Identification of *O-methyltransferase* genes involved in the biosynthesis of 3-alkyl-2-methoxypyrazines in grapevines (*Vitis vinifera* L.)

by

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Abstract

3-alkyl-2-methoxypyrazines (MPs) are a family of potent volatile aroma compounds commonly found in various vegetables. MPs, in particular 3-isobutyl-2-methoxypyrazine (IBMP), can also be found in the grapes and subsequent wines of a small number of grapevine cultivars, including Cabernet Sauvignon and Sauvignon blanc. MPs have odour perceptions commonly described as herbaceous and green capsicum-like, which when present in wines at high concentrations are considered detrimental to wine quality. Controlling the level of MPs in wines is best achieved by manipulating the amount that accumulates in the berry, which is highly variable and influenced by numerous environmental and viticultural factors. Little is known about the biosynthesis of MPs, however a previous study has shown that in grape berries an *O*-methyltransferase (OMT) enzyme catalyses the final step in MP biosynthesis via the methylation of 3-alkyl-2-hydroxypyrazine (HP) precursors (Hashizume *et al.*, 2001a). Furthermore a protein with this activity has previously been purified from the shoots of Cabernet Sauvignon and its N-terminus partially sequenced (Hashizume *et al.*, 2001b).

The aim of this project was to identify the gene encoding the OMT responsible for the final step of MP biosynthesis in grape berries. Using a candidate gene approach the gene *VvOMT1* was identified showing an exact homology to the 22 amino acid sequence of the native protein purified Hashizume et al (2001b) and a second gene *VvOMT2* found with close homology to *VvOMT1*. Kinetic analysis of recombinant VvOMT1 and VvOMT2 revealed that they each possessed methylating activity against HP substrates similar to that reported by

Hashizume et al (2001b), and the expression of *VvOMT1* was found to coincide with the period of MP biosynthesis in early grape development. However, RNAi mediated silencing of both *VvOMT1* and *VvOMT2* in grapevine hairy-roots did not result in a reduction of MPs.

A mapping approach was also used to identify genes responsible for IBMP accumulation. A population of F2 progeny derived from a cross between Cabernet Sauvignon (CS) and a Pinot Meunier dwarf mutant (PM dwarf) was established and found to segregate for the trait of IBMP accumulation in young berries. Using the online genome sequence as a basis, CAPs and dCAPS markers were designed to the genomes of CS and PM, which enabled the identification of a 2.3 Mb locus that segregates with the trait of IBMP accumulation. A search of the online annotated genomic database revealed two putative OMTs, *VvOMT3* and *VvOMT4*, located within this locus. An association mapping study directed to this locus revealed that the gene *VvOMT3* is highly associated (p = 0.005) with the trait of IBMP accumulation in 91 existing grapevine cultivars. Furthermore recombinant VvOMT3 was found to have between 500 - 5,000 fold greater catalytic activity against IBHP than other VvOMTs investigated. The expression of *VvOMT3* also coincided the period of MP accumulation in young grape berries and was associated with IBMP accumulation in a subset of the segregating F2 progeny. Finally the elimination of sunlight from grape bunches was found to significantly reduce the expression of *VvOMT3*, resulting in a reduction of IBMP levels.

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

Jake Dunlevy

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Abbreviations

BLAST	basic local alignment search tool
CAPS	cleavage amplified polymorphic sequence
cDNA	complementary DNA
CS	Cabernet Sauvignon
CSIRO	Commonwealth Scientific and Industrial Research Organisation
dCAPS	derived cleavage amplified polymorphic sequence
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediamine-*tetra*-acetic acid
EST	expressed sequence tag
g	gram(s)
g	relative centrifugal force
GFP	green fluorescent protein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HP	hydroxypyrazine
IBHP	2-isobutyl-3-hydroxypyrazine
IBMP	2-isobutyl-3-methoxypyrazine
IPHP	2-isopropyl-3-hydroxypyrazine
IPMP	2-isopropyl-3-methoxypyrazine
kb	kilobase pairs
kDa	kilo Dalton
L	litre(s)
LB	Luria broth
LD	linkage disequilibrium
M	molar
MES	2-(N-morpholino)ethanesulfonic acid
min	minute(s)
MP	methoxypyrazine
m/z	mass-to-charge ratio
OMT	*O*-methyltransferase
PAGE	polyacrylamide gel electorphoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PM	Pinot Meunier
PN	Pinot noir
PVPP	polyvinylpolyprrolidone
rpm	revolutions per minute
SBMP	2-*sec*butyl-3-methoxypyrazine
SDS	sodium dodecyl sulphate
TBE	tris-borate-EDTA
Tris	tris(hyroxymethyl)aminomethane
v/v	volume per volume
Vv	*Vitis vinifera*
wpf	weeks post flowering
w/v	weight per volume

Chapter 1 - General introduction

1.1 Grapes and Wine

For millennia high quality wine has been prized for its exceptionally pleasurable flavour and alcoholic influences. There is evidence to suggest that wine was being made in northern Iran as early as 5,400 BC (McGovern *et al.*, 1996) and in ancient Greek mythology Dionysus was revered as the God of wine (McGovern, 2004). Today, wine is still a highly valued commodity, so much so that the grapevine (*Vitis vinifera* L.) is the most economically important fruit crop in the world (Myles *et al.*, 2011; Vivier and Pretorius, 2002), with some 8 million hectares of vineyards established worldwide (Vivier and Pretorius, 2000). It has been estimated that there are over 5,000 different cultivars of *V. vinifera* grapes grown worldwide (Alleweldt, 1994). However, only a small number of elite cultivars constitute the vast majority of the global wine market.

The major cultivars of wine grapes grown in Australia are Shiraz (25.5%), Chardonnay (21.6%), Cabernet Sauvignon (13.3%), Merlot (6.8%), Muscat (5.0%), Semillon (4.5%) and Sauvignon blanc (4.4%) with other less common varieties including Pinot noir, Pinot gris, Riesling and Grenache (Source: Australian Wine and Brandy Corporation). Currently, Australia is the 9th largest wine producing country in the world (Souce: http://faostat.fao.org; Food and Agriculture Organization of the United Nations) but is ranked 4th largest in total wine exports. In the 12 year period between 1994-95 and 2006-07 the value of Australian wine exports increased yearly to go from AUD$385 million to a peak of AUD$2.9 billion in 2006-07. However, in the three years following 2006-07 the value of Australian wine exports has steadily declined to $2.2 billion in 2009-

10 (Source: Australian Bureau of Statistics). The reduction in the value of exports in recent years is not reflected in the total volume of wine exported, which in 2009-10 was greater than the volume exported in 2006-07. It is thought that the reduction in export sales is in large due to a current surplus of wine grapes grown in Australia further compounded by reduction in global demand as a result of the global financial crisis. In order for the value of Australian wine exports to remain strong there is a need to ensure that the quality of exported wine remains high or that the product suits new markets. A predominant attribute of wine that is associated with quality is its flavour and aroma.

1.2 Wine flavour and aroma

The flavour of wine, as perceived by the consumer, is determined by the complex mixture of volatile and semi-volatile compounds present. The flavour compounds of a finished wine are commonly grouped into three categories; grape-derived, fermentation-derived and post-fermentation-derived. Fermentation-derived flavour compounds may be produced by yeast or other microbes during the fermentation process from grape berry precursors in the form of secondary metabolites or from more extensive metabolism of grape primary metabolites such as sugars and amino acids. Therefore, the type of yeast strain and/or species used during the primary alcoholic fermentation can greatly affect wine flavour, as can the strain of bacteria used during malolactic fermentation (Swiegers *et al.*, 2005). Post-fermentation treatments such as oak barreling, fining treatments and bottle-ageing also influence the complexity of wine flavour by introducing, removing or altering the array of flavour compounds. However, grape-derived aroma compounds have a significant role to play in determining wine flavour and aroma attributes. This is evident in the fact that wines produced using different grape

varieties have fundamentally different flavour and aroma characteristics, which is due to the differing compositions of the berries (Dunlevy *et al.*, 2009). The ability of different grape varieties to synthesize different aroma compounds is determined by the differences in the genomes of the varieties. Nevertheless, the flavour profile of any given grape variety is also influenced by many factors, such as the environmental conditions during the growing season, management of the vineyard and harvest timing.

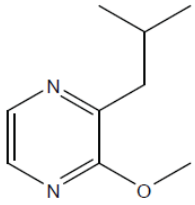
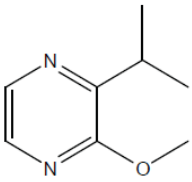
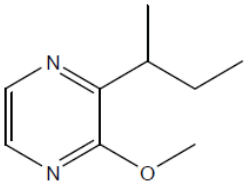
Sensory attributes that are prominent and easily distinguishable in certain varieties are known as varietal characters. Examples of varietal characters include the floral aromas commonly associated with Muscat varieties, which are attributed to high levels of a group of volatile compounds known as monoterpenes (Strauss *et al.*, 1986; Rapp, 1998) and the kerosene-like flavour common to aged Riesling wines. This kerosene-like character has been shown to be due to the presence of the norisoprenioid,1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), which is formed via the acid hydrolysis of grape-derived carotenoids (Simpson, 1978; Winterhalter, 1991). Another prominent varietal character is the herbaceous or vegetative flavour that has long been associated with a number of grape varieties originating from the Bordeaux region, including Sauvignon blanc, Cabernet Sauvignon, Cabernet franc, Carmenere and Merlot (Belancic and Agosin, 2007; Lacey *et al.*, 1991). It has been shown that these distinct herbaceous characters are attributed to the presence of aroma compounds collectively known as methoxypyrazines (Lacey *et al.*, 1991; Noble *et al.*, 1995).

1.3 Methoxypyrazines

1.3.1 Identification and properties of methoxypyrazines

3-Alkyl-2-methoxypyrazines (MPs) are a family of volatile aroma compounds most commonly found in, and associated with, various vegetables. MPs are among some of the most potent aroma compounds known, with extremely low odour detection thresholds (Table 1.1) that have been reported as low as 1-2 $ng.L^{-1}$ in water (Abassi *et al.*, 1998; Buttery *et al.*, 1969; Murray *et al.*, 1970; Seifert *et al.*, 1970). As MPs are extremely potent they are typically found in nature in very low concentrations, which made their initial identification a challenging task.

Table 1.1 – Structures and odour properties of MPs

Name	Structure	Odour Description	Odour Threshold
3-Isobutyl-2-methoxypyrazine (IBMP)		Green capsicum[1, 2, 3] Herbaceous[4] Earthy[4] Musty[3] Leafy[3]	2 $ng.L^{-1}$ in water[1, 3] 2 $ng.L^{-1}$ in model wine[3] 1 $ng.L^{-1}$ in white wine[5] 15,16 $ng.L^{-1}$ in red wine[3, 6]
3-Isopropyl-2-methoxypyrazine (IPMP)		Green capsicum[1, 3] Green peas[2] Asparagus[8] Earthy[3, 4]	1,2 $ng.L^{-1}$ in water[3, 8] 2 $ng.L^{-1}$ in model wine[3] 2 $ng.L^{-1}$in white wine[5] 2 $ng.L^{-1}$in red wine[3]
3-sec-butyl-2-methoxypyrazine (SBMP)		Galbanum oil[2] Moth balls[7] Green peas[2] Musty[3]	1 $ng.L^{-1}$ in water[8]

[1] (Buttery *et al.*, 1969), [2] (Murray and Whitfield, 1975), [3] (Maga, 1989), [4] (Hashizume and Samuta, 1997), [5](Allen *et al.*, 1991), [6] (Roujou de Boubee *et al.*, 2000), [7] (Mihara *et al.*, 1990), [8] (Murray *et al.*, 1970)

3-Isobutyl-2-methoxypyrazine (IBMP) was first identified and isolated from green capsicum (bell pepper), where it was found to be the principle compound responsible for the distinct capsicum odour (Buttery *et al.*, 1969). In this study, 5 kg of capsicums were required to effectively identify IBMP by mass, infrared and UV absorption. Subsequently, the compound 3-*sec*-butyl-2-methoxypyrazine (SBMP) was identified from galbanum oil (Bramwell *et al.*, 1969), in which it is the major odorant and hence SBMP is described as having an odour reminiscent of this oil (Murray and Whitfield, 1975). The perception of SBMP has also been referred to as "moth ball-like" (Mihara *et al.*, 1990). A third MP, 3-isopropyl-2-methoxypyrazine (IPMP), was later identified in green peas (Murray *et al.*, 1970). IPMP is commonly described as "sweet pea-like" and "asparagus-like" (Murray *et al.*, 1970), but it is also sometimes associated with the earthy aroma reminiscent of roots (Gerber, 1977).

A later study utilised early gas chromatographic techniques to survey these three MP compounds in 27 common vegetables (Murray and Whitfield, 1975). This study found that at least one MP compound was detectable in all but four of the vegetables examined and all three compounds were present in 13 of the 27 vegetables. Not unexpectedly, green capsicum was found to contain the highest concentration of IBMP (~50,000 $ng.L^{-1}$), with IPMP the highest in green peas (~3,500 $ng.L^{-1}$) and the greatest amount of SBMP was found in beetroot (~5,600 $ng.L^{-1}$).

1.3.2 Biological function of methoxypyrazines

MPs are widely distributed throughout the biological kingdoms and have been identified in insects, vertebrates, marine organisms, fungi and bacteria (Gallois *et al.*, 1988; Cheng *et al.*, 1991; Gerber, 1977; Murray and Whitfield,

1975). The convergent appearance of these compounds in many unrelated species suggests that MPs have a significant biological function. A major clue as to the possible function of MPs is that they have been found to be present at high levels in many aposematic (warningly coloured) toxic insects, such as the ladybird beetles, the Monarch butterfly and the tiger moth, as well as in toxic plants such as Poppies and Ragwort (Moore *et al.*, 1990; Rothschild, 1987; Rothschild *et al.*, 1984). These findings led ecologists to suggest that MPs act as a deterring odour signal to potential predators, effectively warning of the toxicity of the host plant or insect (Rothschild *et al.*, 1984). This theory was strengthened when a study found that naive hatchling chicks displayed a neophobic (fear of something new) alerting reaction when presented with drinking water to which IBMP had been added, confirming that birds interpret IBMP as an alerting or warning signal (Guilford *et al.*, 1987), and this observation was recently confirmed in wild populations of robins (Siddall and Marples, 2011).

In grape berries MPs accumulation is greatest when the berries are unripe, therefore the role of MPs as a deterring signal seems logical as the MPs are present when it is disadvantageous to the species if the berries are eaten. As the berries ripen, and the seeds within mature, the levels of MPs decrease dramatically (Hashizume and Samuta, 1999; Ryona *et al.*, 2009) thereby making the fruit more palatable to potential seed dispersers. Considering grapes, like most fruits, emit a range of desirable attracting aroma compounds when the fruit is matured and ready to be eaten (Hardie and Obrien, 1988), it is possible that the species has also evolved to emit undesirable aroma compounds when it is not beneficial to the plant for the fruit to be eaten.

Interestingly, as well as acting as a deterrent to predators, IPMP has been found to act as a pheromonal attractant between adult ladybird beetles (Abassi *et*

al., 1998). As ladybird beetles are not herbivorous insects but feed on aphids and scales, it is also possible that grapevines may have evolved to produce IPMP as a way of attracting ladybirds to protect against herbivorous pests. Such tritrophic interactions have been increasingly discovered in the last two decades (for review see (Dicke, 2009)).

1.3.3 Methoxypyrazines in grapes and wine

1.3.3.1 Detection and quantification of MPs

Following the identification of MPs in vegetables, it was proposed that IBMP was responsible for the distinct vegetative/green capsicum flavour of Cabernet Sauvignon grapes and the compound was tentatively identified as a component of grapes from this variety (Bayonove *et al.*, 1975). Later IBMP and IPMP were detected in grapes of the variety Sauvignon blanc, which also produces wines with vegetative characters (Augustyn *et al.*, 1982). The increase in the sensitivity of gas chromatographic techniques, allowed both IBMP and IPMP to be quantified in Sauvignon blanc wines (Harris *et al.*, 1987), and these compounds were later confirmed to be responsible for the vegetative varietal characters of wines made from this variety (Allen *et al.*, 1995; Allen *et al.*, 1991). Subsequently, MPs have also been detected in the grapes and wines of Cabernet Sauvignon, Cabernet franc, Merlot and Carmenere, all of which are varieties that are known to produce wines which can have vegetative sensory characters that are typical of MPs (Belancic and Agosin, 2007; Kotseridis *et al.*, 1999; Lacey *et al.*, 1991). Table 1.2 shows the concentrations of MPs reported in wines of various cultivars. It is evident from these values that IBMP is the predominant MP in wines, where it is commonly found at concentrations of up to 56 $ng.L^{-1}$ in cultivars known to display vegetative characters. IPMP has been

detected less often than IBMP in wines and when present tends to be at concentrations lower than IBMP. SBMP has been rarely detected in wines, and is therefore considered to be of minimal importance to wine flavour.

Table 1.2 Reported MP concentrations ($ng.L^{-1}$) in wines of different varieties. m indicates mean value, n indicates number of samples, nd denotes not detectable.

Variety	IBMP	IPMP	SBMP
Sauvignon blanc	0.5-38 n=22[1] 0.4– 44 n=575[2]	nd-5.0 n=22[1] nd-3.9 n=575[2]	nd -2.0 n=22[1] nd[3]
Cabernet Sauvignon	5-30 m=18 n=37[4] 3.6-56 m=19 n=12[6]	nd[5]	nd-1.9 m=0.35 n=8[6] nd[5]
Cabernet franc	6-43 m=16 n=29[4] m=14 n=6[5]		
Merlot	4-23 m=12 n=23[4] 6-12 m=8 n=12[7]	nd[5]	nd[5]
Carmenere	5-45 m=21 n=30[5]	nd-8.6 n=14[5]	

[1] (Lacey *et al.*, 1991) [2] (Alberts *et al.*, 2009), [3](Schmarr *et al.*, 2010), [4] (Roujou de Boubee *et al.*, 2000), [5] (Hashizume and Umeda, 1996), [6] (Allen *et al.*, 1994), [7] (Kotseridis *et al.*, 1999).

1.3.3.2 Sensory perception of MPs in wine

Wines that contain perceivable levels of MPs are often described as grassy, vegetative, herbaceous, earthy and green capsicum or asparagus-like. It is widely accepted that when the vegetative characters associated with MPs are at intense levels in wine they are undesirable and detrimental to the overall wine quality (Parr *et al.*, 2007). Indeed, one study has shown that in Sauvignon blanc wines the presence of MPs is negatively correlated with more desirable ripe and fruity flavours (Parr *et al.*, 2007). However, when the characters attributed to MPs are subtle and in balance with desirable fruity flavours, some consumers find

their sensory perception to be enjoyable and to add important complexity to wines (Heymann and Noble, 1987). As MPs are only found in a limited number of grape varieties, particularly knowledgeable consumers regard the aromas imparted by MPs to be of great importance to the distinguishing varietal characteristics of these wines (Marais and Swart, 1999; Preston *et al.*, 2008).

The odour detection thresholds for MPs in red, white and model wines have generally been found to be the same as that in water, at 1-2 $ng.L^{-1}$ (Allen *et al.*, 1991; Maga, 1989; Pickering *et al.*, 2007). The only exception to this is IBMP, which in two separate studies was found to have a higher odour detection threshold of 15 and 16 $ng.L^{-1}$ in red wines (Maga, 1989; Roujou de Boubee *et al.*, 2000). A recent study in Cabernet Sauvignon found that when wines were spiked with either bell pepper or fruit flavours (strawberry, raspberry, blackcurrant and cherry) the intensity of these characters increased proportionately. However, when the wine was spiked with a combination of both flavours, the fruity aromas appeared to mask the vegetative aromas (Hein *et al.*, 2009). This finding may explain why in red wines, which typically display fruity characters, the odour detection threshold of IBMP has been reported at 15-16 $ng.L^{-1}$ compared with 1 $ng.L^{-1}$ in white wines and water. Another study found that within 16 Californian Cabernet Sauvignon wines, the bell pepper/capsicum character was not correlated with levels of IBMP or IPMP, suggesting that other wine components can strongly influence the perception of the characters attributed to MPs (Preston *et al.*, 2008).

Thus it appears that when the vegetative characters attributed to MPs are in balance with strong and dominating fruity flavours, their vegetative perception is subtle and adds complexity. However, if a wine is low in fruity flavours or

contains an excessive amount of MPs, the vegetative flavours may become overpowering and detrimental to wine quality.

1.3.3.3 Evaluation of remedial treatments to reduce MP levels in wine

There are a number of techniques that winemakers occasionally use in an attempt to "rescue" a wine displaying undesirable flavour and aroma characteristics. The intention of these treatments is to remove the compounds responsible for the undesirable characters, known as taints. However, in doing so, these non-selective treatments often also remove desirable flavours and are therefore of limited benefit to overall wine quality. A recent study investigated the applicability of such treatments, including the addition of bentonite, activated charcoal or oak chips to wine, as well as exposure to light, for the removal of IPMP from wine (Pickering *et al.*, 2006). This study found that the addition of activated charcoal to young wine for seven days successful reduced the levels of IPMP by 34%. However, the addition of activated charcoal was not successful in reducing the asparagus and bell pepper attributes associated to IPMP and this is likely due to the simultaneous removal of other wine aromas (Lopez *et al.*, 2001). In this study no other refining treatments were successful in reducing IPMP levels, but the addition of oak chips did significantly reduce the asparagus and bell pepper characteristics associated with IPMP (Pickering *et al.*, 2006), probably due to a masking of IPMP by oak-derived flavour compounds introduced via the chips (Perez-Coello *et al.*, 2000).

Another study investigated whether different yeast strains have the ability to reduce IPMP levels during the fermentation of Cabernet Sauvignon juice (Pickering *et al.*, 2008). It was found that 3 of the yeast strains tested produced wines with similar IPMP levels while one strain actually increased the

concentration of IPMP by 11% (Pickering *et al.*, 2008). Another study investigating MP levels during wine storage found there was no consistent effect on the MP levels in wines stored under different light and temperature conditions over a 12 month period (Blake *et al.*, 2010).

These same authors also investigated the effect of different wine closures on Cabernet franc and Riesling wines spiked with MPs (Blake *et al.*, 2009). Natural corks, synthetic corks and screw caps had no significant effect on MP concentrations in the spiked wines. Surprisingly, when the wine was stored in Tetra Pak cartons for 18 months, the levels of IBMP and IPMP in both wines were successfully reduced by 45% and 32% respectively (Blake *et al.*, 2009). However, it is unclear if storage in this medium also resulted in changes in desirable wine aromas, or whether the use of this enclosure would be beneficial to the marketability of a wine as Tetra Pak cartons are not a traditional wine storage container.

To date, there is no known effective treatment for reducing MP levels in wine without the potential to compromise positive attributes of the wine. It has been shown that the concentration of IBMP in a given wine is strongly correlated ($R^2 = 0.97$, $p < 0.0001$) with the concentration of IBMP in the grapes used for vinification (Kotseridis *et al.*, 1999; Roujou de Boubee *et al.*, 2002; Ryona *et al.*, 2009). Therefore, given the lack of a reliable method of removing MPs from wines, the most effective strategies for producing wines with a desirable level of vegetative characters will involve controlling the concentration of MPs present in the grapes at harvest.

1.3.4 Accumulation of methoxypyrazines in grapes

Given the importance of MPs, particularly IBMP, to the flavour and aroma of wines of certain varieties, many studies have focused on understanding the accumulation of these compounds in grapes, and how environmental and viticultural factors affect their concentration at harvest. Table 1.3 lists some concentrations of MPs reported in mature grapes of different varieties.

Table 1.3 Reported MP concentrations ($ng.kg^{-1}$) in mature grapes of different varieties. m indicates mean value, n indicates number of samples, nd denotes not detectable.

Variety	IBMP	IPMP	SBMP
Sauvignon blanc	0.6-78 m=16 n=15[1]	nd-6.8 m=1.1 n= 15[1]	nd-0.6 n=15[1]
	3[2], 3.6[3]	0.7[3]	nd[4]
Cabernet Sauvignon	17-54 m=37 n=12[5]	4-12 m=8.5 n=12[5]	1.2[6]
	2.8-37 m=13 n=5[7]	0.2[3]	
	13[4], 9.1[3]	0.3[6]	
	4.2[10], 3.0[6]		
Cabernet franc	3.6-23.7 m=10 n=13[8]		
	9[2]		
Merlot	14.8[3], 6.7[9]	0.4[3]	
Carmenere	15-100 m=47 n=9[10]	1.1-8.5 m=3.8 n=9[10]	
Semillon	24.2[3], 2[2]	2.1[3]	

[1] (Lacey *et al.*, 1991) [2] (Koch *et al.*, 2010), [3] (Hashizume and Samuta, 1999), [4] (Schmarr *et al.*, 2010), [5] (Battistutta *et al.*, 2000), [6] (Hashizume and Umeda, 1996), [7](Noble *et al.*, 1995), [8] (Ryona *et al.*, 2008) [9] (Scheiner *et al.*, 2010), [10] (Belancic and Agosin, 2007).

1.3.4.1 Location of MPs within grape berries and bunches

A study investigating the location of IBMP within mature Cabernet Sauvignon berries found that grape skins contain IBMP concentrations twice that

of the seeds, while minimal IBMP was found in the flesh (Roujou de Boubee *et al.*, 2002). The location of IBMP primarily within grape skins has implications during wine making as this explains why the free run juice of grapes generally has lower concentrations of MPs than pressed juice or juice macerated in the presence of skins and seeds (Roujou de Boubee *et al.*, 2002; Sala *et al.*, 2004; Sala *et al.*, 2005; Kotseridis *et al.*, 1999).

The stem (rachis) of grape bunches has also been found to contain significant levels of MPs (Hashizume and Samuta, 1997; Hashizume and Umeda, 1996). This can also be a source of MPs in wines as stems are occasionally added to fermentations to add tannin and phenolic compounds that are different from those found in the berry skins and seeds, thereby increasing the astringency and bitterness of wine (Boulton *et al.*, 1995). Interestingly, varieties which lacks MPs in the berries, such as Pinot noir and Muscat Bailey A, have been found to contain IPMP and IBMP at high levels (3.5 - 619.7 $ng.L^{-1}$) in the bunch stems (Hashizume *et al.*, 1998; Hashizume *et al.*, 2001a). The apparent organ-specific accumulation of MPs in grapevines could provide useful comparative information that will be important in understanding the biosynthesis of MPs in grape berries.

