGTACAGTGGCTTATATTCAGAA : 1178
GAGTGGGTTAGATGCAATAATAGCTTAGCTTACACACATGATGTCTTCAGTACAGTGGCTTATATTCAGAA
* 1220 * 1240 * 1260 * 12
CS SCPL1 : GCTCCATGAGAAAGGCTATGGAGGTCTGATTTACAGTGGTGATCATGACATGCTTGTTCCACATATGGGCA : 1177
SCPL1 12x : GCTCCATGAGAAAGGCTATGGAGGTCTGATTTACAGTGGTGATCATGACATGCTTGTTCCACATATGGGCA : 1177
SCPL1 NCBI : GCTCCATGAGAAAGGCTATGGAGGTCTGATTTACAGTGGTGATCATGACATGCTTGTTCCACATATGGGCA : 1249
GCTCCATGAGAAAGGCTATGGAGGTCTGATTTACAGTGGTGATCATGACATGCTTGTTCCACATATGGGCA
80 * 1300 * 1320 * 1340
CS SCPL1 : CACAGGAATGGATAAATTCTCTTAACTTGTCGATTTCCAAAGACTGGGAGCCATGGTTTGTTGATGGCCAA : 1248
SCPL1 12x : CACAGGAATGGATAAATTCTCTTAACTTGTCGATTTCCAAAGACTGGGAGCCATGGTTTGTTGATGGCCAA : 1248
SCPL1 NCBI : CACAGGAATGGATAAATTCTCTTAACTTGTCGATTTCCAAAGACTGGGAGCCATGGTTTGTTGATGGCCAA : 1320
CACAGGAATGGATAAATTCTCTTAACTTGTCGATTTCCAAAGACTGGGAGCCATGGTTTGTTGATGGCCAA
* 1360 * 1380 * 1400 * 1420
CS SCPL1 : GTTGCAGGATTCAGTATCGAGTATTCAAACAGCAAACGTGGTATGACATTTGCAACTGTAAAGGGAGGGGG : 1319
SCPL1 12x : GTTGCAGGATTCAGTATCGAGTATTCAAACAGCAAACGTGGTATGACATTTGCAACTGTAAAGGGAGGGGG : 1319
SCPL1 NCBI : GTTGCAGGATTCAGTATCGAGTATTCAAACAGCAAACGTGGTATGACATTTGCAACTGTAAAGGGAGGGGG : 1391
GTTGCAGGATTCAGTATCGAGTATTCAAACAGCAAACGTGGTATGACATTTGCAACTGTAAAGGGAGGGGG
* 1440 * 1460 * 1480 *
CS SCPL1 : TCACACAGCTCCAGAATACAAACCTAAGGAATGTCTTGCTATGATCTATAGATGGTTGGCTTATTATCCGC : 1390
SCPL1_12x : TCACACAGCTCCAGAATACAAACCTAAGGAATGTCTTGCTATGATCTATAGATGGTTGGCTTATTATCCGC : 1390
SCPL1 NCBI : TCACACAGCTCCAGAATACAAACCTAAGGAATGTCTTGCTATGATCTATAGATGGTTGGCTTATTATCCGC : 1462
TCACACAGCTCCAGAATACAAACCTAAGGAATGTCTTGCTATGATCTATAGATGGTTGGCTTATTATCCGC
CS SCPL1 : TCTAG : 1395
SCPL1 12x : TCTAG : 1395
SCPL1_NCBI : TCTAG : 1467
TCTAG

Appendix N Supplementary data for manuscript in Chapter 5

Table S1 – Information on the characterised BAHD proteins used in phylogenetic tree analysis