1.3.4.2 Effect of grape berry maturity on MP concentration

The concentration of MPs varies greatly according to the developmental stage of the grapes and the general trend is illustrated in Figure 1.1. The accumulation of MPs begins one to two weeks after flowering and they reach a peak in concentration approximately one week prior to véraison (véraison is a French term for colour change, used to signify the onset of grape ripening and sugar accumulation). IBMP concentrations within unripe berries have been reported at 200-247 $ng.L^{-1}$ in Cabernet franc (Ryona *et al.*, 2009; Scheiner *et al.*,

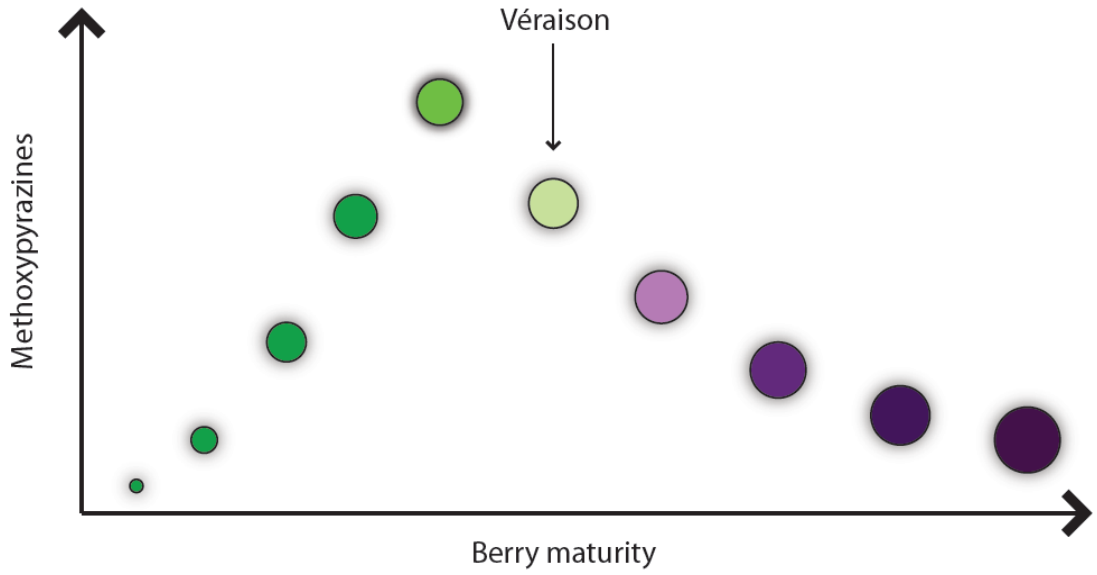


Figure 1.1 – Schematic of the typical accumulation pattern of MPs in developing grape berries. MP accumulation pattern based on the findings of Hashizume and Samuta, 1999; Koch *et al.*, 2010; Ryona *et al.*, 2009 and Scheiner *et al.*, 2010.

2010), 143-157 $ng.L^{-1}$ in Cabernet Sauvignon (Hashizume and Samuta, 1999; Koch *et al.*, 2010), and 211, 104 and 94 $ng.L^{-1}$ in Merlot, Semillon and Sauvignon blanc respectively (Koch *et al.*, 2010). Beginning at the onset of véraison, the MP content of berries declines rapidly and generally continues to decline gradually through until harvest. In mature grapes IBMP concentrations have been reported to be between 0-16% of the respective pre-véraison concentrations, and the size of this decrease is largely dependent on the length of ripening time (Hashizume and Samuta, 1999; Koch *et al.*, 2010; Ryona *et al.*, 2009; Scheiner *et al.*, 2010). In the post-véraison stages of ripening, the physical expansion of berries results in the dilution of many compounds (Conde *et al.*, 2007). However, the dilution of IBMP due to berry expansion only accounts for approximately 10% of the reduction in IBMP concentrations (Ryona *et al.*, 2009), and therefore it appears that other active processes must be involved in the reduction of MP concentrations during the ripening stages of grape berry development. Possible explanations for the decline in MP concentrations during development include further metabolism, volatilization out of the berry and possible degradation by sunlight.

1.3.4.3 Effect of sunlight exposure on the concentration of MPs in grapes

The decrease in MP concentrations during the ripening stage of berry development has largely been attributed to photo-degradation of the compounds, as it has been observed that MPs are degraded by light *in vitro* (Heymann *et al.*, 1986). The effect of sunlight exposure on IBMP levels has received much attention, as this is a factor that can be easily manipulated in the vineyard and therefore provides a potentially effective strategy for manipulating the MP content of grapes.

Indeed a number of studies have shown that increased sunlight exposure within the vineyard leads to reduced levels of IBMP (Marais *et al.*, 1999; Noble *et al.*, 1995; Ryona *et al.*, 2008; Scheiner *et al.*, 2010). The first of these studies showed that grapes from vines with low light intensity in the fruit zone, due to shading from a vigorous canopy, contained approximately 3.5-10 fold higher concentrations of IBMP than grapes from vines within the same vineyard with higher fruit zone light intensity (Noble *et al.*, 1995). However, other variables existed between the vines used for this study, including the water holding capacity, drainage and nutritional content of the soils, which were proposed to be largely responsible for the differences in the vines vigor. Another study shaded Sauvignon blanc vines by training the two adjacent vines to provide increase canopy shading of the target vine (Marais *et al.*, 1999). This study found that over two years and within three growing regions of South Africa grapes of shaded vines contained between 20 and 300% higher levels of IBMP than the non-shaded controls in the 3 weeks proceeding harvest (Marais *et al.*, 1999).

Recently more detailed studies have confirmed that sunlight exposure to berries directly results in a reduction in IBMP levels. Furthermore, these studies demonstrate that its influence is greatest during pre-véraison stages of development as opposed to post-véraison when photo-degradation of the compound was thought to occur (Ryona *et al.*, 2008; Scheiner *et al.*, 2010). Ryona *et al.* (2009) thinned Cabernet franc vines by removing shoots at flowering to produce regions of high and low light exposure to different bunches on the same vine. Exposed grape bunches were found to have 21-44% less IBMP than shaded bunches pre-véraison but no significant differences were observed at harvest. Scheiner *et al.* (2010) investigated this further by determining the effect of removing the basal leaves surrounding grape bunches at different times

throughout the development of Cabernet franc berries. These authors found that removal of basal leaves from around grape bunches had the greatest effect in reducing IBMP levels when performed at 10 days after flowering, which reduced IBMP levels at harvest (125 days after flowering) by approximately 89% compared to controls. Leaf removal at 40 and 60 days after flowering also reduced IBMP levels at harvest by 68% and 46% respectively compared to control bunches. As sunlight exposure appears to have the greatest effect in reducing IBMP levels when applied early in berry development, when IBMP appears to be synthesized, it has been proposed that sunlight exposure may actually decrease IBMP synthesis rather than increase the photo-degradation of the compound (Ryona *et al.*, 2008; Scheiner *et al.*, 2010).

However, results contrary to this have also been observed. A study that used sackcloth to artificially shade Cabernet Sauvignon berries at véraison found no significant differences in IBMP concentration in the pressed juice of the grapes at harvest compared to non-shaded controls (Sala *et al.*, 2004). When the juice from these samples was macerated for one day in the presence of the skins (where the majority of grape IBMP is located, see section 1.3.4.1), the concentrations of IBMP increased approximately 4 fold and the wines produced from these grapes had a significant difference in IBMP concentrations, with the wine made from exposed berries containing 62% more IBMP than the wine made from the shaded berries (Sala *et al.*, 2004).

Another study found that when Cabernet Sauvignon berries were exposed to light, concentrations of IPMP decreased by approximately 40 % over a 120 h period. However, no significant decrease was seen in IBMP levels in the same samples (Hashizume and Samuta, 1999). When unripe grapes were used, light exposure was found to have the opposite effect and actually increased the

concentrations of both IBMP and IPMP by 33% and 20% respectively. When the unripe berries were treated with $CaCl_2$, to inhibit enzymatic reactions that may be involved in the synthesis of MPs, both IBMP and IPMP levels decreased exponentially when exposed to light. These authors suggested that light exposure has two opposite effects: promoting the formation of MPs in unripe grapes; and the photo-degradation of MPs in ripening grapes (Hashizume and Samuta, 1999).

Despite these contradictory results, most of the published data suggests that light exposure in the vineyard leads to a reduction in IBMP content in berries. However, it is unclear whether this reduction is result of photo-degradation of IBMP or possibly due to other factors, such as a decrease in MP biosynthesis or possibly a result of increased thermal-degradation, as exposed berries tend to be of a higher temperature than shaded berries (Smart and Sinclair, 1976).

1.3.4.4 Effect of temperature on the concentration of MPs in grapes

Another environmental factor that influences MP concentrations in grapes is temperature. It has been observed that vines grown in cool climatic regions tend to produce grapes and wines with higher vegetative and herbaceous characters than grapes grown in warmer climates (Heymann and Noble, 1987). Notable examples of this are the Sauvignon blanc wines from New Zealand's Marlborough region which are often characterised by high levels of vegetative flavour (Parr *et al.*, 2007).

Indeed a number of studies have shown that the MP content of grapes is typically greater in cooler regions. One such study found that a sample of Sauvignon blanc wines from New Zealand had mean IBMP and IPMP concentrations of 25.9 and 4.4 $ng.L^{-1}$ respectively, while Sauvignon blanc wines from typically warmer Australia were significantly lower ($p < 0.001$) in IBMP and

IPMP concentrations with means of 6.8 and 1.3 $ng.L^{-1}$ respectively (Lacey *et al.*, 1991) . Similarly a strong negative correlation ($R = 0.754$, $p < 0.005$) was seen between long-term mean January temperatures and IBMP concentrations in Cabernet Sauvignon wines from both Australian and New Zealand (Allen *et al.*, 1994). The effects of temperature on IBMP accumulation has not just been observed in vineyards from different regions but also from the same vineyard over different growing seasons (Belancic and Agosin, 2007; Chapman *et al.*, 2004; Kotseridis *et al.*, 1999; Lacey *et al.*, 1991). One study measured the IBMP content of Sauvignon blanc wines made from the same vineyard over seven consecutive vintages and found that concentrations ranged from 6.4 to 38 $ng.L^{-1}$ with a relative standard deviation of 60% (Alberts *et al.*, 2009).

These studies provide strong evidence that the temperature in different grape growing regions can greatly influence the MP levels in the grapes, with lower temperatures tending to produce grapes with higher MP content. However, it is unclear if the effect of temperature on MP accumulation is through changes in the rate of biosynthesis or degradation of the compounds.

1.3.4.5 Effect of water status on the concentration of MPs in grapes

In an experiment conducted in Spain, Cabernet Sauvignon vines that were not irrigated were found to produced grapes with undetectable levels of IBMP in the juice at harvest, while 3.9 $ng.L^{-1}$ of IBMP was present in the juice of grapes from irrigated vines (Sala *et al.*, 2005). It has also been observed that growing seasons with high levels of summer rainfall are associated with increased grape IBMP accumulation (Belancic and Agosin, 2007; Roujou de Boubee *et al.*, 2000). Belancic and Agosin (2007) found that within three separate regions in Chile the IBMP concentrations of Carmenere grapes in 2004 were between 2.3 and 6.7 fold

higher than in the other years of the study (2003 and 2005), which was attributed to a 3-12 fold increase in summer rainfall in 2004. The authors suggest that the increase in IBMP levels in that year of heavy summer rains was a result of increased vegetative growth leading to decreased sunlight exposure of the grapes (Belancic and Agosin, 2007). Similar observations have been observed in Cabernet Sauvignon vines of differing canopy vigour, arising from differences in the water holding capacity of the vines soil (Noble *et al.*, 1995). These studies display the difficulty in determining the ultimate cause of berry compositional changes as viticultural experiments are often confounded by the interaction between variables.

1.3.4.6 Effect of grape yield on the concentration of MPs in grapes

Grape yield has also shown to have an impact on the IBMP levels of grapes (Chapman *et al.*, 2004). In this study, Cabernet Sauvignon vines were pruned to produce six treatments differing in the number of buds per vine, and thus bunches per vine. Across two seasons significant ($p < 0.001$) negative correlations of $R^2 = 0.7385$ and 0.7007 were seen between buds per vine and the IBMP concentration in the resulting wines. In this study the vines with the lowest yield (12 buds per vine) produced wines with approximately double the IBMP concentration than the highest yielding vines (48 buds per vine). The authors noted that because of the pruning method employed the low yielding vines had fewer shoots and leaves than those with higher yields and consequently the bunches experienced more light exposure than those on the higher yielding vines (Chapman *et al.*, 2004). Therefore, despite the increase in light exposure, which previous experiments suggests leads to a reduction in MP levels (section 1.3.4.3), wines made from low yielding vines still accumulated significantly higher levels

of IBMP than wines made from high yielding vines. This suggests that fruit yield has a direct influence on IBMP levels independent of sunlight exposure. This may also explain the observations made in cooler climates (section 1.3.4.4) where both canopy growth and yield may be reduced.

1.3.4.7 Site of MP biosynthesis within grapevines

As the MP content of berries appears to be, in part, influenced by yield and vine vigor, it had been noted previously that the biosynthesis of MPs could occur in the leaves of grapevines and the compounds translocated into the berries via the xylem (Noble *et al.*, 1995; Ryona *et al.*, 2008). However, this theory was recently disproven by Koch *et al.* (2010). These authors employed a reciprocal grafting experiment using Cabernet Sauvignon vines, which do accumulate MP in the berries, and Muscat blanc vines, which do not accumulate MP in the berries. It was found that when Muscat blanc grape bunches were grafted onto vines of Cabernet Sauvignon the Muscat blanc grapes still did not accumulate IBMP, while the Cabernet Sauvignon grape bunches that were grafted onto Muscat blanc vines retained the ability to accumulate IBMP. These authors concluded that the accumulation of IBMP is dependent on the genotype of the berry, where IBMP is likely to be synthesised, and therefore not translocated to the berry from elsewhere in the vine (Koch *et al.*, 2010).

1.3.5 Ladybird beetle as a source of MP in wine

Although grapes provide the source of MPs in the vast majority of wines that it is encountered in, there is one other known source of MPs in wine. The invasive ladybird beetle *Harmonia axyridis* has been known to infest vineyards where they feed on aphids and coccids living on the grapevine. The ladybird,

which secretes IPMP as a pheromonal attractant (See section 1.3.2) can be incorporated into the winemaking process along with the harvested grapes, thereby tainting the wine with large quantities of IPMP (Pickering *et al.*, 2006). Native to Asia the ladybird beetle is most notorious as a wine taint in North America where it was introduced in 1916 as a biological control for aphids and coccids (Brown *et a*l., 2008). *Harmonia axyridis* is also rapidly spreading in Europe but it is not currently established in Australia (Brown *et a*l., 2008). Although ladybirds can drastically influence a wine by adding large amounts of undesirable IPMP, the number of occurrences is minimal in context of global wine production and therefore will not be expounded on further.

1.3.6 Pathway of methoxypyrazine biosynthesis

Despite the interest in identifying factors that influence MP accumulation in grapes, little work has been done in determining the pathway of MP biosynthesis. This is true in not just grapes but any plant species. However, a biosynthetic pathway for MPs has been proposed (Fig. 1.2). This hypothetical pathway begins with the reaction of an amino acid and an unknown 1,2-dicarbonyl compound leading to the formation of a 3-alkyl-2-hydroxypyrazine (HP) intermediate, which is then enzymatically methylated to form a MP (Leete *et al.*, 1992; Murray *et al.*, 1970). In the bacterial strain *Pseudomonas perolens*, which accumulates high levels of IPMP, feeding experiments have shown that the addition of ^{13}C labelled valine results in the production of ^{13}C labelled IPMP, confirming that amino acids are a precursor to MPs in bacteria (Gallois *et al.*, 1988). It is thought that the amino acids valine, leucine and isoleucine are the precursors of IPMP, IBMP and SBMP respectively, because of similarities in the

alkyl side chains of the compounds, as shown in Figure 1.3 (Murray and Whitfield, 1975).

Despite evidence that amino acids are a precursor to MPs it remains unclear by what mechanisms the given amino acid is converted to the HP intermediate. It has been proposed that the respective amino acid gains a second nitrogen through an unknown amidation reaction before undergoing a condensation reaction with an unknown 1,2-dicarbonyl compound to produce a HP (Murray *et al.*, 1970). Glyoxal is the 1,2-dicarbonyl compound commonly used in the chemical synthesis of MPs, however no reports could be found indicating its presences in grape berries. Two structurally similar compounds, acetaldehyde and glycoaldehyde, which are by products of pyruvic acid metabolism (Sweetman *et al.*, 2009) and tartaric acid biosynthesis (DeBolt *et al.*, 2006) respectively, could potentially be precursors in the formation of HPs in grape berries.

The final step in the proposed pathway, involving the methylation of HP to form MP, is thought to be catalysed by an *O*-methyltransferase enzyme. Indeed, a study in grapevines found that Cabernet Sauvignon berries contain enzymatic activity that is capable of methylating HPs to form MPs *in vitro* (Hashizume *et al.*, 2001a).

1.3.7 Grapes contain a methyltransferase enzyme that methylates HPs

Extensive literature searches found that the only known previous investigations into the biosynthesis of MPs in a plant species were performed in grapevines. In the first of these studies, it was demonstrated that crude protein extracts from unripe grape berries and grapevine shoots contained enzyme activity capable of performing the final methylation step in the putative pathway of MP biosynthesis

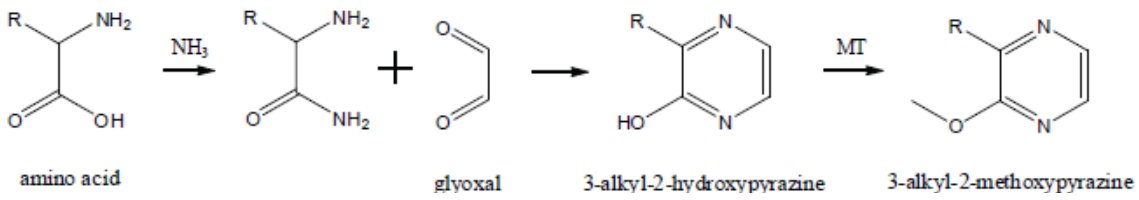


Figure 1.2 - The proposed biosynthetic pathway of MPs (Leete *et al.*, 1992; Murray *et al.*, 1970).

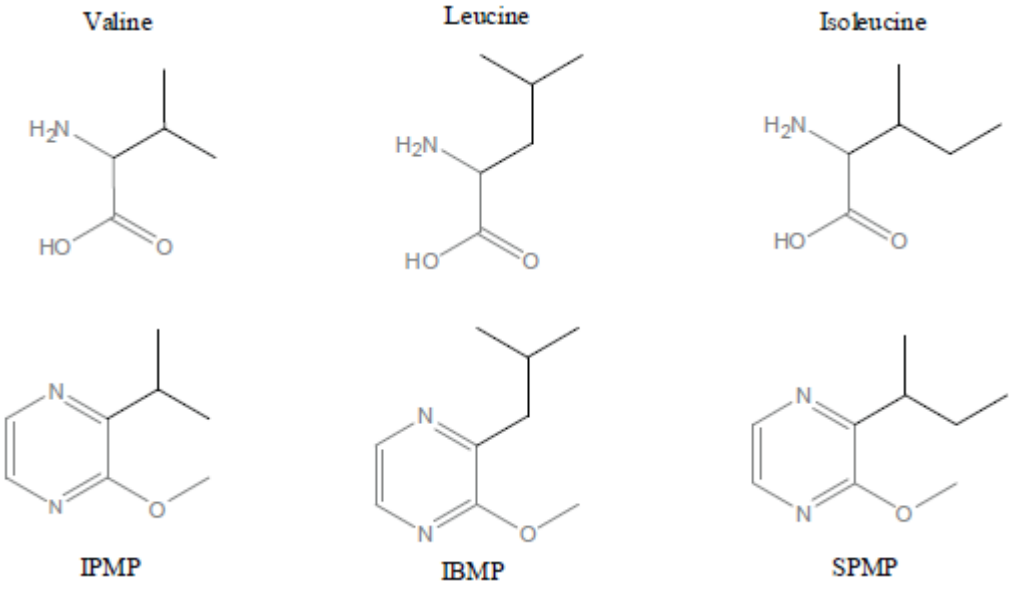


Figure 1.3 - Similarities between the alkyl side chains of MPs and their proposed amino acid precursors.

(Hashizume *et al.*, 2001a). Furthermore, these authors demonstrated that there is a strong association between the amount of HP methylating activity and the subsequent levels of MPs in the unripe berries of eight different cultivars (Fig. 1.4 A&B). Interestingly, the varieties Muscat Bailey A and Pinot noir, which were found to contain only trace levels of MPs, also contained only minimal levels of HP methylating activity (Hashizume *et al.*, 2001a). This finding suggests that some grape varieties do not accumulate MPs in berries because they are unable to perform this final step in MP biosynthesis in berry tissues.

In this same study, 3-isobutyl-2-methoxypyrazine (IBHP) and 3-isopropyl-2-hydroxypyrazine (IPHP) substrate concentrations were quantified in the unripe grape samples and were found to be between 2 and 20-fold higher than the corresponding MP concentrations in all cultivars (Fig. 1.4 C). This suggests that the enzymatic methylation of HP to MP is a rate-limiting step in the production of MPs, making it a good target for the manipulation of the MP content of grape berries.

In a subsequent study, the same authors purified an *O*-methyltransferase enzyme with HP-methylating activity from Cabernet Sauvignon shoots and

sequenced 22 amino acids of its N-terminus (Hashizume *et al.*, 2001b). *In vitro* functional assays of the purified *O*-methyltransferase showed that this enzyme is multifunctional and has the ability to methylate each of the HP precursors of IPMP, IBMP and SBMP. In addition to methylating HP substrates, this *O*-methyltransferase showed activity against a number of other hydroxyl containing molecules and was found to have a ten-fold greater binding affinity against caffeic acid than against HPs (K_ms of 0.3 and 0.032 mM respectively). This implies that HPs are not the preferred substrates of this OMT and that the

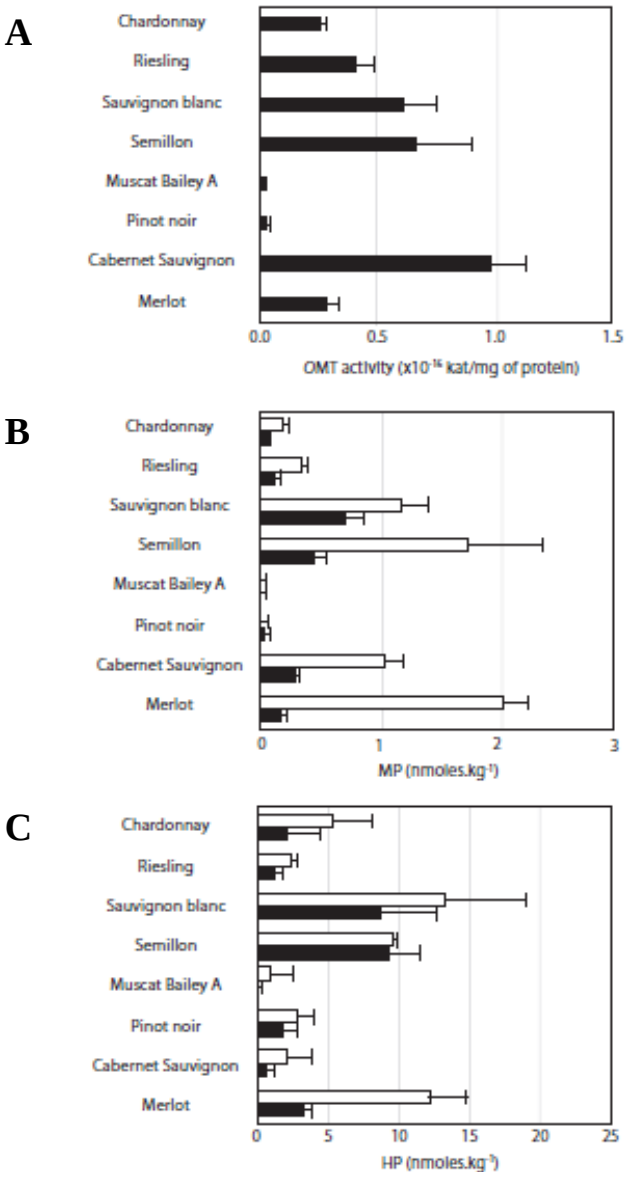


Figure 1.4 – A) HP methylating activity in crude protein extract from immature grapes of eight different varieties. **B**) Concentrations of IBMP (white bars) and IPMP (black bars) measured in the respective varieties, and C) concentrations of the precursors IBHP (white bars) and IPHP (black bars). Figures redrawn from Hashizume *et al.* (2001a).

biosynthesis of MPs in grapes may be a by-product of another metabolic pathway. This could have implications with regards to the flux of MP biosynthesis during grape berry development and in response to external stimuli if the nature of the primary pathway this OMT functions in is known.

1.4 Project aims

MPs are grape derived aroma compounds with highly odorous vegetative characteristics which, when present in wine at high levels, can have a detrimental effect on wine quality. MPs are present in only a few grape cultivars including, Sauvignon blanc, Cabernet Sauvignon, Merlot and Semillon, yet these four varieties account for approximately 29% of the total wine grapes currently grown in Australia (Source: Australian Wine and Brandy Corporation).

The final concentration of MPs in grape berries at harvest appears to be determined by the balance between the amount that is synthesised pre-véraison and the amount that is degraded/metabolised post-véraison. While there is growing knowledge of how viticultural and environmental factors can alter the MP content of berries, the metabolic processes responsible for these changes in MP concentrations during berry development and in response to various stimuli remains unclear.