Abbreviated name	NCBI Genbank accession	Major acyl CoA doner	Major products formed	Species	Reference(s)
Clade 1					
MtMaT6	XP_003601994	malonyl	anthocyanins	*Medicago truncatula*	Zhao et al. 2011
GmIF7MaT	UGT88E3	malonyl	isoflavonoids	*Glycine max*	Dhaubhadel et al. 2008
At5MaT	NP_189600	malonyl	anthocyanins	*Arabidopsis thaliana*	Luo et al. 2007
MtMaT3	ABY91221	malonyl	malonyl isoflavone glucosides	*Medicago truncatula*	Yu et al. 2008
MtMaT4	XP_003608206	malonyl	flavonoid glucosides	*Medicago truncatula*	Zhao et al. 2011
MtMaT2	ABY91222	malonyl	malonyl isoflavone glucosides	*Medicago truncatula*	Yu et al. 2008
MtMaT1	ABY91220	malonyl	malonyl isoflavone glucosides	*Medicago truncatula*	Yu et al. 2008
Ss3AT	AAR28757	hydroxy-cinnamoyl	anthocyanins	*Salvia splendens*	Suzuki et al. 2004b
Pf3AT	BAA93475	hydroxy-cinnamoyl	anthocyanins	*Perilla frutescens*	Yonekura-Sakakibara et al. 2000
Ss5MaT1	AAL50566	malonyl	anthocyanins	*Salvia splendens*	Suzuki et al. 2001
Pf5MaT	AAL50565	malonyl	anthocyanins	*Perilla frutescens*	Suzuki et al. 2001
Lp3MAT1	AAS77404	malonyl	anthocyanins	*Lamium purpureum*	Suzuki et al. 2004b
Vh3MAT1	AAS77402	malonyl	quercetin 3-O-6"-O-malonylglucoside	*Glandularia x hybrida*	Suzuki et al. 2004b
Gt5AT	BAA74428	hydroxy-cinnamoyl	anthocyanins	*Gentiana triflora*	Fujiwara et al. 1998
NtMAT1	BAD93691	malonyl	flavonoid and napthol glucosides	*Nicotiana tabacum*	Taguchi et al. 2005
Dv3MAT	AOO12206	malonyl	anthocyanins	*Dahlia pinnata*	Suzuki et al. 2002

Abbreviated name	NCBI Genbank accession	Major acyl CoA doner	Major products formed	Species	Reference(s)
Dm3MAT1	AAQ63615	malonyl	anthocyanins	*Chrysanthemum x morifolium*	Suzuki et al. 2004a
Dm3MAT2	AAQ63616	malonyl	anthocyanins	*Chrysanthemum x morifolium*	D'Auria et al 2002
Sc3MaT	AOO38058	malonyl	anthocyanins	*Pericallis cruenta*	Suzuki et al. 2003
At3AT1	NP_171890	hydroxy-cinnamoyl	anthocyanins	*Arabidopsis thaliana*	Luo et al. 2007
At3AT2	AEE27579	hydroxy-cinnamoyl	anthocyanins	*Arabidopsis thaliana*	Luo et al. 2007
MtMaT5	XP_003599128	malonyl	anthocyanins	*Medicago truncatula*	Zhao et al. 2011
Clade 2					
ZmGlossy2	CAA61258	unknown	C32 epicuticular waxes	*Zea mays*	Tacke et al. 1995
AtCER2	AAM64817	unknown	C30 epicuticular waxes	*Arabidopsis thaliana*	Negruk et al. 1996 and Xia et al. 1996
Clade 3					
PsSalAT	AAK73661	acetyl	thebaine	*Papaver somniferum*	Grothe et al. 2001
CaPun1	AAV66311	unknown	capsaicin pathway	*Capsicum annuum*	Stewart et al. 2005
CrDAT	AAC99311	acetyl	vindoline	*Catharanthus roseus*	St Pierre et al. 1998
CrMAT	AAO13736	acetyl	minovincinine	*Catharanthus roseus*	Laflamme et al. 2001
RsVS	CAD89104	acetyl	vinorine	*Rauvolfia serpentina*	Bayer and Stockigt 2004
CmAAT4	AAW51126	medium-chain aliphatic	medium-chain and hydroxycinnamoyl acyl esters	*Cucumis melo*	El-Sharkawy et al. 2005
FvVAAT	CAC09062	acetyl	small- to medium-chain aliphatic esters	*Fragaria vesca*	Beekwilder et al. 2004
FaSAAT	AAG13130	acetyl	medium-chain aliphatic and benzyl esters	*Fragaria x ananassa*	Aharoni et al. 2000

Abbreviated name	NCBI Genbank accession	Major acyl CoA doner	Major products formed	Species	Reference(s)
RhAAT1	AAW31948	acetyl	small- to medium-chain aliphatic esters	*Rosa hybrid* cultivar	Shalit et al. 2003
Ss5MaT2	AAR26385	malonyl	anthocyanins	*Salvia splendens*	Suzuki et al. 2004
VvAnAT		acetyl	anthocyanins	*Vitis Vinifera*	This paper
CbBEBT	AAN09796	benzoyl	benzyl benzoate	*Clarkia breweri*	D'Auria et al 2002
NaDH29	CA591847	caffeoyl	dicaffeoylspermidine	*Nicotiana attenuate*	Onkokesung et al. 2012
Clade 4					
DcHCBT	CAB06430	hydroxy-cinnamoyl/ benzoyl	dianthramides	*Dianthus caryophyllus*	Yang et al. 1997
CcHCT	EF137954	hydroxy-cinnamoyl	chlorogenic acids	*Coffea canephora*	Lallemand et al. 2012
NtHCT	CAD47830	hydroxy-cinnamoyl	chlorogenic acid and derivatives	*Nicotiana tabacum*	Hoffmann et al. 2003
AtHCT	NP_199704	hydroxy-cinnamoyl	chlorogenic acid and derivatives	*Arabidopsis thaliana*	Hoffmann et al. 2005
AsHHT1	BAC78633	hydroxy-cinnamoyl	chlorogenic acid and derivatives	*Avena sativa*	Yang et al. 2004
NtHQT	CAE46932	hydroxy-cinnamoyl	chlorogenic acid	*Nicotiana tabacum*	Niggeweg et al. 2004
SlHQT	Q70G32	hydroxy-cinnamoyl	chlorogenic acids	*Solanum lycopersicum*	Sonnante et al. 2010
IbHCBT	Q9SST8	hydroxy-cinnamoyl	chlorogenic acids	*Ipomoea batatas*	Kojima and Kondo 1985
CcHQT1	AM690438	hydroxy-cinnamoyl	chlorogenic acids	*Cynara cardunculus* var. scolymus	Sonnante et al. 2010
CcHQT2	EU839580	hydroxy-cinnamoyl	chlorogenic acids	*Cynara cardunculus* var. scolymus	Sonnante et al. 2010
CcHQT	ABK79690	hydroxy-cinnamoyl	chlorogenic acids	*Cynara cardunculus* var. altilis	Comino et al. 2009

Abbreviated name	NCBI Genbank accession	Major acyl CoA doner	Major products formed	Species	Reference(s)
CsHQT	ABK79689	hydroxy-cinnamoyl	chlorogenic acids	*Cynara cardunculus* var. scolymus	Comino et al. 2009
AtSHT	AEC06845	hydroxy-cinnamoyl	spermidine hydroxycinnamoyl congugates	*Arabidopsis thaliana*	Grienenberger 2009
HvACT	AAO73071	hydroxy-cinnamoyl	hydroxy-cinnamoyl agamatine derivatives	*Hordeum vulgare*	Burhenne et al. 2003
NaAT1	BU494535	caffeoyl	caffeoylputrescine	*Nicotiana attenuate*	Onkokesung et al. 2012
Clade 5					
MsBanAAT	CAC09063	acetyl	cinnamyl acetate and other medium-chain aliphatic acetate esters	*Musa sp.*	Beekwilder et al. 2004
MpAAT1	AAU14879	short- to medium-chain aliphatic	short- to medium-chain aliphatic volatile esters	*Malus x domestica*	Souleyre et al.2005
VlAMAT	AAW22989	anthraniloyl	methyl anthranilate	*Vitis Labrusca*	Wang and De Luca 2005
PhBPBT	AAU06226	benzoyl	benzyl benzoate/ phenethyl benzoate	*Petunia x hybrida*	Boatright et al. 2004
NtBEBT	AAN09798	benzoyl	benzyl benzoate	*Nicotiana tabacum*	D'Auria et al 2002
CmAAT3	AAW51125	medium-chain aliphatic	medium-chain and hydroxycinnamoyl acyl esters	*Cucumis melo*	El-Sharkawy et al. 2005
CbBEAT	AAN09796	benzoyl	benzyl benzoate	*Clarkia breweri*	D'Auria et al 2002
CmAAT1	CAA94432	medium-chain aliphatic	medium-chain and hydroxycinnamoyl acyl esters	*Cucumis melo*	El-Sharkawy et al. 2005