The primary aim of this research project was to identify and characterise the *O*-methyltransferase gene or genes responsible for the final step in the pathway of MP biosynthesis in grapes. In addition, this project aimed to improve our understanding of MP accumulation in grape berries, and to investigate how the final rate limiting methylation step is affected by environmental factors and viticultural management practices known to influence MP concentrations in berries. This knowledge will lead to a greater understanding of the key

determinants of MP biosynthesis and accumulation in grapes, ultimately allowing better management decisions to be made in the vineyard to produce grapes and wines of a desired MP content to meet consumer demands, as well as applications for the breeding of new grape varieties.

Chapter 2 - Characterisation of two *O*-methyltransferases potentially involved in the final step of MP biosynthesis in berries

2.1 Introduction

MPs are a family of potent grape-derived aroma compounds that contribute herbaceous and earthy aromas to wines of some grape cultivars, such as Cabernet Sauvignon and Sauvignon blanc. Consumer appeal of wines produced from these cultivars is greatly influenced by the level of MPs present and this is directly correlated with the concentration of these compounds in the harvested grapes (Ryona et al. 2009). The extent of MP accumulation in berries is a result of the balance between the level of biosynthesis of these compounds and the degree at which they are degraded or metabolized during berry development. Recent studies have largely focused on how different environmental factors and viticultural management practices influence MP levels in grape berries as a tool for manipulating MP levels at harvest. While there is a growing knowledge of how these variables influence MP levels in berries, very little information exists on either the actual pathway of MP biosynthesis or the mechanisms of MP degradation in not just grapes but any plant species. A number of pathways have been proposed and all agree that the final step likely involves the methylation of hydroxypyrazine (HP) precursor to form MP, mediated by an *O*-methyltransferase enzyme (Leete *et al.*, 1992; Murray *et al.*, 1970).

2.1.1 O-methyltransferases are common to plant secondary metabolism

In plants secondary metabolites have vast and varied functions. These range from acting as hormones, interplant signaling molecules, antimicrobial agents or UV and chemo-protectants, to structural support molecules and as attractants of insect pollinators and seed disperser. Over 50,000 diverse secondary metabolites have been indentified throughout the plant kingdom (Hounsome *et al.*, 2008), many of which contain methyl group moieties. *O*-Methyltransferase (OMT) enzymes catalyse the transfer of methyl groups from the donor molecule *S*-adenosyl-L-methionine (SAM) to a hydroxyl group of an acceptor molecule. Methylation of hydroxyl groups is an important modification step in the metabolism of many plant secondary metabolites. *O*-Methylation alters the solubility of a compound which can greatly change its properties, such as its chemical reactivity, mutagenicity, antimicrobial activity, as well as its volatility and aroma. A good example of this is the highly odouriferous and volatile MPs, as their respective HP precursors are non-volatile and odourless.

In plants, OMTs fall into three distinct classes based on protein size, amino acid sequence and substrate preferences (Lam *et al.*, 2007; Ibrahim *et al.*, 1998). Class I OMTs are approximately 23-29 kDa in size and require a bivalent cation such as Mg^{2+} for catalytic activity. Often referred to as CCoAOMTs, class I members specifically methylate the Coenzyme A (CoA) linked esters of the lignin biosynthetic pathway, caffeoyl-CoA and 5-hydroxyferuloyl-CoA (Inoue *et al.*, 1998). As well as methylating CoA-linked substrates, a subgroup of class I OMTs have been shown to also contain activity against various flavonoids and phenylpropanoid compounds and have thus been termed CCoAOMT-like enzymes (Ibdah *et al.*, 2003; Lee *et al.*, 2008; Kim *et al.*, 2010).

Class II OMTs are approximately 38-43 kDa in size and have been found to act on a wide range of substrates. The class II OMTs also tend to be promiscuous, in that individual enzymes have activity against a number of different substrates. However, class II members are still commonly referred to as caffeic acid OMTs as early studies on lignin biosynthesis focused on their ability to methylate this substrate (Bugos *et al.*, 1991; Gowri *et al.*, 1991). As well as acting on caffeic acid and other non-CoA linked precursors to lignin, class II OMTs have been shown to be involved in the synthesis of a diverse range of secondary metabolites including flavonoids, flavonols, stilbenes, phenylpropenes, alkaloids, isoprenoids and various volatile phenolics (Akashi *et al.*, 2003; Gang *et al.*, 2002; Ageorges *et al.*, 2006; Schmidlin *et al.*, 2008; Wein *et al.*, 2002; Nomura and Kutchan, 2010; Pienkny *et al.*, 2009). Interestingly one class II OMT from *Catharanthus roseus* (Madagascar periwinkle) was unexpectedly found to methylate substrates containing sulfhydryl moieties rather than hydroxyl groups (Coiner *et al.*, 2006).

The third class of OMTs consist of members that specifically methlyate substrates containing carboxyl groups. These carboxyl OMTs have been found to specifically methylate compounds important to plant signaling such as benzoic acid (Kolosova *et al.*, 2001), salicylic acid (Ross *et al.*, 1999), jasmonic acid (Seo *et al.*, 2001), indole acetic acid (Qin *et al.*, 2005), and gibberellins (Varbanova *et al.*, 2007). Several members of this family have also been found to methylate the nitrogen moieties of alkaloid substrates involved in the biosynthesis of caffeine (Kato *et al.*, 2000; Ogawa *et al.*, 2001). This family of OMTs has been given the name SABATH, based on the first identified substrates of some of its members - salicylic acid, benzoic acid and theobromine.

A previous study comparing 36 different plant OMT cDNA sequences from all three classes revealed they show between 32-71% sequence homology at the protein level (Ibrahim *et al.*, 1998). This study also identified five conserved regions common to plant OMTs, thought to be involved in SAM and substrate binding. Another study successful determined the crystal structure of two class II OMTs from *Medicargo sativa* and identified specific residues involved in SAM binding, substrate binding as well as residues involved in the catalytic transfer of the methyl group (Zubieta *et al.*, 2001). Knowledge of the residues important for substrate binding has proven useful in predicting potential substrates of plant OMTs (Schroder *et al.*, 2002). However, a study using site-directed mutagenesis of OMTs has shown that even a single amino acid changes can alter an OMTs substrate preference (Gang *et al.*, 2002). Therefore sequences analysis alone can only provide clues to potential substrates of OMTs and *in vitro* activity tests are still required to establish substrate preference and activity.

A number of recent studies have exploited the fact that substrate preferences of OMTs can be altered by as little as single amino acid substitutions. These studies have used directed mutagenesis techniques to engineer OMTs with desired selectivity (Joe *et al.*, 2010; Zhou *et al.*, 2010). Engineering of the lignin pathway by this method has received major interest (Bhuiya and Liu, 2010), as lignin content has major implications in the timber, paper, animal feed and biofuel industries (Brenner *et al.*, 2010; Dixon *et al.*, 1996). Considering the beneficial properties of many plant secondary metabolites there is enormous potential for the use of OMTs in the bioengineering of existing and novel compounds (Willits *et al.*, 2004).

2.1.2 Plant OMTs involved in production or aroma compounds

Many of the class II OMTs previously characterised have come from flowering species in which they have been shown to be involved in the production of floral scent compounds (Wang *et al.*, 1997). In rose, two class II OMTs with 97% identity in their amino acid sequences, methylate orcinol resulting in the production of 3,5-dimethoxytoluene, a major volatile scent compound of Chinese roses (Lavid *et al.*, 2002). In the petals of Chinese rose species the expression of these two genes coincides with 3,5-dimethoxytoluene production, while a lack of expression was seen in two species of European roses that do not produce 3,5-dimethoxytoluene (Scalliet *et al.*, 2006). The two orcinol OMTs also have high activity against this substrate and further methylate it to form 1,3,5-trimethoxybenzene, another key component of Chinese rose odor (Wu *et al.*, 2003).

While an increasing number of studies have identified OMTs involved in floral scent production, few have identified OMTs involved in the production of aroma compounds in fruits. A multifunctional class II OMT has been isolated from strawberries which methylates 2,5-dimethyl-4-hydroxy-3(2H)-furanone to produce 2,5-dimethyl-4-methoxy-3(2H)-furanone, both of which have caramel like aromas important to strawberry flavour (Wein *et al.*, 2002). This OMT also has high activity against protocatechuic aldehyde resulting in the production of vanillin. Vanillin, as its name suggests, is the compound largely responsible for the flavour of vanilla. However, it is only of minor importance to strawberry flavour as it is present in small concentrations in this fruit.

Methyl salicylate is a volatile compound with a sweet aromatic odour and it is found in many flowers and fruits. An OMT belonging to the SABATH family was found to be responsible for the production of methyl salicylate from the

signaling molecule salicylic acid in *Clarkia breweri* (Ross *et al.*, 1999). Recently a carboxyl OMT, named SAMT, was shown to be responsible for the production of methyl salicylate in tomato fruits (Tieman *et al.*, 2010). These authors showed that over-expression of the *SAMT* transcript resulted in an increased level of methyl salicylate while silencing of the transcript resulted in a significant reduction in compound levels. Furthermore in a cross between a domestic tomato variety and a wild tomato, this *SAMT* co-segregates with a QTL associated with higher fruit methyl salicylate emissions.

2.1.3 OMTs characterized in grapevines

An analysis of EST libraries has suggested that at least 29 class I *CCoAOMT* and 56 class II *OMT* genes are present in the grapevine genome (da Silva *et al.*, 2005). Despite this large number only a few grapevine OMTs have been functionally characterised. A cDNA encoding a 27.2 kDa class I CCoAOMT enzyme with activity against both caffeoyl-CoA and 5-hydroxyferuloyl-CoA has been isolated from Pinot noir cell suspension cultures (Busam *et al.*, 1997). The *CCoAOMT* transcript levels were raised when cell cultures where treated with a fungal elicitor suggesting a possible role in cellular defense.

A second grapevine class I OMT, named *VvAOMT*, has been found to have activity against anthocyanins and other flavonol substrates (Hugueney *et al.*, 2009). Typical of class I members, VvAOMT is 26.4 kDa in size and requires the cation Mg^{2+} for activity, but shows greatest homology with the subgroup of CCoAOMT-like members which have activity against flavonoid compounds. It was shown that a rapid increase in *VvAOMT* expression occurs at véraison coinciding with the period of accumulation of methylated anthocyanins during

berry development. Furthermore *VvAOMT* was specifically expressed in the skins of grape berries where accumulation of anthocyanins occurs.

VvROMT is a class II OMT isolated from grapevine with greatest activity against the stilbene resveratrol (Schmidlin *et al.*, 2008). Transient coexpression of *VvROMT* with grapevine stilbene synthase in tobacco leaves resulted in the accumulation of pterostilbene, the methylated product of resveratrol. Both resveratrol and pterostilbene contain powerful antifungal and pharmacological properties and have thus received much research interest. The expression of *VvROMT* in grapevine leaves was found to be stress inducible, consistent with the role of pterostilbene as an antifungal agent. VvROMT shows closest homology to the orcinol OMTs from rose that catalyses the formation of the floral scent compound 3,5-dimethoxytoluene (Lavid *et al.*, 2002). However, VvROMT showed only minimal activity against this substrate.

2.1.4 Grapevine contains an OMT with activity against HPs

The only previous investigations into MP biosynthesis in a plant species that could be found in the scientific literature were performed in grapevines. In one of these studies the authors showed that Cabernet Sauvignon (CS) grape berries, a variety known to commonly accumulate high levels of MPs, contains enzyme activity capable of performing the final step in the putative biosynthetic pathway (Hashizume *et al.*, 2001a). In this study, chemically synthesized IBHP and IPHP substrates were added to crude protein extracts from CS berries which resulted in the formation of IBMP and IPMP respectively. The authors also compared protein extracts from eight different cultivars of varying MP levels and found that a strong correlation exists between HP methylating activity and MP accumulation in the berries (Hashizume *et al.*, 2001a). This implies that the

enzymatic methylation of HPs is a rate limiting step in the pathway of MP biosynthesis. The authors also detected HP methylating activity in CS grapevine shoots and purified a protein with this activity as well as obtaining a 22 amino acid sequence of its N-terminus (Hashizume *et al.*, 2001b). SDS-PAGE of this purified protein suggested that it exists as a dimer, with a native size of approximately 85 kDa and a subunit size of 41kDa typical of class II OMTs (Ibrahim *et al.*, 1998). *In vitro* functional activity test found that the enzyme was multifunctional with the ability to methylate a range of different compounds and does not require a divalent cation for activity, also typical of class II OMTs. The native enzyme showed a ten-fold lower K_m against caffeic acid than against IBHP (0.032 mM and 0.3 mM respectively), which implies that HPs are not the preferred substrates of this OMT. This suggests that the biosynthesis of MPs in grapes could be a by-product of another metabolic pathway, which could have implications with regards to the flux of MP production during berry development and in response to external stimuli.

2.1.5 Aims of the work presented in this chapter

As outlined above, previous work by Hashizume *et al.* (2001a, b) has shown that CS grapes contain an OMT enzyme capable of methylating HPs to form MPs and an enzyme with this activity was purified from CS shoots. Furthermore, this step appears to be rate limiting in the biosynthesis of MPs in grape berries and is thus a good target for manipulating MP content. The aim of the work presented in this chapter was to identify and characterise the gene from CS that encodes the enzyme purified by Hashizume *et al.* (2001b) to gain insights into MP biosynthesis in grape berries. Two candidate *OMT* genes were identified and characterised through recombinant enzyme activity assays and by investigating

temporal and spatial gene expression patterns in grape berries in relation to MP accumulation. RNAi mediated silencing of these two genes was also attempted in grapevine hairy-roots to provide evidence of their *in planta* function.

2.2 Materials and Methods

2.2.1 Chemicals, solutions and media

Solutions and growth media used in this project are described in Table 2.1. All chemicals, other than HP substrates, were purchased from Sigma-Aldrich (St Louis, MO, USA). Restriction endonucleases were obtained from New England Biolabs (Ipswich, MA, USA) or Roche Diagnostics (Basel, Switzerland). All oligonucleotide primers were purchased from Invitrogen (San Diego, CA, USA). The sequences of all primers are given in Appendix A.

The precursors to MPs, IBHP and IPHP, were chemically synthesized using the method of Gerritsma *et al.* (2003). For the synthesis of IBHP a 10 mmol solution of L-leucinamide hydrochloride (Sigma Aldrich) in 20 ml of methanol was cooled to -35°C in a dry ice bath. A 12 mmol solution of aqueous glyoxal was added with rapid stirring followed by the dropwise addition of 2 ml of 12 M sodium hydroxide solution. After 30 min the reaction was removed from the dry ice bath and allowed to warm to room temperature and stirred for a further 2 h. The reaction was then cooled to 0°C and 2 ml of 12 M hydrochloric acid and 2 g sodium bicarbonate then added to neutralize the reaction. The reaction was filtered and 20 ml of water added before the methanol was removed by rotary evaporation. Three 50 ml dichloromethane extractions were performed and the pooled solvent phases dried with 4 g of magnesium sulphate and subsequently removed by filtration. The dichloromethane solvent was removed by rotary evaporation to yield the dried IBHP product. IPHP was synthesized using the

same method but with L-valinamide hydrochloride as the starting material. The identity and purity of synthesized IBHP and IPHP were confirmed by ^{1}H and ^{13}C nuclear magnetic resonance (NMR), and by both low and high-resolution electrospray-ionization mass spectrometry. NMR and mass spectrometry were performed by the Commercial Analytical Service at Monash University School of Chemistry.

Table 2.1 Chemical solutions and media

Solution	Composition
RNA extraction buffer	0.3 M Tris-HCl, 5 M sodium perchlorate, 2% (w/v) PEG 6000, 1% (w/v) SDS, 8.5% (w/v) PVPP and 1% (v/v) β-mercaptoethanol
TE buffer	10 mM Tris-HCl, 1 mM EDTA, pH 7.5
LB	1% (w/v) bacto-tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCL, pH 7.0
ZYM 5052 auto-induction media	1% (w/v) N-Z-amine AS, 0.5% (w/v) yeast extract, 25 mM Na_2HPO_4, 25 mM KH_2PO_4, 25 mM $(NH_4)_2SO_4$, 1 mM $MgSO_4$, 1× trace metals, 2% (v/v) glycerol, 0.05% (w/v) glucose, 0.2% (w/v) α-lactose
His-Trap binding buffer	10 mM Na_2HPO_4, 10 mM NaH_2PO_4, 500 mM NaCl, 20 mM imidazole, pH 7.4
Protein storage buffer	50 mM Tris-HCl pH 7.4, 30 % glycerol and 500 mM NaCl
2× Loading buffer	20% (v/v) glycerol, 4% (w/v) SDS, 500 mM Tris-HCl, 2% (v/v) β-mercaptoethanol, 0.01% (w/v) bromophenol blue, pH 6.8
SDS running buffer	1.9 M glycine, 240 mM tris, 1% (w/v) SDS
Protein staining solution	10% (v/v) methanol, 10% (v/v) acetic acid, 0.1% (w/v) Coomassie blue
Destaining solution	40% (v/v) methanol, 10% (v/v) acetic acid
Western transfer buffer	190 mM glycine, 25 mM tris, 20% (v/v) methanol
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.7 mM KH_2PO_4
Citrate/phosphate buffer	100 mM $NaHPO_4$, 50 mM citric acid, pH 5.0

MGL media	0.5% (w/v) mannitol, 0.5% (w/v) tryptone, 0.25% (w/v) yeast extract, 0.1% (w/v) L-glutamate, 85 mM NaCl, 1.1 mM KH_2PO_4, 400 μM $MgSO_4$, 0.1 × Fe/EDTA, 82 μM biotin, pH 7.0
200× Fe/EDTA	100 μM EDTA, 55 μM $FeSO_4$
LG0 media	2.5% (w/v) sucrose, 0.5% (w/v) phytoagar, 0.23% (w/v) Murashige and Skoog Salts Mixture (Invitrogen), 0.05% (w/v) casein hydrolysate, 1× MS macros, 1× C1 vitamins, pH 5.7
1× MS macros	20.6 mM NH_4NO_3, 3 mM $CaCl_2.6H_2O$, 1.5 mM $MgSO_4.7H_2O$, 19 mM KNO_3, 1.25 mM KH_2PO_4
1× C1 vitamins	550 μM *myo*-inositol, 25 μM thiamine, 8 μM nicotinic acid , 5 μM pyridoxine 4.4 μM D-pantothenic acid, 40 nM biotin

2.2.2 Plant material

Cabernet Sauvignon grapes were sampled from a commercial vineyard in Waikerie, South Australia (34^o 06' S, longitude 139^o 50' E) during the 2007-2008 growing season. Samples were taken in triplicate from three areas of the vineyard at fortnightly intervals after flowering (flowering defined as 50% cap fall). Leaves, tendrils, rachis and flowers were sampled from another commercial vineyard in Willunga, South Australia (latitude 35^o 15' S, longitude 138^o 33' E). Root material was obtained from plantlets grown in tissue culture. In all cases samples were snap frozen in liquid nitrogen and stored at -80ºC until required. Skin, flesh and seed samples were obtained from whole berries by lightly breaking the berries while still frozen and the individual tissues separated by hand before the tissue could thaw.

2.2.3 RNA extraction and cDNA synthesis

Total RNA was isolated from grape tissue using a protocol based on the perchlorate method of Rezaian and Krake (1987) with modifications to remove any contaminating genomic DNA. A 2 g sample of finely ground frozen tissue

was added to 16 ml of RNA extraction buffer (Table 2.1) and stirred rapidly for 30 min at room temperature. This slurry was then squeezed through a 20 ml syringe plugged with approx $2cm^3$ of glass wool wrapped in 2 pieces of Miracloth. The eluate was collected and 2.5 volumes of chilled ethanol added and kept at -20°C for 2 hours to precipitate nucleic acids which were then pelleted by centrifugation at 4,100 × *g* for 20 min at 4°C. This pellet was rinsed twice with 70% (v/v) ethanol, air-dried and resuspended in 4ml of TE buffer (Table 2.1) containing 0.2% (v/v) β-mercaptoethanol. The suspension was extracted twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and the phases separated by centrifugation at 4,100 × *g* for 10 min at 4°C. The aqueous phase was collected and the RNA precipitated by adding 0.1 volumes of 3M sodium acetate and 2.5 volumes of ethanol and incubating at -20°C for 2 hours. Precipitated RNA was pelleted by centrifugation at 4,100 × *g* for 15 min at 4°C, washed with 70% (v/v) ethanol, air dried and resuspended in 200 µl of TE buffer. The sample was then centrifuged at 7,700 × *g* for 5 min at room temperature and the pellet discarded to remove polysaccharides. The RNA was further purified by precipitation with 0.44 volumes of 10 M LiCl at -20°C for 3 h after which the pellet was resuspended in 100 µL TE buffer. Genomic DNA was removed from 100 µL aliquots of total RNA from each sample by digestion with RNAse-free DNAse following the protocol supplied with the RNeasy mini kit (Qiagen, Venlo, The Netherlands). DNAse-treated RNA (1 µg) was used as template for cDNA synthesis by reverse transcription using the SuperScript III Reverse Transcriptase (Invitrogen) using an oligo$(dT)_{20}$ primer according to manufacturer's instructions.

2.2.4 Isolation of full length VvOMT cDNA clones

The partial N-terminal amino acid sequence of a previously purified grape protein with HP methylating activity (Hashizume et al. 2001b), was used to search EST and genomic databases for corresponding gene sequences. Full-length sequences of the two grape genes identified from this search (*VvOMT1* and *VvOMT2*) were amplified from Cabernet Sauvignon cDNA by PCR using the primer pairs MT1F1/MT1R1 and MT2F1/MT2R1 respectively (Appendix A). PCR products were cloned into the vector pDRIVE (Qiagen) and the constructs sequenced with the M13F and M13R3 primers (Appendix A) using the BigDye Terminator® v3.1 Cycle Sequencing Kit (Applied Biosystems, Victoria, Australia). Sequencing reads were performed by the Australian Genomic Research Facility (AGRF, Adelaide, Australia).

2.2.5 Phlyogenetic analysis of VvOMT encoded proteins

Amino acid sequence alignments were generated using the AlignX program in the VectorNTI software (Invitrogen). Phylogenetic analysis was conducted by first aligning sequences using the ClustalW program (Thompson et al. 1994) and the Seqboot program (Felsenstein 1989) was then used to produce 1,000 data sets. Maximum-parsimony phylogenetic trees were constructed and the final tree selected using the Protpars and Consensus programs (Felsenstein 1989).

2.2.6 Recombinant protein production

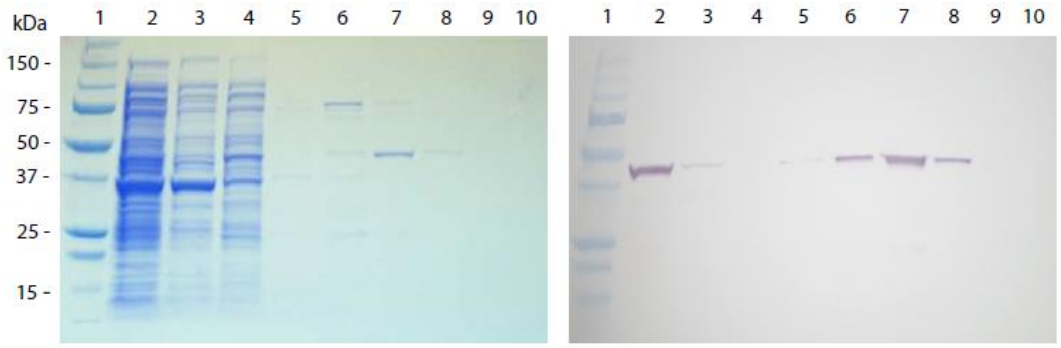
Recombinant VvOMT1 and VvOMT2 were synthesised in *Escherichia coli* as N-terminal polyhistidine-tagged proteins. *VvOMT1* and *VvOMT2* coding sequences were each cloned into the expression vector pET30a (Novagen, Darmstadt, Germany) by incorporating *Bam*HI restriction endonuclease sites to

the above forward primers and *Xho*I sites to the reverse primers (Appendix A). *Bam*HI and *Xho*I digested PCR products were ligated into pET30a vector digested with the same restriction endonucleases. Sequencing of constructs with the T7 promoter and T7 terminator primers (Appendix A) was performed to confirm the inserted sequences were in frame with the pET30a start codon. The resulting constructs co-transformed with the tRNA accessory plasmid pRIL (Stratagene, La Jolla, CA, USA) into One Shot® BL21 cells (Invitrogen) according to manufacturer's instructions. Positive transformants were selected on LB plates (Table 2.1) containing 25 $\mu g.mL^{-1}$ kanamycin (pET30a) and 25 $\mu g.mL^{-1}$ chloramphenicol (pRIL). Cells containing both vectors were cultured in ZYM-5052 auto-induction media (Table 2.1; Studier, 2005) containing 25 $\mu g.mL^{-1}$ kanamycin and 25 $\mu g.mL^{-1}$ chloramphenicol, at 18°C for 96 hours. Cells were harvested by centrifugation and resuspended in His-Trap binding buffer (Table 2.1) containing 5 mM DTT, 0.2 $mg.mL^{-1}$ lysozyme (Roche), 20 $\mu g.mL^{-1}$ DNase I (Roche) and complete EDTA-free Protease Inhibitor (Roche). Cells were disrupted by sonication for 15 min and cell debris removed by centrifugation at 7,700 × *g* for 20 min. The crude protein extract was loaded onto His GraviTrap columns (GE Healthcare, Buckinghamshire, UK) previously equilibrated with 10 mL of binding buffer. After washing with a further 10 mL of binding buffer, bound proteins were sequentially eluted with 3 ml of binding buffer supplemented with 50, 100, 200, 300, 400 or 500 mM imidazole. The 200 mM imidazole fraction, which contained the most recombinant protein, as determined by Western blot analysis (described below and Figure 2.1), was then passed though a PD-10 desalting column (GE Healthcare) to remove imidazole, and then mixed with an equal volume of protein storage buffer (Table 2.1) and kept at -80°C pending use in assays.

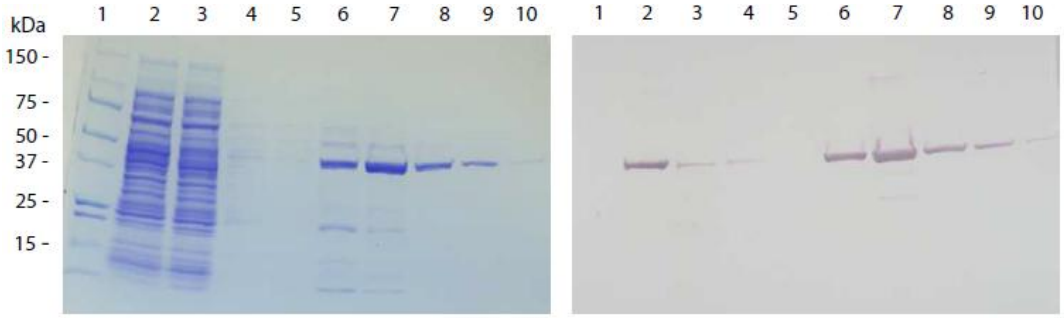
2.2.7 SDS-PAGE and western blot analysis

SDS-PAGE and western blot analyses using antibodies raised against polyhistidine were performed to determine the eluted fractions containing the most recombinant protein. Protein extracts were mixed with an equal volume of 2× Loading buffer (Table 2.1), heated to 90°C for 5 min and then loaded into the wells of two individual pre-set 4-20% acrylamide gels (iGels; NuSep, Sydney, Australia) submerged in SDS running buffer (Table 2.1). Electrophoretic separation of the proteins was performed at 90 V for 4 h. One of the gels was then submerged and incubated overnight in protein staining solution (Table 2.1), followed by five 1 h washes in destaining solution to remove unbound stain and reveal protein bands (Table 2.1). The proteins in the second gel were transferred onto 0.45 µm Biotrace™ polyvinylidene fluoride membrane (Pall Life Sciences, NY, USA) using a mini-gel transfer apparatus (BioRad) and western transfer buffer (Table 2.1) at 35 V overnight at 4°C. The membrane was removed and placed in phosphate buffered saline (PBS, Table 2.1) and 10 % low fat milk powder with gentle agitation for 2 h followed by three 5 min washes in PBS plus 0.05% (v/v) Tween20 (PBS-T). The membrane was then incubated with a 1:3000 dilution of monoclonal anti-polyhistidine mouse antibody (Sigma) in PBS-T with 5% bovine serum albumen (BSA) for 2 h followed by three 5 min washes in PBS-T. The membrane was then incubated with a 1:5000 dilution of anti-mouse IgG alkaline phosphatase conjugated antibody (Promega, Madison, WI, USA) in PBS-T for 2 h followed by three 5 min washes in PBS-T. The liquid substrate BCIP/NBT (Sigma) which reacts with alkaline phosphatase to form a blue/brown precipitate, was pipetted onto the membrane for visual detection of secondary antibodies and thus the HIS-tagged recombinant proteins.

A - VvOMT1



B - VvOMT2



C - Empty pET30 vector

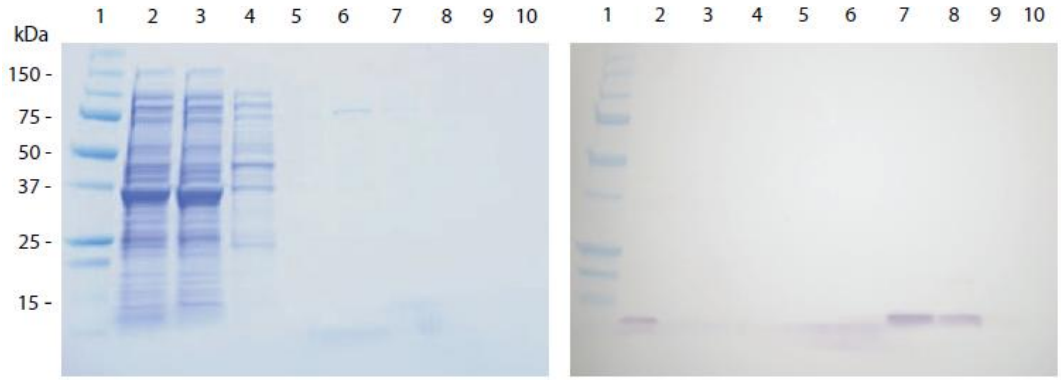


Figure 2.1 - SDS-Page gel (left) and corresponding western blot (right) of samples collected during HIS-trap column purification of recombinant VvOMT1 (**A**), VvOMT2 (**B**) and empty pET30 vector (**C**). Lane (1) Protein size marker; (2) crude protein extract before purification; (3) flow through after addition of crude extract to HIS column; eluates after the addition of elution buffer containing: (4) 20mM imidazole; (5) 50mM imidazole; (6) 100mM imidazole; (7) 200mM imidazole; (8) 300mM imidazole; (9) 400mM imidazole and (10) 500mM imidazole.

2.2.8 OMT enzyme assays

Standard enzyme assays were performed in 1.5 mL microcentrifuge tubes. The 100 µL reaction mixtures consisted of 50 mM Tris-HCl pH 7.5, 1mM $MgCl_2$, 1 mM DTT, 0.1 mM SAM, purified recombinant enzyme and varying HP concentrations. Controls with heat denatured enzymes (100°C for 5 min) or purified protein from empty pET30 vector control cultures were conducted to confirm the purity of the HP substrate and eliminate the possibility of methyltransferase activities from contaminating bacterial proteins contributing to MP production. Reactions were incubated at 30°C for 40 min from the addition of SAM and the reactions stopped by the addition of 100 µL of methanol containing deuterated IBMP as internal standard for quantification of MPs by SPME-GC-MS as describe below (section 2.2.9). Assays to determine pH optima of VvOMT1 and VvOMT2 against the HP substrates were conducted essentially as described above, except that buffers at different pH were used. For pH levels between 5.5 and 6.5, 200mM MES was used, 200mM HEPES was used for between pH 7.0 and 7.5 and 200mM Tris-HCl was used for the pH range 7.5 to 9.0. Again the reactions were stopped by the addition of 100 µL of methanol containing deuterated IBMP and the MP products assayed using SPME-GC-MS. For determination of kinetic values, assays were run in triplicate at six different concentrations of HP (100-1000 µM) with a saturating concentration of SAM. Lineweaver-Burk plots were produced to calculate kinetic values (V_{max} and K_m) which were verified using the non-linear regression analysis package from the SigmaPlot® 11 software (Systat Software Inc, San Jose, CA, USA). Protein quantification was conducted using the *DC* Protein Assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions and enzyme

concentrations then used to calculate k_{cat} and specific activity values for recombinant VvOMT1 and VvOMT2.

To determine the activity of VvOMTs against HPs and other potential substrates, the SAM265 methyltransferase assay (G-Biosciences, St Louis, MO, USA) was used. The SAM265 methyltransferase assay is an enzyme-coupled assay that uses UV absorbance to indirectly measure the formation of *S*-adenosylhomocysteine, the by-product of methyltransferase reactions resulting from the removal of a methyl group from *S*-adenosyl-L-methionine (Dorgan et al. 2006). Methyltransferase activity assays (180 µL) were performed using the conditions outlined above but with a final substrate concentration of 100 µM. Reactions were incubated at 30°C for 60 min before transferring to a 96 well UV-Star microplate (Greiner bio-one, Frickenhausen, Germany). SAM265 enzyme mix (20 µL) was added and the UV absorbance at 265 nm was measured at 30 sec intervals for 120 min using a FLUOstar Omega plate-reader (BMG Labtech, Offenburg, Germany). Absorbance change at 265nm was subtracted from that of a blank assay and plotted against a standard curve to quantify adenosylhomocysteine formation in the assays and thus the methyltransferase reaction rate.

2.2.9 SPME-GC-MS quantification of MPs

MPs were quantified in both grape tissue and enzyme assays using a stable isotope dilution assay (SIDA) modified from that of Chapman *et al.* (2004). For grapes, frozen tissue was ground to a powder and 1 g added to 10 mL of citrate/phosphate buffer (Table 2.1). A 100 µL aliquot of 5 $\mu g.L^{-1}$ D_3-IBMP (CDN Isotopes, Quebec, Canada) was added as an internal standard before the sample was centrifuged at 1000 × *g* for 10 min. The supernatant was then transferred to a

20 mL brown-glass headspace vial and 3 g of NaCl added to increase partitioning into the headspace. For quantification of MPs in enzyme assays, 100 µL of 5 $\mu g.L^{-1}$ D_3-IBMP and 2 mL of citrate/phosphate buffer was added to each reaction mixture before being transferred to a 20 mL brown-glass headspace vial and 600 mg of NaCl added. Samples were analysed immediately as it was found that overnight equilibration did not enhance sensitivity or affect the MP quantification.

Solid phase micro extraction gas chromatography-mass spectroscopy (SPME-GC-MS) was carried out using an 6890 gas chromatograph (Agilent, Santa Clara, CA, USA) equipped with a Gerstel (Mülheim an der Ruhr, Germany) MP2 auto-sampler and using an Hewlett-Packard 5973N mass spectrometer for peak detection and compound identification. The auto-sampler was operated in SPME mode utilizing a divinylbenzene-carboxen-polydimethylsiloxane fiber (2 cm, 23-Gauge, 50/30 µm DVB-CAR-PDMS, Supelco, Bellefonte, PA, USA) for extraction. The samples were equilibrated at 40°C for 5 min and then extracted at 40°C for 30 min with agitation at 250 rpm. Analytes were desorbed in the injector for 2 min at 250°C after which the purge was switched on to 20 $mL.min^{-1}$. Chromatography was performed using a ZB-Wax column (length 30 m, 0.25 mm i.d., film thickness 0.25 µm) and helium as a carrier gas at a constant flow of 1.2 $mL.min^{-1}$. After an initial hold at 30°C for 2 min, a temperature gradient of $3°C.min^{-1}$ from 30°C to 80°C was applied and then held isothermically for 14 min before a gradient of $25°C.min^{-1}$ was applied from 80°C to 230°C, and the final temperature held for 5 min. Mass spectrometry was performed with a transfer line temperature of 250°C, source temperature of 230°C, quadrapole temperature of 150°C, ionising potential at 70eV using selective ion monitoring. Mass channels were *m/z* = 124, 137 and 152 for IPMP with 50 ms dwell times from 0 to 25.5 min. Then after 25.5 min mass channels were *m/z* = 94, 124 and 151 for IBMP

and 95, 127 and 154 for D_3-IBMP with 50 ms dwell times. MPs were quantified using the 124 ion for IBMP, 137 for IPMP and 127 for D_3-IBMP, while 124 and 137 were qualifying ions for IPMP, 94 and 151 for IBMP and 95 and 154 for D_3-IBMP. The limit of detection of both IPMP and IBMP was calculated at 15 $ng.L^{-1}$ which was the minimum concentration in which the peak signal to baseline noise ratio was greater than 3:1. All GC-MS analysis was performed in triplicate and quantification of target MP was achieved by relating ion peak areas with that of the internal standard then calculated using a standard curve. No carry-over was observed between samples.

2.2.10 Real-time PCR analysis

VvOMT gene expression was analysed by Real-Time PCR using a Rotor-Gene 3000 thermo cycler (Corbett Research, Sydney, NSW, Australia) and Absolute™ QPCR SYBR® Green reagent (ABgene, Epsom, Surrey, UK). All experiments were performed in triplicate and each 15 µl PCR reaction consisted of 2 µL of cDNA sample (1:100 dilution of the synthesis reaction), 70 nM of each primer and 1× Absolute™ QPCR SYBR® Green mix. Thermal cycling conditions used were 95°C for 15 min followed by 45 cycles of 95°C for 20 sec, 58°C for 20 sec and 72°C for 30 sec, followed by a melt ramp of 0.2°C per sec from 65°C to 95°C. Primers were designed to each gene of interest (described in Appendix A) and melt curves were analysed to ensure the absence of primer dimers or spurious amplification products. PCR standards consisted of serial dilutions of linearised plasmid constructs (pET30a vector plus target gene, described in Section 2.2.6) of known DNA concentration, determined using the Picogreen® quantification kit (Invitrogen). Plasmid DNA concentrations were converted to approximate copy number and used to produce a linear standard curve of cycle threshold versus

copy number. Quantification of transcript copy number of cDNA samples was obtained by plotting the cycle threshold values against the standard curve and then normalised against an average value obtained from three housekeeping genes, *ubiquitin*, *actin* and *elongation factor 1* (Genbank accessions CF406001, AF369524 and BQ799343 respectively).

2.2.11 Construction of VvOMT targeted hairpin constructs

RNAi hairpin constructs targeted to a conserved 300 bp sequence of *VvOMT1* and *VvOMT2* were designed to allow simultaneous silencing of both genes in transformed grapevine hairy-roots. Forward and reverse primers flanked by the 28 bp attB1 or attB2 sequences respectively (Appendix A), were designed to amplify a 300 bp region homologous to both *VvOMT1* and *VvOMT2*. The amplified PCR fragment was recombined into the vector pDonar using the AR clonase reaction of the Gateway® transformation system (Invitrogen). Recombined constructs were transformed into the chemically competent One Shot® OmniMAX™ 2 Ti Phage-Resistant Cells (Invitrogen) and selected for on LB plates containing 25 $\mu g.mL^{-1}$ kanamycin. Positive colonies were picked, transformed plasmids purified from overnight cultures and confirmed as containing the correct *VvOMT* sequence by *Xho*I and *Xba*I restriction digests and sequencing with M13 forward and reverse primers (Appendix A).

The pDonar:VvOMT construct was then recombined into the pHellsgate12 vector (Helliwell and Waterhouse, 2003) using the LR clonase reaction of the gateway system resulting in an inverted repeat sequence. The recombined pHellsgare12:VvOMT construct was transformed into chemically competent One Shot® OmniMAX™ 2 Ti Phage-Resistant Cells (Invitrogen) and selected on LB plates containing 50 $\mu g.mL^{-1}$ spectinomycin. Plasmids were purified from

overnight cultures and *Xho*I and *Xba*I restriction digests performed to confirm the presence of the *VvOMT* fragment in the construct. The hairpin cassette of pHellsgate12:VvOMT was then excised by *Sbf*I digestion and ligated into pCLB1301, also digested with *Sbf*I, to generate pCLB1301:hpVvOMT. The binary vector pCLB1301 is derived from pCAMBIA1301 (Hajdukiewicz *et al.*, 1994) with the original 35S promoter and gusA replaced by *gfp*-5-ER for visual detection of transformed plant lines expressing genes in the T-DNA cassette. pCLB1301:hpVvOMT was transformed into *Agrobacterium rhizogenes* A4 cells by electroporation and plated onto MGL plates (Table 2.1) containing rifampicin (25 $\mu g.mL^{-1}$) and kanamycin (25 $\mu g.mL^{-1}$) selection. A control construct pCLB:hpMLO was obtained from Dr Ian Dry (CSIRO), encoding a hairpin sequence targeted to the grapevine *MLO17* gene thought to be involved in powdery mildew susceptibility (Feechan *et al.*, 2008).

2.2.12 RNAi mediated silencing of VvOMT genes in grapevine hairy-roots

The transformation of grapevine to produce hairy roots was based on the protocol of Torregrosa and Bouquet (1997) and with modifications described by Terrier *et al.* (2009). Leaves from 2-5 month old CS plantlets grown in tissue culture were cut at the petiole and placed adaxial surface down onto LG0 growth media (Table 2.1). Sterilized forceps were dipped into overnight culture of *A. rhizogenes* A4 cells (OD_{600} of 0.5) containing either pCLB1301:hpVvOMT or the control plasmid pCLB:hpMLO and the leaf explant inoculated by gently squeezing and crushing the tips of the leaf petioles. Inoculated leaves were incubated in the dark at 28°C for 48 h before being transferred to LG0 plates containing 100 $\mu g.mL^{-1}$ kanamycin and 1,000 $\mu g.mL^{-1}$ timentin for the selection of positive transformants and to inhibit further *A. rhizogenes* growth respectively.

Inoculated leaves were incubated at 27°C with a regime of 16 h light and 8 h dark for 2-4 weeks until callus developed on the petioles. Callus tissue was removed and plated onto LG0 media containing 100 $\mu g.mL^{-1}$ kanamycin and 1,000 $\mu g.mL^{-1}$ timentin and continually grown under tissue culture conditions. After 1-2 months the young roots sprouting from the calluses were scored for presence or absence of GFP fluorescence. Lines displaying GFP fluorescence, and thus expressing the T-DNA insertion cassette, were selected and transferred to fresh plates. After 6 months of growth tissues were harvested and analyzed for MP and mRNA transcript analysis as described in sections 2.2.9 and 2.2.10 respectively.

2.3 Results

2.3.1 Identification of two OMT genes encoding proteins with similarity to an enzyme with HP methylating activity

Previous work by Hashizume *et al.* (2001a) demonstrated that CS grape berries contain enzyme activity that is capable of methylating HPs to produce MPs. Subsequently, an enzyme with such activity was purified from grape and 20 amino acids of the proteins N-terminal sequence was obtained (Hashizume *et al.* 2001b). A search of grapevine EST sequences revealed a CS cDNA clone encoding a protein with a 100% match to the N-terminal amino acid sequence of this purified protein. Using data from ESTs collections and the grapevine genomic sequence (Jaillon *et al.*, 2007), suitable primers were designed and used to amplify a full length cDNA encoding this methyltransferase gene, subsequently named *VvOMT1* (Genbank accession: GQ357167). When the protein encoded by *VvOMT1* was used to search the grapevine genome sequence, a similar gene sequence, *VvOMT2* (Genbank accession: GQ357168), was identified that showed 87% amino acid identity to *VvOMT1* from the predicted translation product.

VvOMT2 possesses 19 of the 20 N-terminal amino acids of the native protein purified by Hashizume *et al.* (2001b). Both *VvOMT1* and *VvOMT2* possess a 77 bp intron in the coding region of the gene and a comparison of genomic and matching EST sequences suggests the presence of another 108 bp intron in the 3'-untranslated region of *VvOMT2*. Both *VvOMT* genes were found to be located in a 22 kb region on chromosome 12 of the grapevine genome (Fig. 2.2). Interestingly, there are two gene fragments located between *VvOMT1* and *VOMT2* that show homology to these methyltransferases, but both are truncated at the 5'-end (Fig. 2.2). No ESTs matching either of these truncated sequences were found in grapevine databases.

Sequence alignment with other plant OMTs (Fig. 2.3) showed that the proteins predicted to be encoded by *VvOMT1* and *VvOMT2* contain five conserved domains characteristic of plant OMTs (Ibrahim et al. 1998). Phylogenetic analysis of VvOMT1 and VvOMT2 with other plant OMTs reveals both are class II OMTs. Subsequent phylogenetic analysis with previous characterised members of the class II OMT family shows that these OMTs can be grouped into two distinct clades (Fig. 2.4). VvOMT1 and VvOMT2 fall into clade II and show closest homology to an OMT from *Rosa chinensis* (Wu et al. 2003). OMTs of clade I generally have activity against phenolic compounds involved in lignin biosynthesis such as caffeic acid and 5-hydroxyferulic acid and their respective aldehydes and alcohols. OMT members of clade II tend to be active against a range of substrate molecules including flavonoids (Christensen *et al.*, 1998), isoflavonoids (Akashi *et al.*, 2003) resveratrol (Schmidlin *et al.*, 2008), orcinol (Lavid *et al.*, 2002), eugenol and chavicol (Gang *et al.*, 2002).

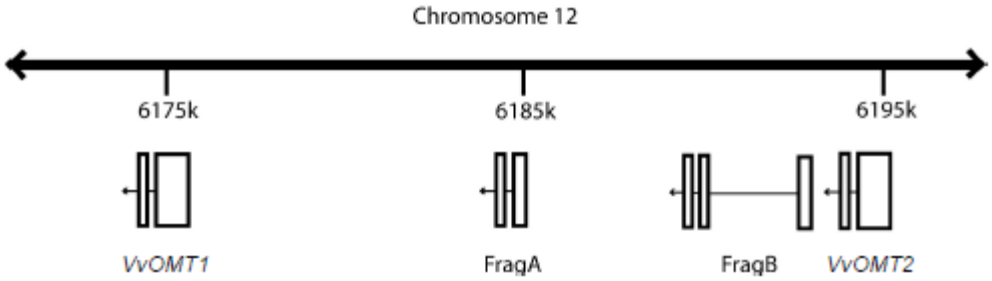


Figure 2.2 - *VvOMT1* and *VvOMT2* are linked and located on chromosome 12 of the grape genome. There also exist two homologous DNA sequences, both truncated at the 5' end, located between *VvOMT1* and *VvOMT2* and these are denoted as FragA and FragB. Boxes represent putative amino acid coding sequences and arrows represent the predicted non-coding sequences of the genes.

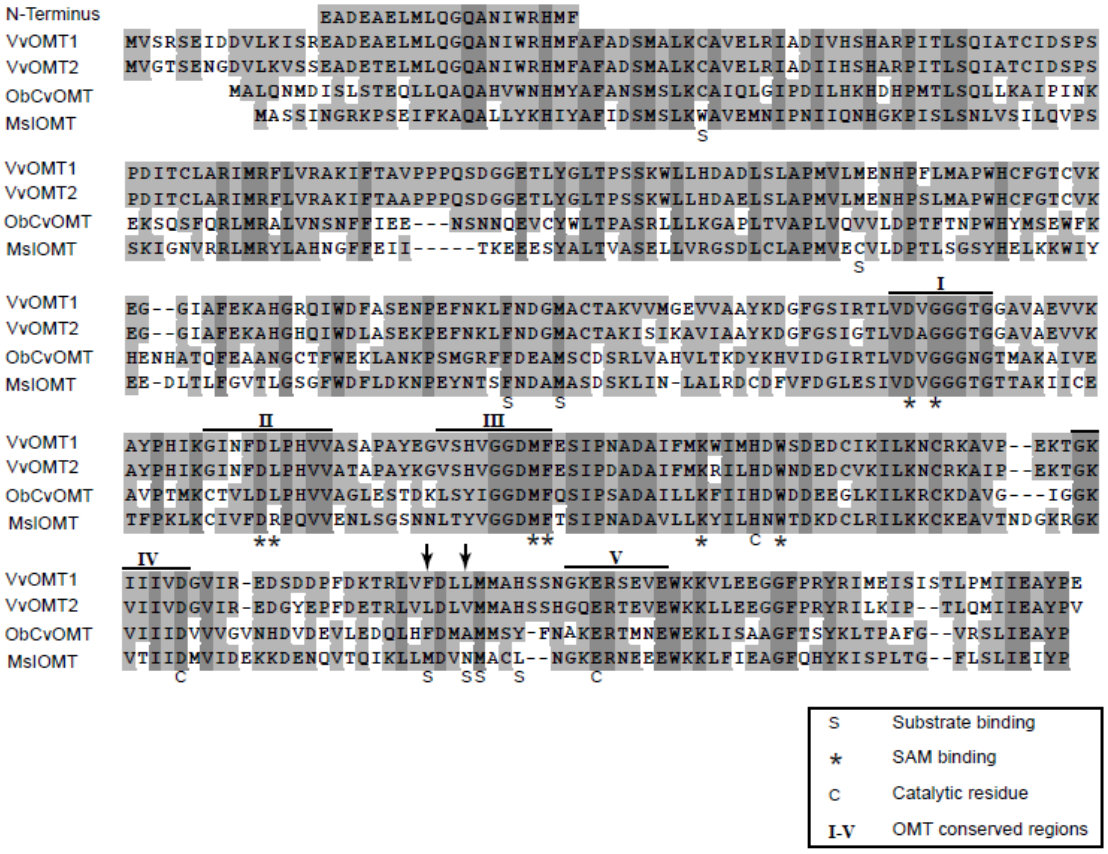


Figure 2.3 - Alignment of the predicted amino acid sequences of VvOMT1 and VvOMT2 with the partial N-terminal sequence of a grape OMT previously reported to have HP-methylating activity (Hashizume *et al.* 2001b) and two plant OMTs where important residues have been identified (Gang *et al.*, 2002; Zubieta *et al.*, 2001). Regions I-V are domains conserved among plant OMTs. C, signifies catalytic residues; *, SAM binding residues; and S, substrate binding residues of MsIOMT as determined by Zubieta *et al.* (2001); ↓ highlights amino acid differences between VvOMT1 and VvOMT2 in substrate binding positions. Black and grey shading represents identical and similar amino acids respectively. ObCVOMT is an *Ocimum basilicum* chavicol OMT (AF435007) and MsIOMT is a *Medicago sativa* isoflavanoid OMT (AF023481).

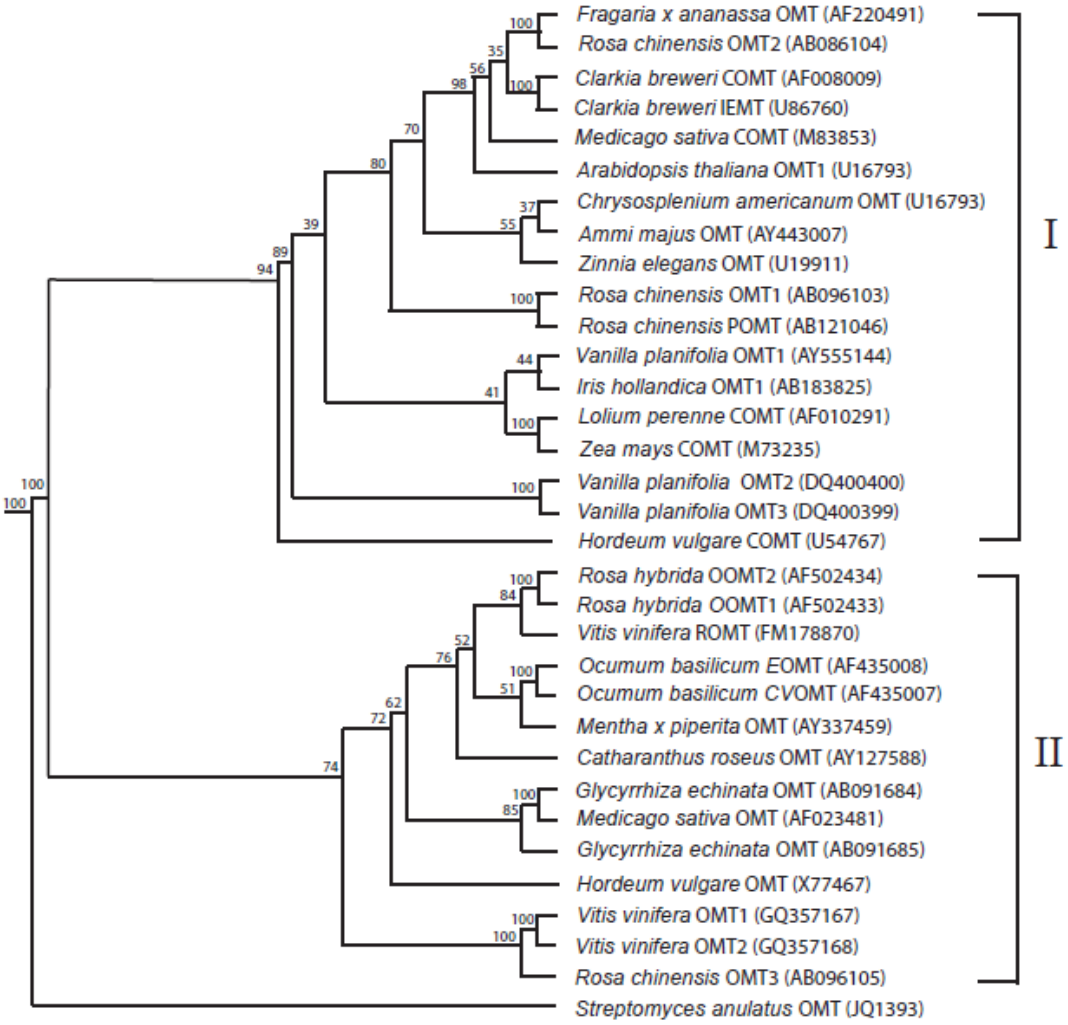


Figure 2.4 Phylogenetic tree of VvOMT1 and VvOMT2 and other previously characterised plant class II OMTs reveals two distinct clades. Numbers at branch points are bootstrap values representing the confidence level as a percentage based on 1,000 repeats. The *Streptomyces anulatus* OMT was used as an outgroup.