Abbreviated name	NCBI Genbank accession	Major acyl CoA doner	Major products formed	Species	Reference(s)
CmAAT2	AAL77060	medium-chain aliphatic	medium-chain and hydroxycinnamoyl acyl esters	*Cucumis melo*	El-Sharkawy et al. 2005
LaHMT/HLT	BAD89275	tigloyl	quinolizidine alkaloids	*Lupinus albus*	Okada et al. 2005
AtCHAT	AAN09797	acetyl	(Z)-3-hexen-1-yl acetate	*Arabidopsis thaliana*	D'Auria et al 2002
AtSCT	NP_180087	coumaroyl	coumaroylspermidine	*Arabidopsis thaliana*	Lou et al. 2009
AtSDT	NP_179932	sinapoyl	disinapoyl spermidine	*Arabidopsis thaliana*	Lou et al. 2009
TcDBNTBT	AAM75818	benzoyl	2'-deoxytaxol	*Taxus canadensis*	Walker et al. 2002
TcDBAT	AAF27621	acetyl	baccatin III	*Taxus cuspidata*	Walker and Croteau 2000b
TcTAT	AAF34254	acetyl	taxa-4(20),11(12)-dien-5a-yl acetate	*Taxus cuspidata*	Walker et al. 2000
TcDBBT	Q9FPW3	benzoyl	7,13-diacetylbaccatin III	*Taxus cuspidata*	Walker and Croteau 2000a
TcBAPT	AAL92459	β-phenylalanoyl	N-debenzoyl-2'-deoxytaxol	*Taxus cuspidata*	Walker and Fujisaki et al. 2002
AtDCF	AAQ62868	hydroxy-cinnamoyl	medium-chain and hydroxycinnamoyl acyl esters	*Arabidopsis Thaliana*	Rautengarten et al. 2012
AtHHT	AED94628	hydroxy-cinnamoyl	suberin	*Arabidopsis thaliana*	Gou et al. 2009

Table S2 – MS parent ion and MS2 major daughter ions detected by LC/MS/MS and used to identify acylated anthocyanins in recombinant VvAnAT bioassays and transgenic tobacco flowers

Peak identity	MS Parent ion	MS2 major ion
Malvidin-3-acetylglucoside	535	331
Malvidin-3-coumaroylglucoside	639	331
Malvidin-3-caffeoylglucoside	655	331
Malvidin-3-malonylglucoside	579	331
Cyanidin-3-acetylglucoside	491	287
Delphinidin-3-acetylglucoside	507	303
Peonidin-3-acetylglucoside	505	301
Cyanidin-3,5-acetylglucoside	653	287
Cyanidin-3-rutinoside	595	287
pelargonidin-3-rutinoside	579	271
cyanidin-3-*O*-caffeoylglucoside	611	287
cyanidin-3-*O*-coumaroylglucoside	595	287
pelargonidin-3-*O*-acetylglucoside	475	271
pelargonidin-3-*O*-caffeoylglucoside	595	271
pelargonidin-3-*O*-coumaroylglucoside	579	271

Table S3- Details of primers

Primer Name	Purpose	Primer sequence
VvBAHD17.47qPCR_F1	qPCR	AGTGAGTCGCGAGGATGTGTTGT
VvBAHD17.47qPCR_R1	qPCR	TCCAAGCAGGATTTCCCCAACCA
VvBAHD17_F1	Sequencing gDNA	ATGGAGGTCAAAATACTGTCAAAG
VvBAHD17_R1	Sequencing gDNA	TCAAGGAGCTCCATTGGAACTG
VvBAHDNotI_F1	Ligation to pET30A expression vector	GCGGCCGCATGGAGGTCAAAATACTG
VvBAHDXhoI_R1	Ligation to pET30A expression vector	CTCGAGTCAAGGAGCTCCATTGG
VvBAHDXhoI_F1	Ligation to plant expression vector	CTCGAGATGGAGGTCAAAATACTG
VvBAHDAsp718_R1	Ligation to plant expression vector	GGTACCTCAAGGAGCTCCATTGG
VvBAHDPrF2_SacI	Promoter ligation into pLUC vector	GAGCTCGGAGTATAGAGAGTACAGG
VvBAHDPrR1_BglII	Promoter ligation into pLUC vector	AGATCTTGACGCTACCAGCTTCAGG
35SF	PCR screen of tobacco transformants	TTCGCAAGACCCTTCCTCTA
OCS rev	PCR screen of tobacco transformants	GGCGGTAAGGATCTGAGCTA

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