2.3.2 Recombinant VvOMT1 and VvOMT2 methylate HPs

To investigate the methyltransferase activity of *VvOMT1* and *VvOMT2* products, recombinant HIS-tagged fusion proteins were produced in *E. coli* and purified using Ni-affinity columns (Fig. 2.1). Purified recombinant VvOMT1 and VvOMT2 were assayed against the precursors of IBMP and IPMP, 3-isobutyl-2-hydroxypyrazine (IBHP) and 3-isopropyl-2-hydroxypyrazine (IPHP) respectively, to provide evidence of their functional activity against these substrates and to determine their substrate preferences. GC-MS analysis of the products produced from incubation of the enzymes with individual HP substrates showed that both VvOMT1 and VvOMT2 are able to methylate both IBHP and IPHP (Fig. 2.5). An optimum pH of 7.5 was determined for the activity of both VvOMT1 and VvOMT2 against HPs (Fig. 2.6).

The kinetic parameters of the activity of VvOMT1 and VvOMT2 against HPs were determined by calculating the reaction rate at different HP substrate concentrations (Fig. 2.7 & 2.8). These kinetic values show that VvOMT1 has a similar preference for IBHP and IPHP with K_m values of 539 µM and 493 µM respectively (Table 2.2). However, VvOMT1 has a higher turnover number (k_{cat}) and therefore a greater catalytic efficiency (k_{cat}/K_m) with IBHP compared to IPHP. VvOMT2 showed a higher affinity for IBHP (K_m = 628 µM) than for IPHP (K_m = 1264 µM) but conversely had a lower turnover number against IBHP than IPHP resulting in similar catalytic efficiencies for both substrates (Table 2.2). Comparison of the k_{cat}/K_m of VvOMT1 and VvOMT2 reveals that VvOMT1 is approximately 9-fold more efficient at methylating IBHP and approximately 5-fold more efficient at methylating IPHP than VvOMT2.

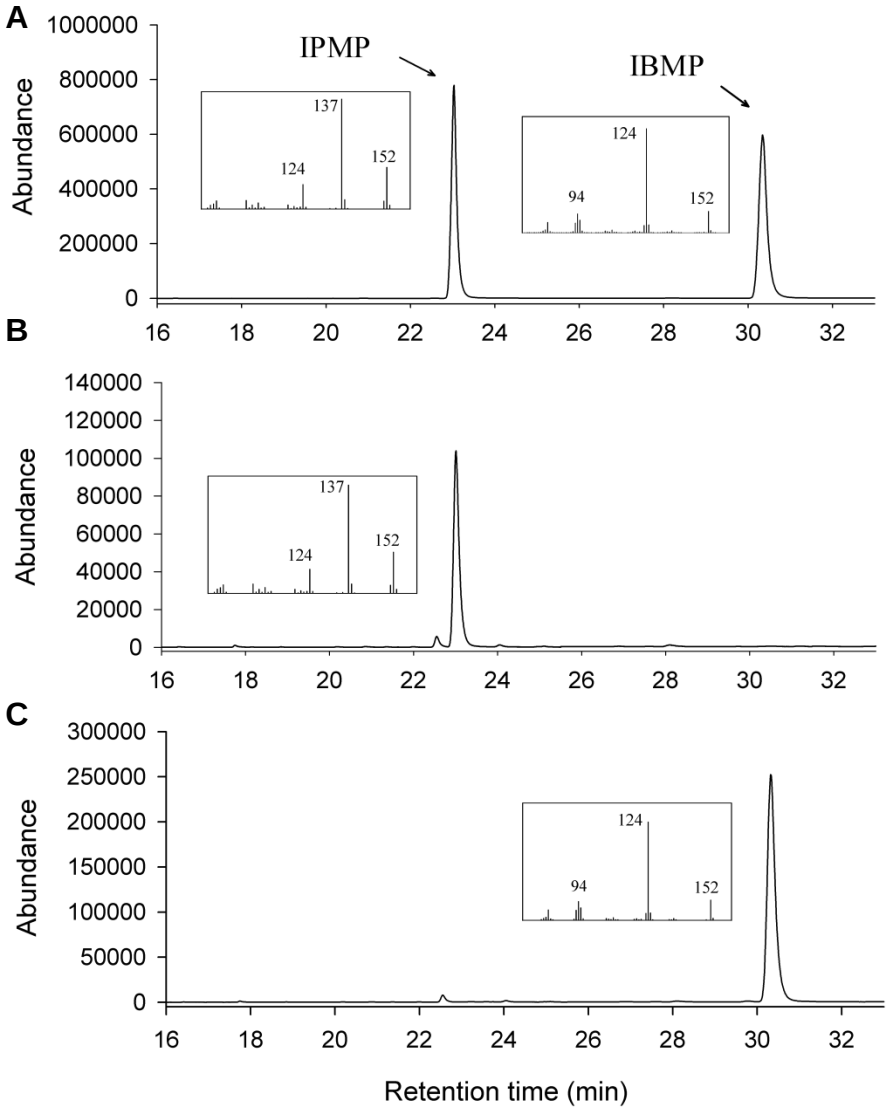
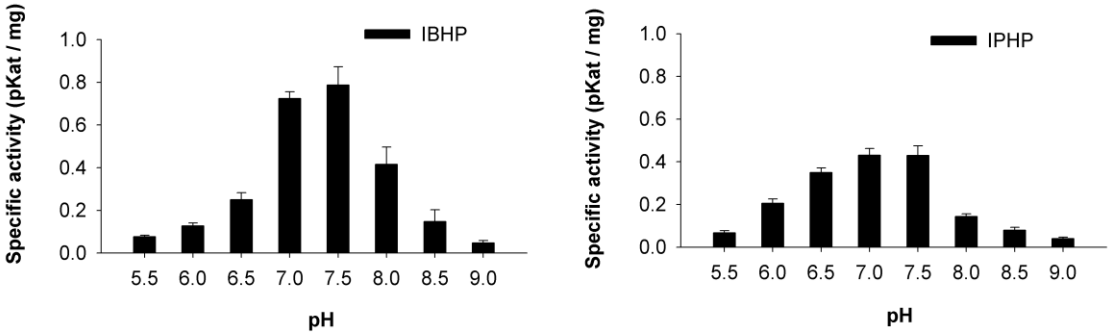


Figure 2.5 - Gas chromatography-mass spectrometry (GCMS) analysis of products produced by recombinant VvOMT1. (**A**) Total ion chromatogram of authentic IPMP and IBMP standards showing the mass spectra of each peak inlaid. Total ion chromatogram of the reaction products of VvOMT1 assay against the substrates IPHP (**B**) and IBHP (**C**) respectively**.** Mass spectra of peaks are inlaid and confirm the identity of VvOMT1 reaction products as IPMP and IBMP respectively. The results when recombinant VvOMT2 was used in assays with IPHP and IBHP were identical to those obtained for VvOMT1.

A - VvOMT1



B - VvOMT2

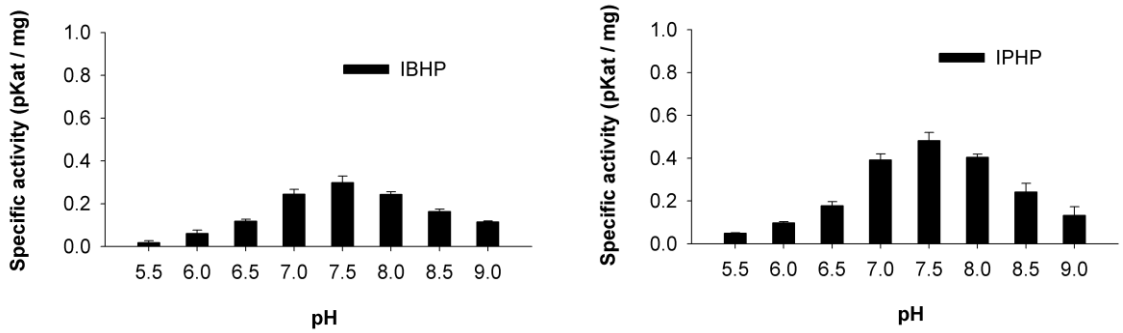
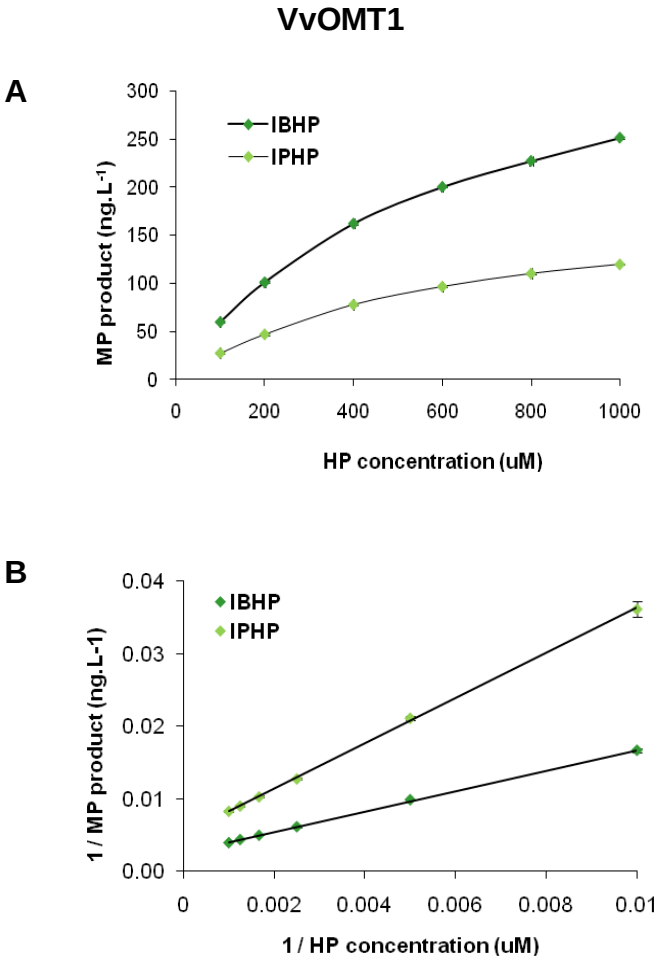
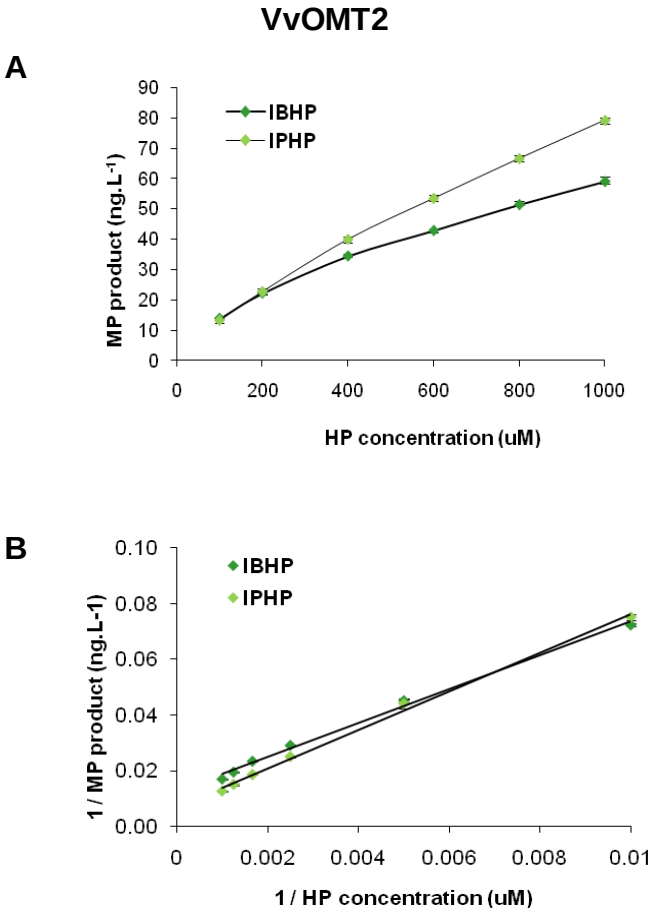


Figure 2.6 - Determination of pH optima for VvOMT1 (**A**) and VvOMT2 (**B**) activity against IBHP (left) and IPHP (right). The buffer MES was used for pH ranges 5.5-6.5, HEPES used for 7.0-7.5 and Tris-HCl used for 8.0-9.0.



Substrate	r^2	Intercept	Slope	K_m (µM)	k_{cat} x 10^{-6} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
IBHP	0.9996	0.0052	3.111	539 ± 31	58.6 ± 2.8	0.109 ± 0.005
IPHP	0.9996	0.0027	1.408	493 ± 28	32.4 ± 1.4	0.066 ± 0.003

Figure 2.7 Calculation of the kinetic values of VvOMT1 against HP substrates. (**A**) Michaelis-Menton curve displaying changes in MP formation with various HP substrate concentrations and the same data presented as a Lineweaver-Burk plot (**B**) used to calculate kinetic values. V_{max} is equal to 1/intercept and indicates the maximum velocity of the enzymatic reaction. K_m is equal to V_{max} / slope and is the concentration of substrate at which reaction velocity is half its maximum. k_{cat} equals V_{max} / moles of enzyme per assay × 40 min × 60 sec and calculates to the number of substrate molecules converted to product per enzyme site per second. k_{cat} / K_m is also called the specificity or rate constant and gives a measure of total catalytic efficiency.



Substrate	r^2	Intercept	Slope	K_m (µM)	k_{cat} x 10^{-6} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
IBHP	0.9950	0.0127	6.080	628 ± 29	7.5 ± 0.2	0.012 ± 0.0002
IPHP	0.9967	0.0070	6.920	1,264 ± 120	16.9 ± 1.1	0.013 ± 0.001

Figure 2.8 Calculation of the kinetic values of VvOMT2 against HP substrates. (**A**) Michaelis-Menton curve displaying changes in MP formation with various HP substrate concentrations and the same data presented as a Lineweaver-Burk plot (**B**) used to calculate kinetic values. V_{max} is equal to 1/intercept and indicates the maximum velocity of the enzymatic reaction. K_m is equal to V_{max} / slope and is the concentration of substrate at which reaction velocity is half its maximum. k_{cat} equals V_{max} / moles of enzyme per assay × 40 min × 60 sec and calculates to the number of substrate molecules converted to product per enzyme site per second. k_{cat} / K_m is also called the specificity or rate constant and gives a measure of total catalytic efficiency.

Table 2.2 - Comparison of kinetic parameters determined for recombinant VvOMT1 and VvOMT2 against HP substrates.

Enzyme	Substrate	Apparent K_m (μM)	k_{cat} x 10^{-6} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
VvOMT1	IBHP	539 ± 31	58.6 ± 2.8	0.109 ± 0.005
	IPHP	493 ± 28	32.4 ± 1.4	0.066 ± 0.003
VvOMT2	IBHP	628 ± 29	7.5 ± 0.2	0.012 ± 0.0002
	IPHP	1,264 ± 120	16.9 ± 1.1	0.013 ± 0.001

2.3.3 Recombinant VvOMT1 and VvOMT2 can methylate many substrates

To further investigate the activity of the recombinant VvOMTs, a SAM methyltransferase assay (Dorgan *et al.*, 2006) was used to assay methylating activity of these enzymes against HPs and other potential substrates. VvOMT1 and VvOMT2 showed greatest activity against the flavonol quercetin with specific activity values of 196 and 110 pkat.mg^{-1} respectively (Table 2.3). This activity is more than 100-fold greater than that seen against the HP substrates (Table 2.3). Both enzymes displayed methylating activity when resveratrol, caffeic acid or epicatechin (6.7-10.1 pkat.mg^{-1}) were used as substrates. VvOMT2 showed activity against catechin (8.0 pkat.mg^{-1}) while no activity could be detected for VvOMT1 against this compound. No activity was detected for either enzyme against the compounds eugenol, isoeugenol and orcinol (Table 2.3).

Table 2.3 - Methylating activity of recombinant VvOMT1 and VvOMT2 with potential substrates. Standard error values are given in parentheses. n.d. indicates not detectable.

	Specific Activity (pkat.mg^{-1})	
Substrate	VvOMT1	VvOMT2
Quercetin	195.6 ± 11.2	110.2 ± 3.3
Resveratrol	9.5 ± 1.3	10.1 ± 0.6
Caffeic acid	6.7 ± 0.3	10.1 ± 0.5
Epicatechin	6.9 ± 1.4	8.7 ± 1.0
Catechin	n.d.	8.0 ± 0.1
IBHP	1.4 ± 0.07	0.2 ± 0.01
IPHP	0.8 ± 0.03	0.4 ± 0.02
Eugenol	n.d.	n.d.
Isoeugenol	n.d.	n.d.
Orcinol	n.d.	n.d.

2.3.4 Expression of VvOMT1 coincides with MP levels in berry skin and flesh

The expression of *VvOMT1* and *VvOMT2* and MP levels were analysed in grapes to see if the presence of transcripts from these genes was associated with the production of MPs. Using SPME-GC-MS, MP levels were measured in CS berries sampled at fortnightly intervals throughout development (Fig. 2.9A). The amount of IBMP in the berries was always greater than that of IPMP. IBMP levels increased from 20 pg.g^{-1} fresh weight at 2 weeks post flowering (wpf) to a peak of 204 pg.g^{-1} at 8 wpf and then declined to 25 pg.g^{-1} at 16 wpf. IPMP was only detected in samples taken 4, 6 and 8 wpf, where levels ranged from 13 to 20 pg.g^{-1}. The expression levels of *VvOMT1* and *VvOMT2* in the berry samples were measured by real-time PCR (Fig. 2.9B). In the whole berry samples, both *VvOMT1* and *VvOMT2* reach a peak in expression at 4 wpf after which the transcript levels of both genes declined. Whilst the peak in expression of both *VvOMT* genes coincides with the period of greatest increase in MP accumulation in the berries, it was surprising that higher expression levels were not maintained

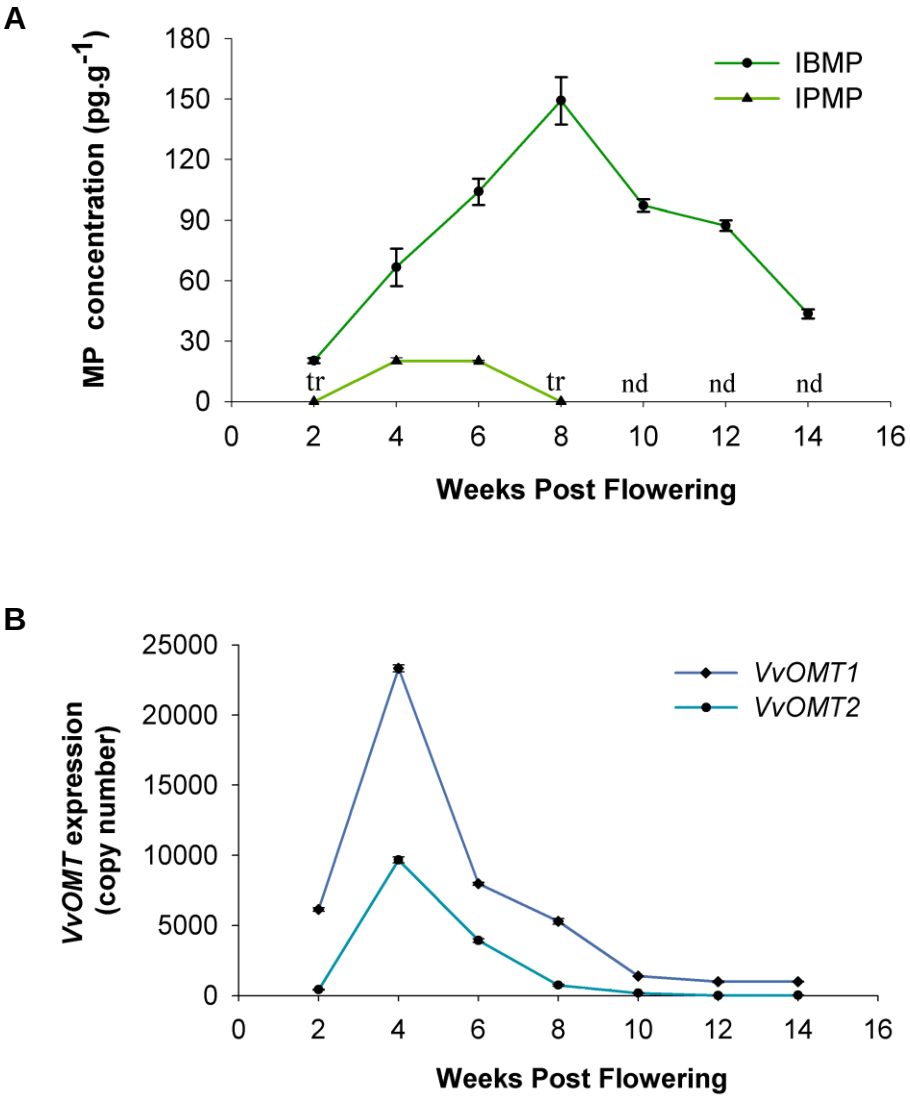


Figure 2.9 - MP concentrations and *VvOMT* expression in whole berries throughout development. (**A**) IBMP and IPMP concentrations, expressed as $ng.kg^{-1}$ of fresh weight, in developing grape berries; tr signifies that only trace levels were detected and nd signifies not detectable. (**B**) Relative expression of *VvOMT1* and *VvOMT2*, expressed as copy number, in the berry samples as quantified by Real-Time PCR.

throughout this period of MP accumulation in developing berries (Fig. 2.9). It was thought that this may be due to variation between MP concentrations and *VvOMT* gene expression in different berry tissues.

To investigate this hypothesis, CS berries from the same samples were separated into skin, flesh and seeds to determine those berry tissues that accumulated methoxypyrazines and to analyse *VvOMT* gene expression in the separated samples (Fig. 2.10). IBMP was found in the greatest concentrations in the skin and the lowest levels were detected in the seeds at all the stages of berry development that were examined (Fig. 2.10A). Skin IBMP concentrations increased from 276 $pg.g^{-1}$ at 4 wpf to a peak of 490 $pg.g^{-1}$ at 8 wpf and then declined to 135 $pg.g^{-1}$ at 14 wpf. Flesh IBMP concentrations increased from 102 $pg.g^{-1}$ at 4 wpf to 189 $pg.g^{-1}$ at 8 wpf and then declined to 17 $pg.g^{-1}$ at 14 wpf. Seed IBMP concentrations remained relatively low in all samples (between 19 and 34 $pg.g^{-1}$). IPMP was only found above trace amounts in the berry skin tissue at 4 and 6 wpf respectively (Fig. 2.10B). No IPMP was detected in the flesh or seeds at any time point.

Surprisingly, the highest level of *VvOMT1* expression was found in the seed at 4 wpf after which it then decreased dramatically (Fig. 2.10C). In both the skin and the flesh, expression of *VvOMT1* was greatest during the period of MP accumulation in these tissues, which occurred between 4 and 8 wpf. After 8 wpf, *VvOMT1* expression in the skin and the flesh decreased to lower levels similar to that observed in the seed during this post-véraison stage of berry development. The pattern of *VvOMT2* expression in the seed was similar to that of *VvOMT1*, with maximum expression observed at 4 wpf following which transcript levels decreased markedly by 8 wpf and

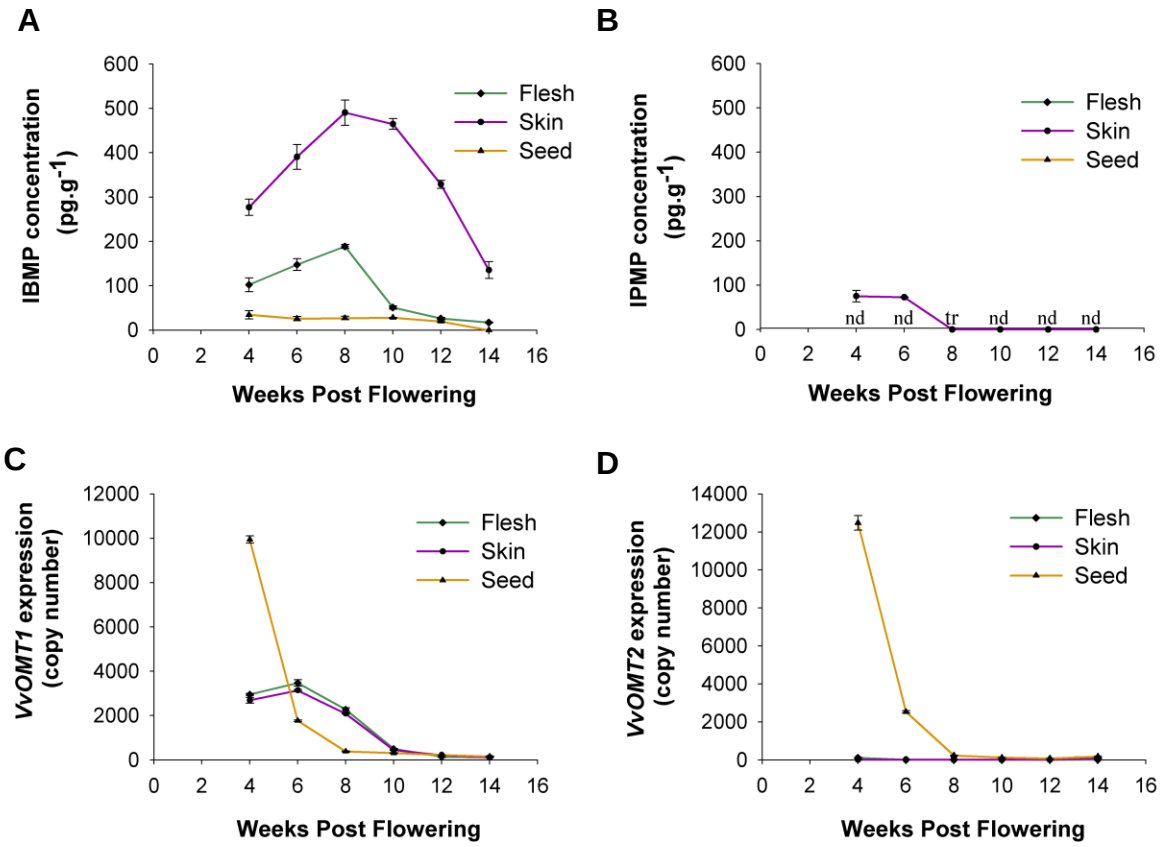


Figure 2.10 - MP concentrations and *VvOMT* expression in the flesh, skin and seed of developing berries. IBMP (**A**) and IPMP (**B**) concentrations respectively in the flesh, skin and seed of developing berries, expressed as $ng.kg^{-1}$ of fresh weight. Only trace (tr) levels of IPMP were detected in the 8wpf skins, and it was not detected (nd) in any seed or flesh samples, or skin samples taken after 8wpf. Relative expression levels of *VvOMT1* (**C**) and *VvOMT2* (**D**) in the flesh, skin and seed of developing berries

were maintained at low levels throughout the rest of development. However, unlike *VvOMT1*, the levels of *VvOMT2* expression were very low in both the skin and flesh samples taken throughout berry development (Fig. 2.10D). The lack of *VvOMT2* expression in the skin and flesh and the concurrence of *VvOMT1* expression and MP accumulation in these tissues imply that *VvOMT1* provides the methyltransferase activity responsible for MP accumulation in grape berries.

Other grape tissues were analysed to determine if there was a relationship between MP levels and *VvOMT* gene expression (Fig. 2.11). Rachis (bunch stem), flowers and tendrils all contained higher levels of IBMP than IPMP, while neither compound could be detected in leaves (Fig. 2.11A). Interestingly, roots accumulated substantially higher levels of methoxypyrazines than other tissues with 2,100 $pg.g^{-1}$ of IBMP and 8,270 $pg.g^{-1}$ of IPMP. Expression of *VvOMT2* was highest in the roots which was the only tissue tested that had more IPMP than IBMP (Fig. 2.11B). In the rachis, flower and tendril samples, *VvOMT1* expression levels showed a similar pattern to the levels of IBMP found in these tissues.

2.3.5 RNAi silencing of VvOMTs in grapevine hair-roots

The above results are suggestive of an association between MP accumulation and the expression of *VvOMT1* and *VvOMT2* in various tissues. This supports the hypothesis that these genes are responsible for MP accumulation in grapevines. However, *in planta* evidence is needed to prove this conclusively. Stable transformation to produce knockout lines using a hairpin construct is one approach for obtaining *in planta* evidence of gene function in plants, but stable transformation of grapevines is a long, labor-intensive and costly process and was not feasible for this project.

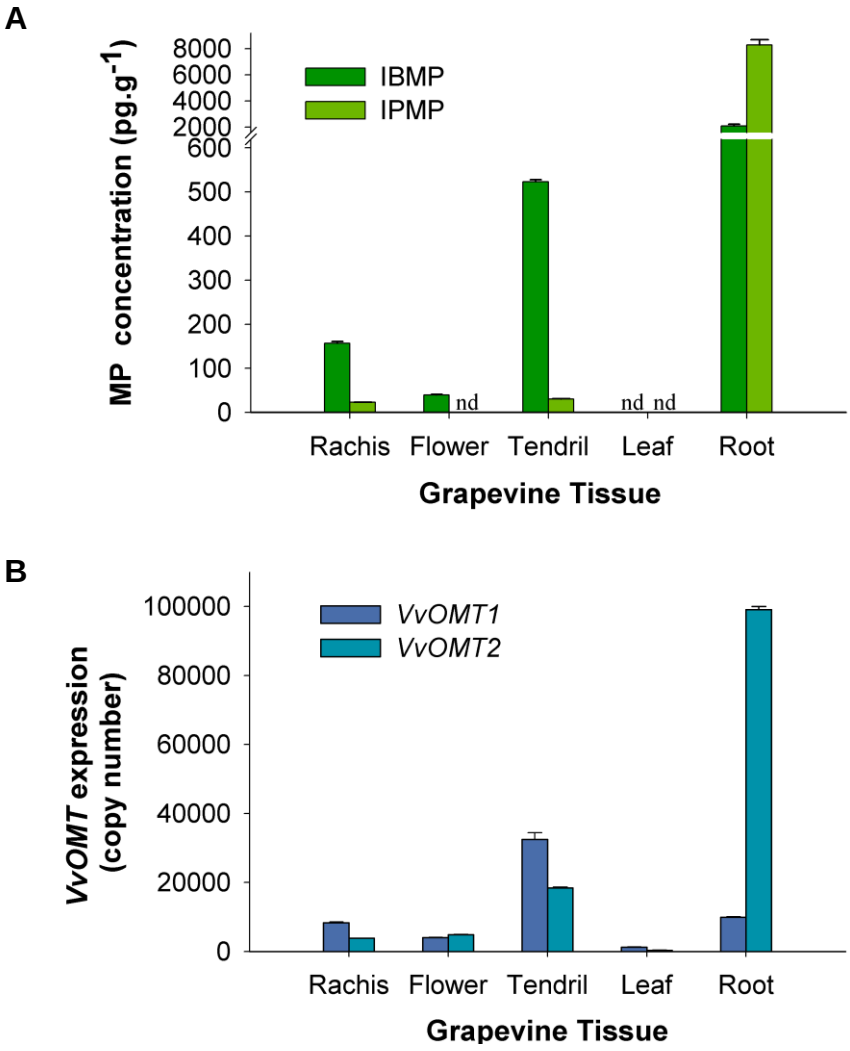


Figure 2.11 - IBMP and IPMP concentrations in various grapevine tissues (**A**), and the relative expression of *VvOMT1* and *VvOMT2* in those tissues (**B**). nd signifies not detectable.

Previous studies on grapevine OMTs have used transient over-expression in tobacco leaves to provide *in planta* evidence of gene function (Hugueney *et al.*, 2009; Schmidlin *et al.*, 2008), a method that is quicker and cheaper than stable transformation. However, these studies required the co-expression of genes involved in the biosynthesis of the presumed OMT substrate to detect enzyme activity. As it is unknown if the biosynthetic pathway leading to HP accumulation is active in tobacco leaves this approach may not be suitable.

An alternative to generating whole transformed grapevines is the production of transformed hairy-roots, which take a fraction of the time to produce and generate tissue for assays. Grapevine roots were found to contain high levels of MPs (Fig 2.11A) and thus provide a good target for silencing *VvOMT* genes to provide evidence of gene function. In this experiment, the aim was to silence both *VvOMT1* and *VvOMT2* simultaneously using a hairpin construct targeted to a 300 bp sequence homologous to both genes to provide *in planta* evidence of *VvOMT1* and *VvOMT2* gene function. Transformation of leaf explants with constructs targeted to *VvOMTs* resulted in the generation of 43 independent hairy-root cultures. Another RNAi construct targeted to a *VvMLO17* gene was used as a control and resulted in 26 hairy-root cultures. After 1 month of growth, roots were checked for GFP fluorescence to ensure they were positively transformed. GFP fluorescence was present in 24 of the 43 *VvOMT* cultures, while 15 of the 26 control cultures were positively transformed and expressing GFP. However, after 6 months of growth only 10 *VvOMT* and 5 control lines were sizable enough for both MP quantification and mRNA transcript analysis.

RNA was extracted from these hairy-root lines to determine the relative levels of *VvOMT1* and *VvOMT2* expression and thus the effectiveness of the silencing procedure. Real-time PCR analysis revealed that the five control lines

had comparable levels of *VvOMT1* and *VvOMT2* gene expression but variation of up to 60% was still seen between individuals (Fig 2.12A). The levels of both *VvOMT1* and *VvOMT2* were significantly reduced ($p < 0.05$ and $p < 0.001$) in the targeted lines compared to the controls. In 8 of the 10 targeted lines (A-H) the expression of both *VvOMT1* and *VvOMT2* ranged from 23% to 63% of that of the lowest control line, while 2 lines (I and J) actually showed an increase in *VvOMT1* and *VvOMT2* expression. These results suggests that the hairpin construct targeted to both *VvOMT1* and *VvOMT2* simultaneously was effective in reducing the expression of both genes albeit only partially and not consistently as two lines (I and J) showed no reduction in expression.

Although a complete silencing of *VvOMT* genes was not achieved, a partial reduction in expression was seen and if the activity of the enzymes encoded by these genes is responsible for MP accumulation in grapevine roots then a similar reduction of MPs might be expected. However, analysis of MP levels revealed no statistical difference between the control and these partially silenced target lines (Fig 2.12B). In both the control and *VvOMT*-targeted lines the highest levels of both IBMP and IPMP were seen in line E which showed low levels of *VvOMT1* and *VvOMT2* expression (56% and 28% respectively). Conversely the lowest levels of both IBMP and IPMP were seen in line I which showed no reduction in expression of *VvOMT1* or *VvOMT2* (Figure 2.12B). These results suggest that neither *VvOMT1* nor *VvOMT2* are responsible for the accumulation of MPs in grapevine roots.

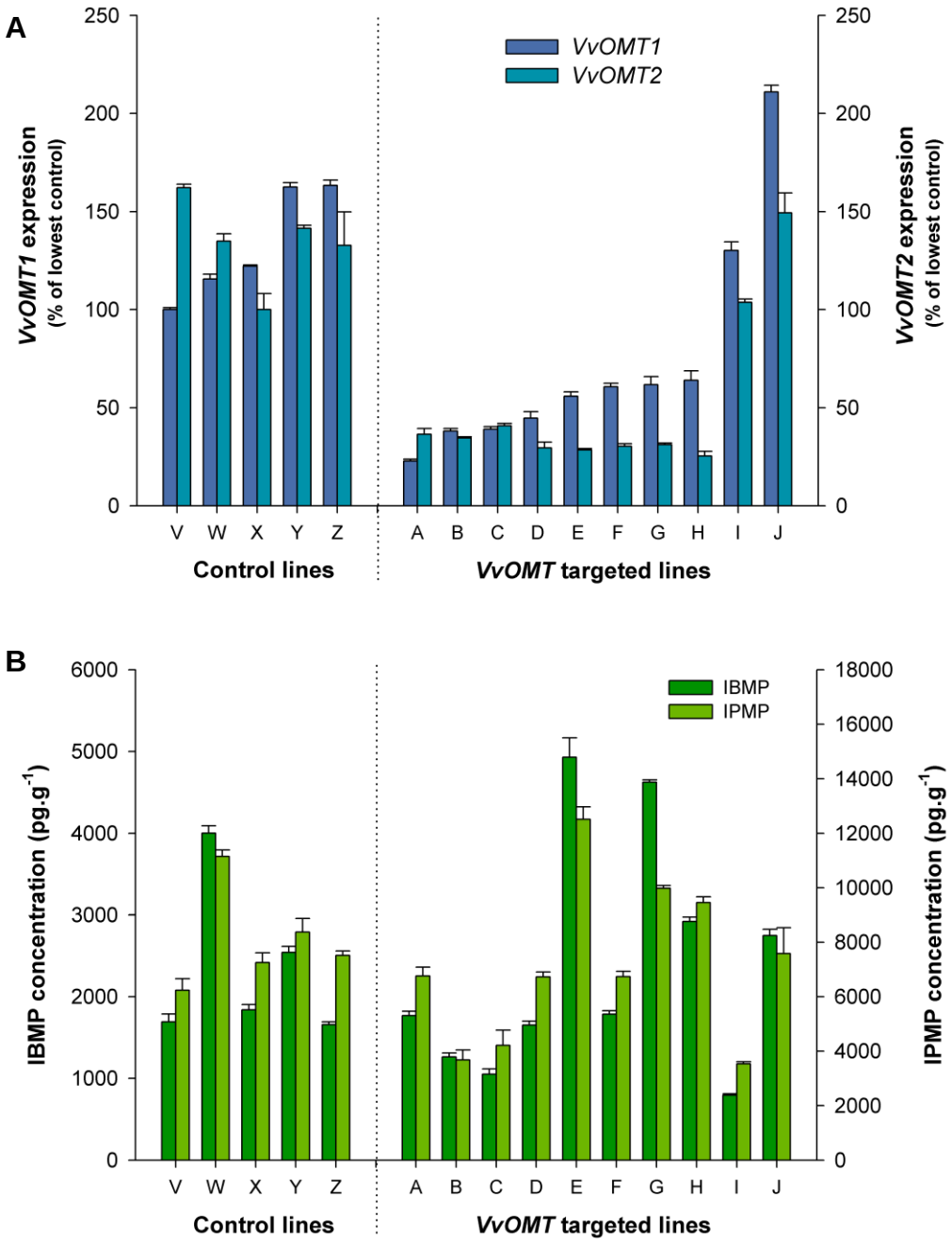


Figure 2.12 RNAi mediated silencing of *VvOMT* genes in grapevine hairy roots has no effect on corresponding MP levels. (**A**) The expression of *VvOMT1* and *VvOMT2* is markedly reduced in eight (A-H) of the ten RNAi targeted lines compared to 5 control lines (V-Z), transformed with a *VvMLO17* targeted construct. (**B**) The *VvOMT* RNAi targeted lines reveal no consistent reduction in IBMP or IPMP concentrations compared to the control lines.

2.4 Discussion

A search of grapevine genomic and EST databases resulted in the identification of two gene sequences, *VvOMT1* and *VvOMT2*, both encoding proteins with sequence homology to the N-terminal sequence of the protein purified by Hashizume and co-workers (2001b; Fig. 2.2). The predicted sizes of the proteins encoded by *VvOMT1* and *VvOMT2* are 40.8 kDa and 40.3 kDa respectively, both very similar to the 41 kDa molecular mass estimated by Hashizume *et al.* (2001b). The presence of two similar genes encoding enzymes with HP-methylating activity may explain the presence of two active fractions detected by Hashizume *et al.* (2001b) during purification of the native enzyme. Both *VvOMT1* and *VvOMT2* genes were found to be localised within a 22kb section of chromosome 12 of the grape genome (Fig. 2.1) suggesting that a possible duplication event followed by mutations may have led to the divergent evolution of the two genes. A similar occurrence has been reported in the legume *Medicago truncatula* in which three OMTs with different substrate specificities are located within a 20kb region of the genome (Deavours *et al.*, 2006). Truncated gene fragments with homology to methyltransferases were located between *VvOMT1* and *VvOMT2*, although no ESTs matching these sequences were found in the public databases.

Recombinant protein generated from both *VvOMT1* and *VvOMT2* was capable of methylating IBHP and IPHP *in vitro* to form IBMP and IPMP respectively (Fig. 2.5). Kinetic analysis suggests that VvOMT1 has greater activity against IBHP than IPHP, whereas VvOMT2 has higher activity against IPHP than IBHP (Table 2.2). However, both VvOMT1 and VvOMT2 show greatest activity against the flavonol compound quercetin (Table 2.3). Indeed the activity of the VvOMTs against HPs was in the order of 100 to 500 times less than

the methylating activity measured when quercetin was used as the substrate. These kinetic parameters of recombinant VvOMT1 and VvOMT2 against HPs are in accord with the trace levels of MPs that accumulate in grapes, and are similar to that of the native protein determined by Hashizume *et al.* (2001b), further evidence that these genes are responsible for the grape HP-methylating activity previously reported. Sequence alignment (Fig. 2.3) reveals that VvOMT1 and VvOMT2 differ in the amino acids at position 319 and 322, residues which have been shown to be important for substrate binding in plant OMTs (Zubieta *et al.*, 2001). This may be the reason for the differences seen in the kinetic activities of the two enzymes against HPs (Table 2.2). However a recent study performed detailed docking simulations of VvOMT1 and VvOMT2 with HP substrates to investigate the differences in activity of the two enzymes (Vallarino *et al.* 2011). These authors suggest that differences in the proximity of residues H272 and M182 in relation to the HP substrates could explain the differences in catalytic efficiency of the two enzymes (Vallarino *et al.* 2011).

VvOMT protein sequences align closely with OMTs shown to have greatest activity against various flavonoid molecules, which together with the results of activity assays using other compounds (Table 2.3), suggest that flavonols may be a preferred substrate of these enzymes *in planta*.

When the expression of *VvOMT1* and *VvOMT2* was analysed in whole grape berries, the peak in expression of both genes preceded the peak in MP levels by 4 weeks (Fig. 2.9). However, when berry skin, flesh and seeds were analysed separately the results showed that most of the expression of both *VvOMT* genes observed in the 4 wpf berries came from the seeds, which accumulate only trace levels of MPs (Fig. 2.10). The berry skin and flesh tissues accumulate MPs between 4 and 8 wpf and this coincides with the highest levels of *VvOMT1*

expression observed in these tissues during berry development (Fig. 2.10). Expression of *VvOMT2* was much lower than *VvOMT1* in the skin and flesh samples (Fig. 2.10), suggesting that *VvOMT1* is responsible for most of the HP-methylating activity in grape berries. Furthermore, the higher activity of VvOMT1 against IBHP compared to IPHP may play a role in the predominance of IBMP over IPMP in Cabernet Sauvignon berries, although substrate availability and product stability could also play a role in the ratio of these products at harvest.

Similarly, in all other grape tissues studied, excluding the seeds, there appears to be an association between the expression of *VvOMT1* and IBMP accumulation and between *VvOMT2* expression and IPMP accumulation. This suggests that the relative amount of each enzyme may be involved in determining MP composition in grape tissues. This hypothesis is supported by the kinetic parameters determined for each enzyme which suggest that VvOMT1 has greater catalytic activity towards IBHP than IPHP with the converse true for VvOMT2 (Table 2.2). Interestingly, the highest levels of MPs in any of the grape tissues tested were found in the roots (Fig 2.11A). The level of IPMP in grapevine roots was found to be above 8,000 $pg.g^{-1}$ (roughly 4,000 times higher than its odour detection threshold), and so it is not surprising that the aroma of IPMP has previously been reported as “like roots” (Gerber, 1977) and that the term “earthy” is often used to describe wines with high MP concentrations. The assumed biological role of MPs as a deterring odourant could suggest that extremely high levels of MPs are present in the roots to deter foraging herbivorous animals. Many pyrazine derivatives possess antimicrobial properties (Dolezal *et al.*, 2008; McGovern, 2004). While this has not been shown for either IBMP or IPMP, they may exhibit similar activities or act as precursors to more complex pyrazine derivatives which provide defense against microbial pathogens of grapevine roots.

The high levels of MP present in roots made it a desirable tissue for attempting to silence *VvOMT* genes to provide evidence of their function *in planta*. Transformation of grapevine explant with hairpin constructs targeted to both *VvOMT1* and *VvOMT2* resulted in hairy-root lines that had significantly lower levels of *VvOMT1* and *VvOMT2* expression compared to control lines (Fig. 2.12A). However complete silencing of either *VvOMT1* or *VvOMT2* was not observed in any of these 10 lines. This may be because either or both of these genes are vital to root growth and the complete silencing of these genes results in lethality or slowed growth, as only the fastest growing of the transformed roots were selected for MP and transcript analysis. Nevertheless the significantly reduced level of *VvOMT* transcription in the transformed lines did not result in any significant reductions in MP levels (Fig. 2.12B). This result indicates that other *VvOMTs* beside *VvOMT1* and VvOMT2 are likely to be responsible for MP accumulation in grapevine roots.

This is likely to be the reason why the high level of MP found in the roots was not reflected in high levels of *VvOMT1* or *VvOMT2* expression compared to other tissues. The reason roots contain high levels of MPs could be due to high expression of other *VvOMTs* or enzymes with greater catalytic activity against HPs. However, it is also possible that the roots contain high levels of HP substrates thus explaining the high level of MPs. Another possibility is the absence of competitive phenolic substrates of VvOMTs such as quercetin, which is not present in the roots of grapevines (Jeong *et al.* 2008). Seeds were found to contain high expression of both *VvOMT1* and *VvOMT2* in early grape development yet this was not associated with MP accumulation, providing another disconnect between expression of these genes and MP levels. This could be due to a lack of HP substrates in the seeds or may be the result of high levels of

substrates that compete for VvOMT activity, such as caffeic acid and related compounds involved in the production of lignin or the condensed tannin precursors catechin and epicatechin which are found at high levels early in seed development (De Freitas and Glories 1999; Rodriguez Montealegre *et al.*, 2006).

Despite the evidence suggesting VvOMT1 and VvOMT2 are not responsible for MP accumulation in roots and that *VvOMT* expression in seeds does not correspond to MP production, this does not rule out the possibility that these genes function in MP biosynthesis in grape berries. A strong association exists between *VvOMT1* expression and MP accumulation in the skin and flesh of developing berries and the VvOMT1 enzyme is capable of methylating HPs to produce MPs. These initial results together with the previous work of Hashizume *et al.* (2001b) suggest that *VvOMT1* is involved in MP biosynthesis in grapes, however further *in planta* evidence in berries is still required to prove this conclusively.

Chapter 3 - Towards mapping the gene responsible for methoxypyrazine accumulation in Cabernet Sauvignon berries

3.1 Introduction

3.1.1 Genetic diversity of cultivated grapevines

The domestication of the grapevine is thought to have occurred approximately 8,000-6,000 years ago (McGovern *et al.*, 1996; Zohary, 2004) and now over 5,000 different cultivars of *Vitis vinifera* are known to exist worldwide (Alleweldt, 1994). This large genetic variation is the result of thousands of years of human selection for grapevines possessing desirable traits such as hermaphrodite flowers, high fruit yield, large berry size and content such as increased sugars and flavour characteristics (This *et al.*, 2006).

The presence of new grapevine genotypes can arise by either sexual reproduction or through vegetative propagation of a vine sporting a new phenotype resulting from a somatic mutation. The grapevine genome is highly heterozygous, therefore the production of new varieties through conventional breeding and sexual reproduction results in progeny that have highly variable phenotypes. Desirable varieties are maintained by the clonal propagation of vegetative cuttings to maintain their valuable phenotypes. Somatic mutations occasionally occur that can result in a growth shoot displaying a different phenotype to the rest of the vine and propagation of these cuttings should lead to the establishment of a new cultivar. Somatic mutations can arise through small single base mutations or by insertions or rearrangement of larger genetic elements such as transposons. For example, it has been found that many white skinned grape varieties arose via a disruptional insertion of a retrotransposon into the

promoter region of *VvMybA1*, a gene which regulates anthocyanin biosynthesis in berry skins (Walker *et al.*, 2007). Another example of a variety arising from a somatic mutation in an existing cultivar is Pinot Meunier, which is considered to be a sport of Pinot noir (Vivier and Pretorius, 2000). Genetic variation is not only invaluable for crop improvement but also provides important diversity which can be utilised in mapping studies to understand gene functions.

3.1.2 Genetic mapping

The aim of genetic mapping is to detect and locate the gene(s) responsible for a given phenotypic trait within a variable population. By assessing correlations between genetic markers and phenotypic traits, genetic mapping not only has the power to locate genes but also provides strong evidence of *in planta* gene function. Two different methods of genetic mapping can be employed, family or linkage mapping and association mapping. Both mapping approaches exploit recombination's ability to break up the genome into fragments allowing associations of phenotypic traits with genomic loci, yet each differ in the population used to identify these associations and each has its advantages and disadvantages.

Linkage mapping utilizes variations in populations of known and controlled relatedness, such as a progeny of an experimental cross, whereas association mapping involves selecting naturally varying individuals, such as plants from a germplasm collection, to provide a population in which marker-trait associations are sought. Linkage mapping has traditionally been used for studying plants where the generation of large segregating progenies is relatively straight forward. The method of association mapping on the other hand grew from human studies where the creation of large related progenies is not achievable. In plant

studies where both approaches are applicable, a major advantage of association mapping is that it does not require the establishment of specific progenies, but utilizes pre-existing populations. The differences between the populations used in the two approaches also results in markedly different levels of possible mapping resolution. As individuals in family-based populations are only one or a few meiotic generations removed from the parents, only a relatively small number of recombination events results in a low resolution of mapping. In populations of association mapping studies however, the individuals are often very distantly related with many generations of recombination events occurring in the ancestry of each individual. This results in individuals having genomes which tend to be highly shuffled compared to each other and allows for a high level of mapping resolution (Myles *et al.*, 2009).

The extent of genome fragmentation within a given population is known as linkage disequilibrium and is a measure of the nonrandom association between alleles at different loci. The lower the LD, the more highly fragmented a genome is. LD is important as it reflects the resolution of mapping possible from a given population as well as the density of genetic markers required to identify associations between traits and the underlying functional genetic variation. For example, family-based populations have high LD and therefore only allow a low resolution of mapping, which in turn means fewer genetic markers are required, compared with association populations which show low LD, allowing a higher resolution of mapping, but requires many genetic markers. Knowledge of LD is especially crucial in association mapping populations in which relatedness of individuals is usually unknown. The extent of LD in different plant species and populations can vary greatly as LD is influenced by many factors such as mating system (selfing versus outcrossing), natural and artificial selection, recombination

rates (hot spots) and population size and structure (Flint-Garcia *et al.*, 2003). As many of these factors affect the structure and interrelationships of plant populations used for association mapping, complex statistical methods are often required to control for false positive associations (Mackay and Powell, 2007). Consequently the analysis of data from association mapping is far more intricate and time consuming than in a simple bi-parental cross of a linkage mapping experiment. However an advantage of association mapping is that all of the existing phenotypic variation within a population can potentially be mapped, whereas linkage mapping is limited to the variations existing between the original parents.

3.1.3 Genetic mapping in grapevine

As in most plant species, linkage mapping strategies have previously been the mapping method of choice in grapevines. However, certain physiological traits of grapevine hamper the establishment of mapping progenies and therefore only a limited number of mapping studies have been performed compared to other plant species such as *Arabidopsis*, maize, rice and wheat. Unlike these small annual species, grapevine is a large woody perennial with a long generation time of 3-5 years and requires large spaces to grow and maintain. Unless the appropriate populations already exist, these constraints make typical genetic mapping studies of grapevines logistically difficult, time consuming and often not feasible. As such, linkage mapping studies are often not pursued past the F1 generation, therefore only traits that are heterozygous in parents can be mapped. Nevertheless the limited linkage mapping studies performed in grapevine have been successful in identifying major single gene loci as well as QTLs responsible for a range of important phenotypic traits including flower sex (Dalbo *et al.*, 2000), berry

colour (Doligez *et al.*, 2002), fleshlessness (Fernandez *et al.*, 2006), seedlessness (Mejia *et al.*, 2007) and resistance to the pathogens Pierce's disease (Krivanek and Walker, 2005; Krivanek *et al.*, 2006) and powdery and downy mildew (Donald *et al.*, 2002; Hoffmann *et al.*, 2008). Of particular interest to this project are two previous studies which used linkage mapping to investigate QTLs responsible for berry monoterpene content, the compounds responsible for the distinctive Muscat flavour in certain varieties. In a pre-existing F1 progeny of Muscat Hamburg x MTP2687-85 (Olivette x Ribol), a major QTL located on linkage group 5 was found responsible for 17-55% of the total variance of the three major monoterpene compounds in grape berries (Doligez *et al.*, 2006). A similar study using two F1 progenies from Italia x Big Perlon and Moscato BIanco x *V. riparia* confirmed the QTL on linkage group 5 and found it accounted for 26-84% of the variation in berry monoterpene content (Battilana *et al.*, 2009). Further analysis or this QTL revealed the presence of a gene encoding a putative 1-deoxyxylulose-5-phosphate synthase (DXS). In other plant species DXS has been shown to be the first enzyme of the non-mevalonate pathway, one of two pathways that produce the five carbon precursors required for monoterpene biosynthesis (Dunlevy *et al.*, 2009).

Because the creation of mapping populations in grapevine is not an easy task and there is an increasing access to quality genomic data, there has been an emerging interest in the potential of association mapping in this plant species. Association mapping in grapevines is still in a developmental stage, but is likely to soon be a powerful tool for genome-wide studies targeted to multiple traits. The focus of recent studies has been to establish the extent of LD in grapevine populations to determine the feasibility of genome-wide association studies. One study assessing 38 microsatellite markers across 5 chromosomes in 141 cultivated

grapevine varieties suggested a decay in LD after approximately 650-1,080 kb (Barnaud *et al.*, 2006). Using the same markers in 85 wild *Vitis vinifera* L. subsp. *silvestris* the same authors found LD decays more rapidly at approximately 351-583 kb in wild grapevines (Barnaud *et al.*, 2010). In a separate study between 11 cultivated and 2 wild grapevines LD was found to decay after only 100-200 bp (Lijavetzky *et al.*, 2007). It has been suggested that the large discrepancy between the two findings is due to the differences in the type of markers used (Costantini *et al.*, 2009), however, the major differences in population size and structure between the studies is also likely to have had a major effect. Based on these LD values it can be estimated that at least 2,000 and possibly as many as 2 million genetic markers would be required to effectively cover the 475-500 Mb genome of grapevine (Myles *et al.*, 2009), but this will ultimately depend on the structure of the population under investigation. Thus genome-wide association studies in grapevine are currently unfeasible because of the extensive costs and time associated with the identification of polymorphisms for genetic markers. However, with the rapid advancement of sequencing technologies, access to large numbers of genetic markers required for genome-wide association studies may soon be possible.

A directed approach of association mapping targeted to specific candidate genes only requires a small number of markers around a target locus. Two recent studies performed in grapevine have demonstrated the enhanced mapping resolution of directed association mapping over traditional linkage mapping. However, both these studies first utilised linkage mapping experiments to indentify the locus of interest. In the first study, a single QTL was identified that accounted for 62% of the variation in anthocyanin content in an F1 population from a Shiraz × Grenache cross (Fournier-Level *et al.*, 2009). Located on this

QTL were four *Myb*-type genes which have previously been shown to be responsible for berry anthocyanin content (Deluc *et al.*, 2008; Lijavetzky *et al.*, 2006; This *et al.*, 2007; Walker *et al.*, 2007; Ageorges *et al.*, 2006). Association mapping targeted to this cluster of *Myb* genes was performed in 141 grapevine varieties and successfully identified four polymorphisms responsible for 84% of the variation in anthocyanin content in that population (Fournier-Level *et al.*, 2009). In a second study, the gene 1-deoxy-D-xylulose 5-phosphate synthase, located on a QTL responsible for the Muscat flavour of grapes mentioned above (Battilana *et al.*, 2009), was sequenced in 95 Muscat-flavoured and 53 non-Muscat flavoured cultivars. A single SNP was found to be present in 95% of the Muscat-flavoured cultivars (Emanuelli *et al.*, 2010).

Each of these studies demonstrates the advantages of performing both traditional linkage mapping and association mapping approaches to complement each other, which can increase the mapping resolution from QTLs to QTNs (quantitative trait nucleotides).

3.1.4 The Pinot Meunier dwarf provides a tool for rapid grapevine genetics

Pinot Meunier (PM), which is considered to be a sport of Pinot noir (PN; Vivier and Pretorius, 2000) originates from the Champagne region in France. It is thought to have been selectively propagated because it has a later budburst than PN which improves fruit set and increases yield in frost-prone sites (Dry and Gregory, 1988). The name Meunier (French for miller) was given to the cultivar because of its tomentose leaves and shoot tips, giving the impression that it has been dusted with flour. It has been shown that PM is a chimera, containing a mutation in the plants L1 outer cell layer which causes the tomentose phenotype (Boss and Thomas, 2002). This mutation is located in a grapevine homologue of

the *GA Insensitive* gene (*VvGAI1*) which reduces the response of the plants to gibberellins (GAs), plant growth regulators which play important roles in cell elongation and floral development. When plants were regenerated by somatic embryogenesis from the PM L1 cell layer, they were found to have a dwarfed phenotype as a result of the altered GA response (Boss and Thomas, 2002). The markedly small size of the PM dwarf allows as many as 34 plants to be grown per m^2 in a glasshouse (Chaib *et al.*, 2010) compared to regular vines which can be grown at approximately 4 per m^2. Unlike wild-type vines, which have a long generation time of 2-5 years (depending on cultivar and environmental factors), the PM dwarf has the ability to flower rapidly, producing fruit as quickly as 6 months after germination (Chaib *et al.*, 2010). These traits make the PM dwarf especially suited to mapping experiments as large numbers of progenies can be established quickly and maintained under controlled growth room or glasshouse conditions. The short generation time of the PM dwarf means progenies can easily be taken past the F1 generation allowing the mapping of traits that are recessive in a parent (Chaib *et al.*, 2010). Under conditions favourable for floral induction, the PM dwarf is also capable of producing fruit all year round, unlike seasonal wild-type grapevines that only offer a limited seasonal window of time during which grape berries can be collected for phenotyping.

Many of these physiological traits that hamper mapping studies also make transformation of grapevines a tedious, time-consuming and expensive operation with no guarantee of success (Dhekney *et al.*, 2009; Iocco *et al.*, 2001). As an alternative to transformation to provide functional *in planta* evidence of gene function, the PM dwarf offers a valuable tool for rapid linkage mapping studies.

3.1.5 Aims

With the aim of obtaining *in planta* evidence for the role of *VvOMT1* in IBMP accumulation in berries, a genetic mapping approach was employed. The work presented in this chapter describes the generation, phenotypic evaluation and genetic mapping of a segregating F2 population resulting from a bi-parental cross between the PM dwarf and Cabernet Sauvignon (CS). A locus showing co-segregation with IBMP accumulation in berries is further investigated with a directed association mapping study utilizing the genetic variation within a collection of existing grapevine cultivars.

3.2 Materials and Methods

3.2.1 Chemicals and solution

Solutions and growth media used are described in Table 3.1. All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA). Restriction endonucleases were obtained from New England Biolabs (Ipswich, MA, USA), and Roche Diagnostics (Basel, Switzerland).

Table 3.1 Composition of solutions and growth media

Solution	Composition
Grapevine soil mix	2 parts coarse pine bark, 1 part coarse river sand, 0.3% (w/v) dolomite, 0.1% (w/v) lime, 0.1% (w/v) gypsum and 0.1% (w/v) $FeSO_4$
Murashige & Skoog (MS) solution	1× MS macros, 1× MS micros, 0.5 μM EDTA1 and 0.3 μM Fe/EDTA
1× MS macros	20.6 mM NH_4NO_3, 2.99 mM $CaCl_2.6H_2O$, 1.5 mM $MgSO_4.7H_2O$, 18.79 mM KNO_3, 1.25 mM KH_2PO_4
1× MS micros	100 μM H_3BO_3, 100 μM $MnSO_4.4H_20$, 29.9 μM $ZnSO_4.7H_20$, 0.1 μM $CuSO_4.5H_20$, 0.11 μM $CoCl_2.6H_20$

3.2.2 Plant material

A total of 91 different varieties (Table 3.2 and Appendix D), were used for association mapping and were sampled at 7 wpf sampled from the Coombe vineyard at the University of Adelaide's Waite Campus in Adelaide, South Australia (latitude 34^{o} 56' S, longitude 138^{o} 36' E). From each variety 50-100 berries were sampled at approximately 7 wpf for IBMP analysis. In all cases samples were snap frozen in liquid nitrogen and stored at -80ºC until required.

3.2.3 Generation of an F2 population from a cross between CS and the PM dwarf

The cross between CS and the PM dwarf vine was performed by Dr Paul Boss, and the F1 progeny had been established prior to the commencement of this project. The parents of this cross consisted of 12 CS vines and 12 PM dwarf vines grown under glasshouse conditions. The cross was conducted using both genotypes as either the male or female parent.

For the establishment of the F2 progeny the berries from F1 plants were grown to maturity and the seed then collected and sterilized by shaking 20-100 harvested seeds in 40 ml of 0.5 M hydrogen peroxide at 60 rpm overnight. Sterilized seeds were stored on cotton wool at 4°C for at least 2 months, then placed onto moist filter paper and left to germinate under growth room conditions (27°C, 16 hours light/ 8 hours dark). Once germinated, 2-4 week old sterile seedlings were transferred to a soil mixture of 50% fine sand and 50% grapevine soil mix (Table 3.1) which had been pre-sterilized by autoclaving. Seedlings were supplemented with a 1 in 20 dilution of MS solution (Table 3.1) and continued to be grown under growth room conditions for approximately 1 month. Mature

seedlings were then transferred to grapevine soil mix in small 8 cm diameter pots, covered with a clear plastic bag to maintain humidity and grown under natural lighting conditions in a glasshouse. To acclimatize the plants to the glasshouse humidity, each week a corner of the covering bag was cut, followed by the complete opening and then removal of the bag. After 2-3 months of growth, dwarfed plants were easily identifiable and were selected from wild-types which were discarded. Dwarf plants were transferred to large 20cm diameter pots containing grapevine soil mix and a teaspoon of Osmocote® (Baulkham Hills NSW, Australia) added. Plants were irrigated with drippers on a twice daily 10 min watering regime. As the dwarf vines do not show the usual synchronized ripening patterns of wild-type grapevines but set fruit at different times, individual bunches were tagged at the date of flowering (cap-fall) and harvested at 7 wpf.

3.2.4 Methoxypyrazine quantification

IBMP was quantified by a stable isotope dilution assay using HS-SPME-GC-MS as described in section 2.2.9. All samples were analysed in triplicate. Samples that had trace levels of IBMP quantified below or near the limit of detection were repeated using 5 g of tissue (instead of 1 g) to increase sensitivity.

3.2.5 DNA extraction and purification

For purification of DNA, a small leaf (1-3 cm in diameter) was sampled from each of the 91 varieties, plants from the F1 and F2 populations as well as the parents. DNA extractions were performed at the Australia Genome Research Facility (Urrbrae, SA, Australia) using a NucleoSpin® 96 Plant II kit (Macherey-Nagel, Düren, Germany).

3.2.6 SNP identification and design of CAPS and dCAPS markers

Potential single nucleotide polymorphisms (SNPs) between the CS and PM genomes were identified using online genomic databases. The annotated genomic database at NCBI (Jaillon *et al.*, 2007) was used to select genes of interest based on chromosome number and position. CS ESTs showing homology to the selected genes were identified using BLAST (Altschul *et al.*, 1990) and aligned with the corresponding PN genomic sequence to identify any potential SNPs using clustalW (Thompson *et al.*, 1994). Primers were then designed to DNA sequences that showed polymorphisms between the PN genome and CS ESTs and PCR performed to amplify DNA fragments from CS and PM DNA. Amplified products were then sequenced with the same primers to verify the existence of SNPs and to assure homozygousity in each of the parent genomes. SNPs were then assessed for suitability as a CAPS marker using the online tool, dCAPS Finder 2.0 (Neff *et al.*, 2002), which identifies differential restriction enzyme sites between the polymorphic sequences. In the absence of a restriction enzyme site suitable for a CAPS marker, primers were designed with a single nucleotide mismatch to introduce a restriction enzyme site to one of the polymorphic sequences, producing a dCAPS marker (Neff *et al.*, 1998). Full descriptions of the markers developed and used in this study are given in Appendix C and the respective primers listed in Appendix A.

3.2.7 Mapping using the CAPS and dCAPS markers

For each genetic marker, PCR using marker specific primers (Appendix A) was used to amplify DNA from each mapping plant individually. PCR products (10 µl) were digested with the appropriate restriction enzyme (Appendix C) in a total volume of 30 µl for 3-6 h. Digested fragments were then run on a 1.5-2.5%

(w/v) agarose gel to visualize the separation of digested and/or non-digested fragments. Depending on the pattern of restriction digests, individuals were scored as either homozygous CS, homozygous PM or heterozygous at the respective marker loci (Appendix D). The Fisher's exact test was used to calculate the significance of marker-trait associations within the cultivar population used in the association mapping study.

3.3 Results

3.3.1 Phenotypic analysis of the progeny from a CS × PM dwarf cross

As outlined in the introduction to this chapter, the small growth habit and rapid flowering phenotype of the PM dwarf make it an ideal tool for grape genetic experiments (Fig. 3.1). However, its usefulness as a crossing parent also depends on the phenotype of the PM dwarf and whether it will provide genetic variation that can be utilized in subsequent populations. To determine the suitability of the PM dwarf as a crossing parent in this study, berries were analyzed at 7 wpf for IBMP content. Analysis of the berries from 12 PM dwarfs revealed no detectable levels of IBMP whereas the CS parents contained IBMP at concentrations ranging from 110 to 162 $pg.g^{-1}$ when grown under the same glasshouse conditions (Fig. 3.2). Therefore the PM dwarf is ideal to cross with a variety (in this case CS) that does accumulate IBMP with the aim of producing progeny that segregate for the trait of IBMP-accumulation. At the commencement of this study a cross between the PM dwarf and CS had been performed by Dr Paul Boss, resulting in a population of 94 dwarfed F1 progeny. The dwarfed phenotype is a semi-dominant trait and is heterozygous in the PM dwarf parents (Boss and Thomas, 2002), therefore a 1:1 ratio of dwarfed to wild-type plants was seen in the F1 progeny although only dwarfed plants were retained. Of the 94 dwarfed F1 plants

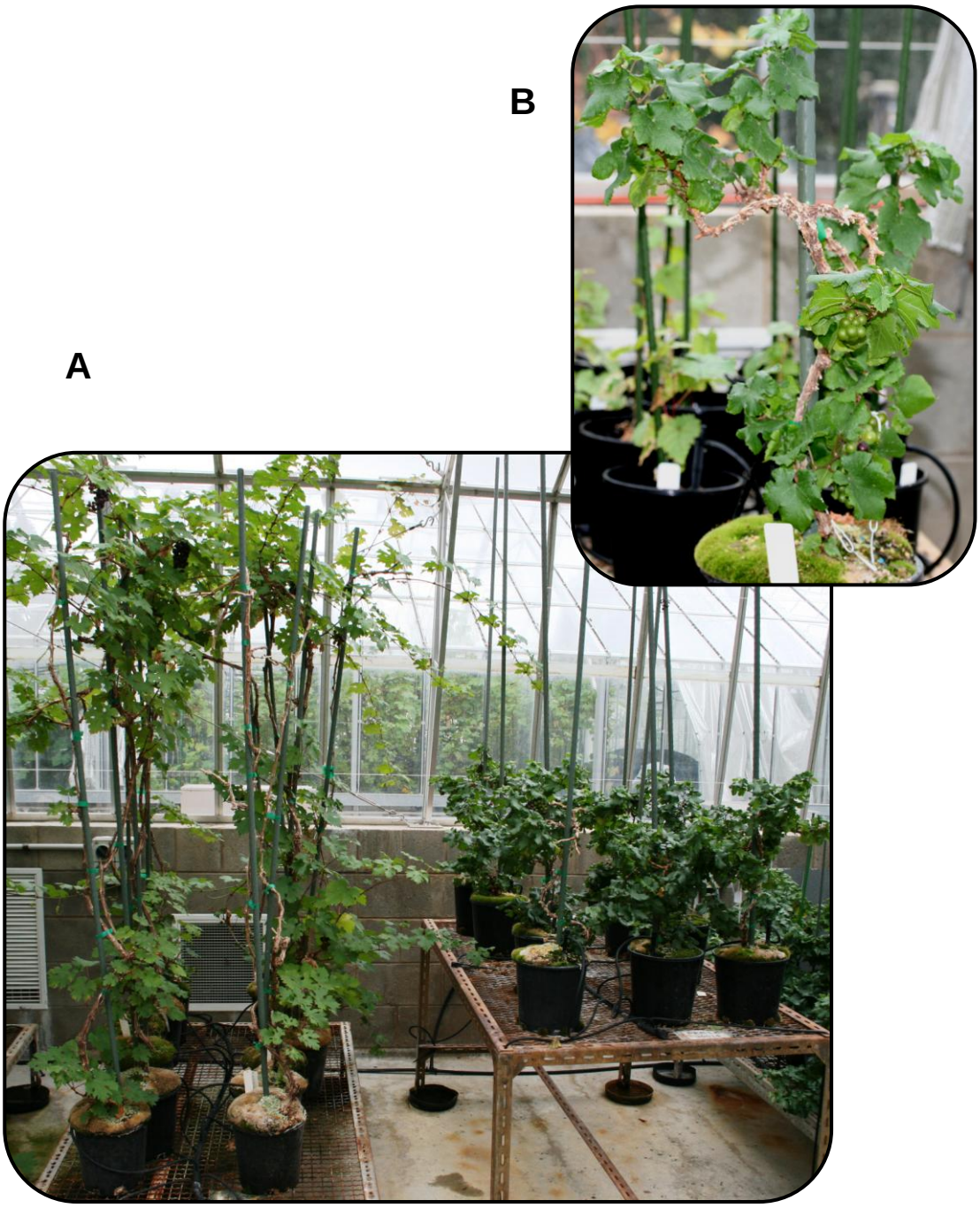


Figure 3.1 A - Wild-type CS grapevines (left) compared with the PM dwarf parents (right). **B -** A F1 dwarf offspring from the CS × PM dwarf cross, which is producing bunches shortly after pruning.

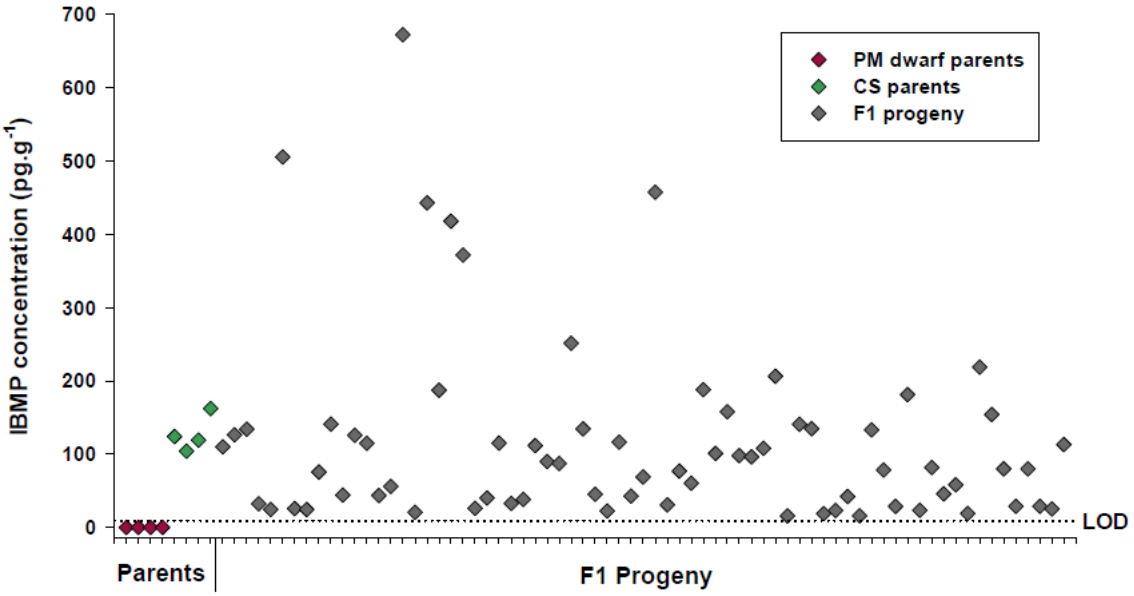


Figure 3.2 - IBMP concentrations in the PM dwarf and CS parents and the resulting F1 progeny. IBMP concentration is expressed as $pg.g^{-1}$ of berry fresh weight at 7 wpf. The dotted line represents the limit of detection (LOD) of the GC-MS method of quantification. All PM dwarf parents (red) contain no detectable levels of IBMP. All CS parents (green) contain detectable levels of IBMP as do all of the F1 progeny (grey).

established, only 71 had produced berries at the time of sampling. All of these 71 F1 plants contained IBMP at 7 wpf at concentrations varying from 15.7 to 672.2 $pg.g^{-1}$ (Fig. 3.2). As all the F1 progeny accumulate IBMP in the berries, it is hypothesised that this is a dominant trait which is homozygous in the CS parents. As the F1 population does not segregate for the trait of IBMP-accumulation in the berries, it is not suitable for genetic mapping studies. With the aim of producing a population that does segregate for IBMP, an F2 population was established.

3.3.2 Phenotypic analysis of the CS × PM dwarf F2 population

Assuming that the F1 plants are all heterozygous for the trait of IBMP-accumulation, a 3:1 ratio of accumulating to non-accumulating plants would be expected in an F2 population. Seeds from mature F1 berries were collected, germinated and dwarfed vines identified. A population of 92 F2 dwarfed plants was generated of which only 62 had produced berries by the end of this project. Metabolite analyses revealed that the population did in fact segregate for the trait of IBMP-accumulation in the berries. At the 7 wpf stage, 42 plants contained IBMP at levels between 14.3 and 1,178.6 $pg.g^{-1}$ and 20 plants were found to contain no detectable levels of IBMP (Fig. 3.3). Chi-square analysis reveals there is a 20% probability that the deviation of observed ratios from the expected 3:1 ratio is due to chance alone. Therefore it can be accepted that the segregation follows a 3:1 ratio and, furthermore, conclude that the F1 plants are heterozygous and that the PM dwarf parents are homozygous recessive for the trait.

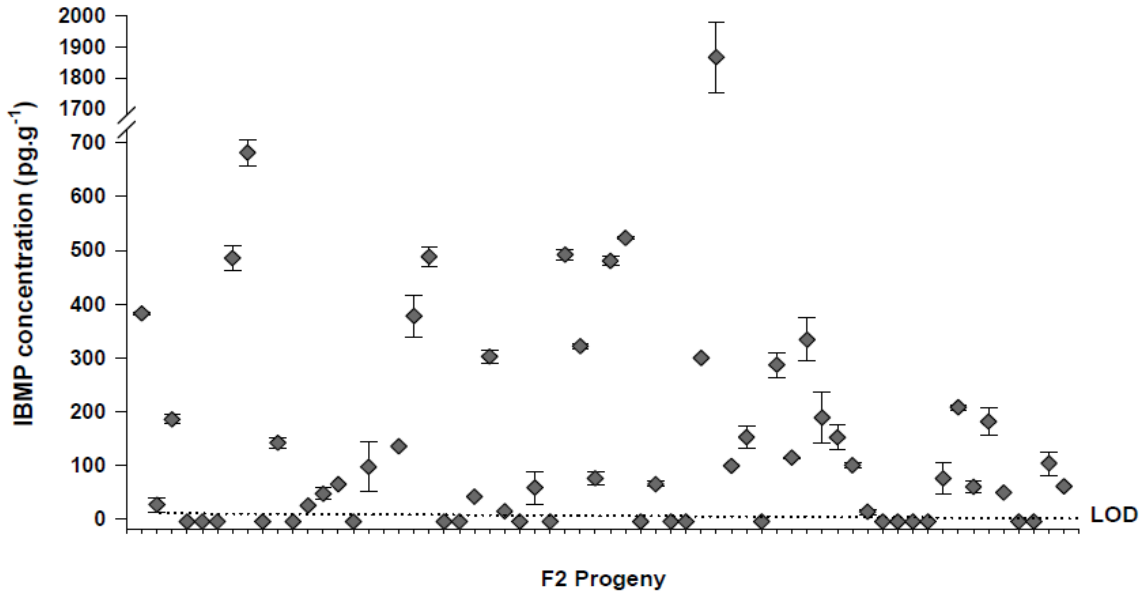


Figure 3.3 - Segregation of IBMP accumulation in the F2 progeny. IBMP concentration is expressed as $pg.g^{-1}$ of berry fresh weight at 7 wpf. Of the 62 F2 progeny producing berries, 42 contain detectable levels of IBMP and 20 contain levels below the limit of detection (LOD) shown as the dotted line. Error bars represent standard error based on triplicate analysis.

3.3.3 Association of IBMP accumulation with genetic markers in the PM dwarf × CS F2 population

The original hypothesis to be tested with this cross was that *VvOMT1* alleles from CS would segregate with the IBMP berry accumulation phenotype. However, sequence analysis of both the *VvOMT1* and *VvOMT2* genes from CS and the PM dwarf revealed that for both genes there was one shared allele and one unique allele in both varieties (Appendix A). Given the segregation of the trait in the F1 and F2 populations which suggests that the genotype is homozygous in both parents, it is unlikely that *VvOMT1* or *VvOMT2* are the genes responsible for IBMP accumulation in the berries of CS.

Therefore, genetic markers were designed to map the locus associated with the trait of IBMP-accumulation in the berries of the segregating F2 population. In this study IBMP accumulation was treated as a simple qualitative trait, i.e. positive or negative for IBMP accumulation, rather than a quantitative trait concerning the extent of IBMP accumulation. CAPS and dCAPS markers were designed based on SNPs between the CS and PM dwarf parent genome sequences (Appendix C). The individual plants of the F2 population were scored for parental origin as each marker was developed, and the third marker tested showed an association with the trait of IBMP-accumulation in berries (Appendix D). This marker was located on chromosome 3, and so the design of further markers focused on this region to confirm the initial marker-trait associations and to identify the borders of this locus.

The results of the mapping indicate that, in this population, the trait of IBMP-accumulation in berries is strongly associated with a locus located near the top of chromosome 3. Of the 62 F2 plants that produced fruit, 60 show perfect associations between the parental origin of this locus and the observed IBMP

phenotype (Fig. 3.4). All of the 40 plants that were either heterozygous or homozygous for the CS allele at this locus accumulated IBMP in berries and all 20 plants that lacked IBMP were homozygous for the recessive PM allele (Appendix D). However two plants, #3 and #42, were homozygous for the PM allele yet accumulated IBMP in the berries, albeit at small levels (26.8 and 64.7 $pg.g^{-1}$ respectively). Therefore, this locus appears to be the major one responsible for the IBMP-accumulation in berries, but one or more other loci may be involved in this process to a lesser degree. Recombination events between the markers at 0.31 and 1.6 Mb as well as between 2.4 and 2.6 Mb allowed the borders of this locus to be established at 0.31 and 2.6 Mb (Fig. 3.5). A search of the online annotated genomic database (Jaillon *et al.*, 2007) revealed 261 gene sequences located between these markers. Analysis of the putative identities of these genes resulted in the identification of two new putative *O*-methyltransferase genes, subsequently named *VvOMT3* and *VvOMT4*. Of the 261 genes residing in this locus, these two putative OMTs showed the greatest potential for a direct role in IBMP biosynthesis.

3.3.4 IBMP-accumulation in the berries of different grapevine cultivars

To further investigate the genetic basis of IBMP-accumulation in grape berries, a collection of 91 different grapevine cultivars from the same vineyard were assessed for phenotypic suitability as an association mapping population. Previous studies have identified a number of cultivars that contain detectable levels of IBMP at harvest (Belancic and Agosin, 2007; Koch *et* al., 2010; Lacey *et al.*, 1991) but little information exists on whether other varieties that lack IBMP at harvest contain any detectable levels at time of the pre-véraison peak in

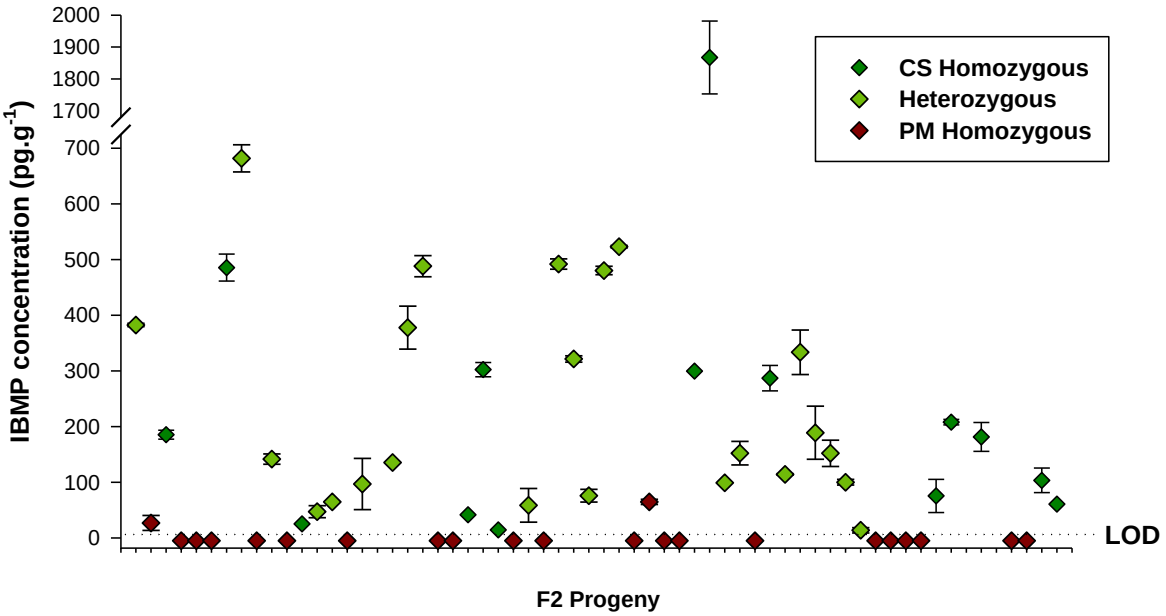


Figure 3.4 - Association of a locus near the top of chromosome 3 with the trait of IBMP-accumulation in the F2 progeny. IBMP concentration is expressed as $pg.g^{-1}$ of fresh weight berry at 7 wpf. Individuals homozygous for the CS locus are shown in dark green, individuals heterozygous at this locus are shown in light green and individuals homozygous for the PM locus are shown in red. All F2 individuals that are either homozygous CS or heterozygous contain detectable levels of IBMP. All but two F2 individuals that are homozygous PM lack detectable levels of IBMP. Two plants contain low concentrations of IBMP but are homozygous for the PM locus.

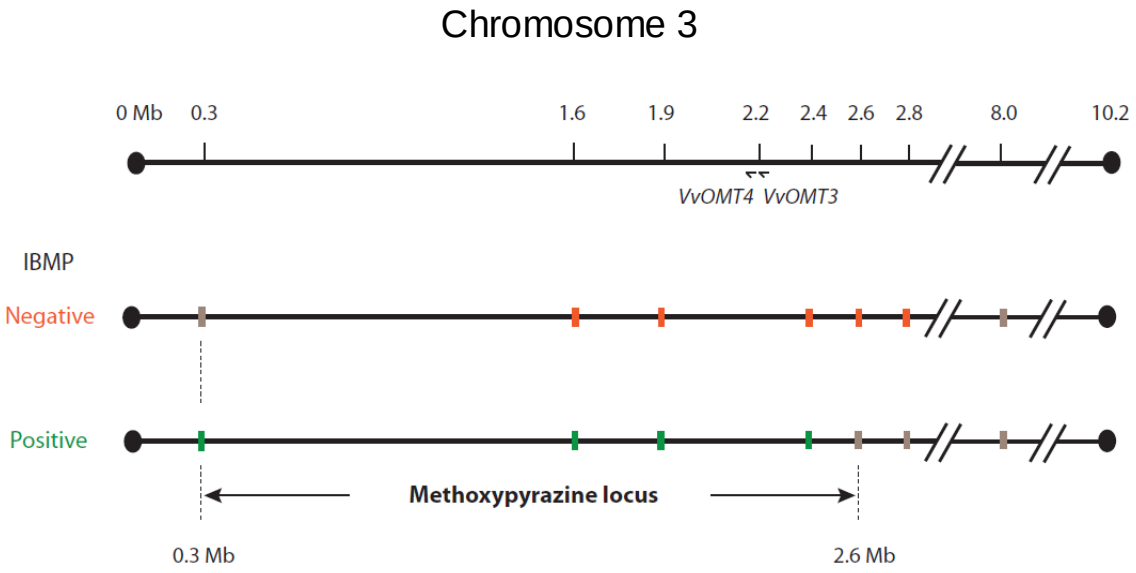


Figure 3.5 - Schematic of the genomic locus on chromosome 3 that segregates with IBMP accumulation in the F2 progeny. Dashes represent the location of CAPS/dCAPS markers used to assess the genetic origin of F2 individuals. Distances from the top of the chromosome are given in megabase pairs (Mb). Red dashes represent situations where all individuals were homozygous PM, green dashes represent situations where all individuals were CS or heterozygous and grey dashes represent recombination at a marker thus breaking the association between genotype and phenotype. All F2 individuals that were negative for IBMP were homozygous PM at this locus. A recombination event between marker 0.3 Mb and 1.6 Mb established the upper border of this locus. With the exception of #3 and #42 all F2 individuals that were positive for IBMP accumulation were either CS homozygous or heterozygous at this locus. A recombination event between marker 2.4 Mb and 2.6 Mb established the lower border of this locus. Two putative *O*-methyltransferase gene sequences, *VvOMT3* and *VvOMT4* reside within this locus.

accumulation that was observed in CS. To investigate this, berries were sampled at approximately 7 wpf from the 91 different varieties. IBMP was detectable in only 11 of the 91 varieties with the highest concentrations found in varieties previously reported to accumulate IBMP, namely, Cabernet Franc (464 $pg.g^{-1}$), Semillon (205 $pg.g^{-1}$), Sauvignon blanc (175 $pg.g^{-1}$) , Merlot (136 $pg.g^{-1}$) and Ruby Cabernet (120 $pg.g^{-1}$). The varieties Roussane, Aleatico, Biancone, Durif, Tinta amarella and Tinta Cao also contained detectable but low levels of IBMP (15 – 57 $pg.g^{-1}$), while all of the other 80 varieties did not contain detectable levels of IBMP at 7 wpf (Table 3.2).

3.3.5 Association of the IBMP-accumulating phenotype with genetic markers in the cultivar collection

Having phenotyped the cultivar collection, the locus identified in the F2 population was further investigated for its involvement in IBMP-accumulation using a candidate gene based association study within this population of grapevine cultivars. Theoretically this diverse population should contain higher linkage disequilibrium than the closely related F2 population, allowing for a greater resolution of mapping. The CAPS and dCAPS markers at the locus between 0.3 and 2.6 Mb were used for association mapping of the individuals in the cultivar collection. Analysis of the genetic markers within the cultivars revealed a strong association between the trait of IBMP-accumulation and the marker 2.20 Mb located in the *VvOMT3* gene (Appendix D). Of the 11 cultivars that accumulate IBMP, 10 were either homozygous CS or heterozygous at the 2.20 Mb marker. Conversely 75 of the 76 cultivars that were homozygous PM at marker 2.20 Mb contained no detectable IBMP, while one cultivar, Tinta amarella, contained a small level of IBMP. Five cultivars, Terret noir, Nebbiolo, Crouchen, Souzao and

Table 3.2 - IBMP concentrations in berries from the Coombe vineyard cultivar collection. Berries were sampled at 7 wpf and assessed for IBMP by GCMS analysis. Eleven wine grape cultivars showed varying levels of IBMP and the values represent the mean ± the standard error based on duplicate analyses. In all other cultivars, IBMP concentrations were below the limit of detection (nd).

Wine Grapes						Table Grapes	
Cultivar	IBMP ($pg.g^{-1}$)	Cultivar	IBMP ($pg.g^{-1}$)	Cultivar	IBMP ($pg.g^{-1}$)	Cultivar	IBMP ($pg.g^{-1}$)
Cabernet Franc	464 ± 39	Grenache	nd	Valdiguié	nd	Sugarone	nd
Semillon	205 ± 4.5	Pinot noir	nd	Kadarka	nd	Gold	nd
Sauvignon blanc	175 ± 37	Malbec	nd	Raboso Piave	nd	Merbein Seedless	nd
Merlot	136 ± 4.1	Gamay	nd	Black Frontignac	nd	Italia	nd
Ruby Cabernet	120 ± 1.6	Zindfandel	nd	Muscadelle	nd	Dawn Seedless	nd
Roussanne	57 ± 2.9	Mataro	nd	Bastardo	nd	Perlette	nd
Aleatico	42 ± 1.5	Sangiovese	nd	Souzão	nd	Sultana	nd
Tinta Cão	31 ± 1.8	Barbera	nd	Touriga	nd	Waltham Cross	nd
Tinta Amarella	27 ± 0.8	Canocazo	nd	Nebbiolo	nd	Calmeria	nd
Biancone	23 ± 8.0	Rkaziteli	nd	Malvasia Bianca	nd	Christmas Rose	nd
Durif	15 ± 1.6	Ondenc	nd	V. candicans	nd	Cardinal	nd
Riesling	nd	Palomino	nd	Parthenocis-sus	nd	Monukka	nd
Chardonnay	nd	Clairette	nd	Tempranillo	nd	Flame Seedless	nd
Viognier	nd	Farana	nd	Verdicchio	nd	Ruby Seedless	nd
Verdelho	nd	Trebbiano	nd	Tannat	nd	Red Globe	nd
Marsanne	nd	Chasselas	nd	Arneis	nd	Blush Seedless	nd
Colombard	nd	Doradilo	nd	Chambour-cin	nd	Emperor	nd
Chenin blanc	nd	Crouchen	nd	Rubired	nd	Ribier	nd
Müller-Thurgau	nd	Petit Verdot	nd	Kyoho	nd	Barlinka	nd
Traminer	nd	Carignan	nd	Pedro Ximénes	nd	Zante Current	nd
Muscat Gordo	nd	Bonvedro	nd	Concord	nd	Carina	nd
Shiraz	nd	Cinsault	nd	Muscat Hamburg	nd	Marro Seedless	nd
		Terret noir	nd			Beauty Seedless	nd
		Dolcetto	nd				
		Mondeuse	nd				

Raboso Piave, were heterozygous at the 2.20 marker but did not contain detectable levels of IBMP. However, population structure (relatedness) can greatly affect statistical analysis of association mapping studies by increasing false positive associations (Mackay and Powell, 2007; Aranzana *et al.*, 2005). Analysis of the marker data revealed a high level of relatedness with many individuals displaying identical haplotypes (combination of alleles) at this locus. A total of 40 non-IBMP accumulating varieties all showed the same PM haplotype across this region, while 7 of the IBMP-accumulating varieties all contained the same CS haplotype at this region (Appendix D). While these varieties are likely to vary greatly throughout the entire genome, at this locus of interest they essentially represent replicates of each other. To avoid false positive associations attributed to population structure a total of 60 cultivars with non-unique haplotypes were removed from statistical analysis. Of the 31 individuals displaying unique haplotypes at this region, 5 were positive and 26 negative for IBMP-accumulation. Fisher's exact test was used to calculate the significance of association at each of the six markers at this locus with the trait of IBMP-accumulation (Fig. 3.6). The marker 2.20 Mb located in the *VvOMT3* gene showed the greatest significance of association ($p = 0.005$) while the marker 2.19 Mb located in the *VvOMT4* gene was not significant ($p = 0.093$). Markers either side of this locus showed no association with IBMP-accumulation in the berries, with markers 1.6 Mb, 2.4 Mb and 2.6 Mb having p values of 1.000, 0.583 and 1.000 respectively. The marker at 1.9 Mb was ineffective as the primers were unable to amplify a PCR product in many of the cultivars, likely due to other polymorphisms existing in those cultivars. Nevertheless there is a strong association between this locus and IBMP-accumulation in berries of various

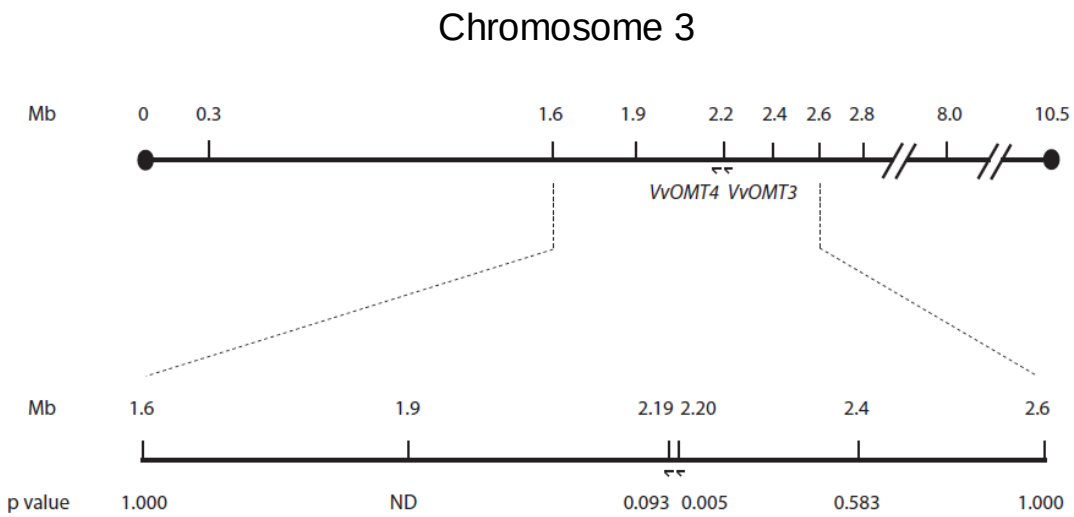


Figure 3.6 - Association mapping using the cultivar collection and adjusting for relatedness confirms the association of this locus with IBMP accumulation. Fisher's exact test demonstrates that the strongest marker-trait association is at marker 2.20 Mb, located within the *VvOMT3* sequence ($p = 0.005$). The adjacent marker at 2.19 Mb located within the *VvOMT4* sequence was only significant at the $p < 0.1$ level ($p = 0.093$). While no significant association was seen between the surrounding markers 1.6 Mb and 2.4 Mb, 2.6 Mb ($p = 1.000$, 0.583 and 1.000 respectively). Marker 1.9 Mb could not be amplified from many cultivars and therefore the marker-trait association was not determined (ND).

cultivars. Furthermore it appears that the association is greatest at the marker located in the *VvOMT3* gene, suggesting it functions in IBMP biosynthesis.

3.4 Discussion

3.4.1 Linkage mapping of the IBMP-accumulation trait in the CS × PM dwarf F2 progeny

Genetic mapping is commonly used in plant species as a tool for identifying genomic loci responsible for a given phenotypic trait. Linkage mapping studies have been widely used in *Arabidopsis*, maize, rice and wheat which are fast growing annual plants in which progenies can be established relatively easily and quickly. Grapevine however is a large woody perennial which takes 2-5 years to produce fruit, which makes the establishment of mapping progenies both time and space consuming. The recently generated PM dwarf lacks many of the characteristics that have previously hindered the establishment of large grapevine progenies. The PM dwarf is small in size and is well suited to growth in glasshouse conditions, in which it often produces fruit within 6-12 months after germinating. Unlike the seasonal wild-type grapevine, the PM dwarf is able to set fruit year round which is a major advantage for studying berry characteristics.

In this study, the PM dwarf was utilised to create a population of F2 dwarf plants that segregate for the trait of IBMP-accumulation. First, a bi-parental cross performed between the PM dwarf and CS resulted in an F1 population of which all 71 plants that produced berries contained detectable levels of IBMP at 7 wpf (Fig. 3.2). Thus, it appears that the trait of IBMP-accumulation in berries is homozygous dominant in CS. The subsequent F2 population did segregate for IBMP-accumulation with 42 individuals positive for the trait and 20 individuals

negative for the trait (Fig. 3.3). Chi square analysis accepts these observed numbers as a 3:1 ratio, thus we can conclude that the trait of IBMP-accumulation in fruit is heterozygous in the F1 progeny and therefore homozygous recessive in PM dwarf parents. CAPS and dCAPS markers designed to distinguish the parental genomes of CS and PM allowed the identification of a locus atop of chromosome 3 that is strongly associated with the trait of IBMP production in berries in the segregating F2 progeny (Fig. 3.4). Furthermore two putative methyltransferase genes, *VvOMT3* and *VvOMT4*, reside within this locus and are potentially responsible for the methylation of IBHP to produce IBMP in berries.

Of the 62 fruit-bearing F2 plants only two individuals did not show the expected association of this locus with the trait of IBMP-accumulation (Fig. 3.4). Both plants, #3 and #42, were homozygous for the PM allele yet accumulated small concentrations of IBMP in pre-véraison fruit. This may be because IBMP-accumulation was treated as a qualitative trait (based on the limit of detection), when in reality it may be better treated as a quantitative trait. It can be seen in the F1 and F2 populations that there is up to 50 and 100-fold variation in the extent of IBMP accumulation in pre-véraison berries (Figs. 3.2 and 3.3). It is likely that this variation is due to the segregation of other genetic factors involved in IBMP biosynthesis or degradation such as transcriptional regulation of *VvOMT3* or *VvOMT4*, enzymes involved in IBHP biosynthesis, unknown factors responsible for IBMP degradation or changes in berry development that may alter the timing of the peak of IBMP accumulation. Although *VvOMT3* or *VvOMT4* may represent the major genetic factor involved in IBMP biosynthesis (i.e. the major QTL), despite the non-functional PM allele at this locus in plants #3 and #42, it is possible that other methyltransferases, possibly VvOMT1, are capable of synthesizing small amounts of IBMP which reach levels above the limit of

detection when the right combination of other genetic factors that favor IBMP accumulation are present.

Grapevine has a small diploid genome of 475-500 Mb located on 19 chromosomes (This *et al.*, 2006). In this study, marker-trait associations on chromosome 3 were detected early in the process of marker design and priority was then given to identifying markers surrounding this region of interest. The design of further markers on other chromosomes was made a low priority, as the random segregation of chromosomes means other associations of genomic loci with IBMP-accumulation are extremely unlikely. However, it would be prudent for future work to eliminate any other linkage groups by designing and testing markers across all 19 chromosomes for their association with this trait. *VvOMT1* and *VvOMT2* are located on chromosome 12. It was also found that PM and CS share an allele of each of these genes, and so while markers where not designed to this chromosome it is safe to presume that no association exists between these genes and IBMP-accumulation given the homozygousity of this trait in PM and CS. The *VvGAI1* gene conferring the dwarfed phenotype is located on chromosome 1. Therefore the selection of only dwarfed F2 progeny should have no skewing effect on the number of plants positive for the trait of IBMP accumulation.

In linkage mapping studies it is also desirable to have more than one recombination event to establish the borders of a locus associated with a trait. However, due to the small population size in this study (limited by glasshouse availability) only single recombination events between markers 0.31 Mb and 1.6 Mb and between 2.4 Mb and 2.6 Mb allowed the borders for this locus to be determined (Fig. 3.5). However, the results of the association mapping validate the findings of the linkage study and demonstrate the advantages of combining

both mapping approaches. There were six F2 plants positive for IBMP that showed recombination from a heterozygous state to homozygous CS between various markers within this locus. Unfortunately, these recombination events in one allele have no mapping power while next to the dominant CS allele. However, if these plants are taken to the F3 stage, segregation of these alleles should result in a quarter of the progeny being homozygous for the recombined allele. Thus, the progeny should contain recombinants between the markers at this locus that segregate for the IBMP-accumulating phenotype, allowing the resolution of the mapping to be increased. Another way to increase mapping resolution and recombination replicates is to germinate a large number of F2 seedlings from all F1 plants and screen the germinating seedlings for recombination events at this locus using the genetic markers. Marker-assisted screening of F2 plants at the seedling stage solves issues of space availability as only individuals displaying useful recombination events are grown to maturity.

Amongst the plants that accumulated IBMP in berries, the amount measured in the samples varied by approximately 100-fold (Figs. 3.2 and 3.3). This suggests that other genetic or environmental factors play a role in determining the levels of IBMP that the berries have accumulated. These unknown factors could include any genetic elements involved in the transcriptional regulation of the *VvOMT* genes responsible for the methylation of the substrate IBHP and genes involved in IBHP biosynthesis or the degradation or further metabolism of IBMP (discussed further in Chapter 5). The berry samples for this study were not harvested at the same date, but were taken at the first possible opportunity as the plants began to flower. So, although the plants were maintained in the same glasshouse, which was under a constant temperature and lighting regime, it is possible that seasonal differences, such as light intensity, still

had an effect on IBMP levels in the berries. Nevertheless, the F1 and F2 populations would be suitable for a second mapping study treating IBMP-accumulation as a quantitative trait rather than a qualitative trait. Such a study would help identify quantitative trait loci (QTLs) responsible for the variations seen in IBMP levels, and eventually provide a greater understanding of the pathways that influence both IBMP biosynthesis and degradation.

3.4.2 Association mapping of the IBMP-accumulation trait in the cultivar collection

Association mapping has traditionally been used in human studies where the establishment of large progenies or families is not possible. The rapid decay in LD seen in these distantly removed human populations means a large number of genetic markers are required to identify associations between phenotypic traits and the underlying functional genetic variation. In plant species, limited access to the high number of genetic markers that are required has meant that association mapping studies have been limited. However, the increasing availability of quality genomic data in recent times has seen growing interest in association mapping as it bypasses the need to establish large progenies. This makes it a highly attractive approach to assigning functions to genes, especially when appropriate populations are difficult to establish and/or score for the trait of interest. In grapevines two recent studies have demonstrated the enhanced mapping power achieved by using directed association mapping to investigate a locus of interest (Emanuelli *et al.*, 2010; Fournier-Level *et al.*, 2009).

In this study, the identification of the locus responsible for IBMP-accumulation in the F2 progeny allowed a directed approach to association mapping to be performed in a collection of existing cultivars. Previous studies in

plants have shown that a high level of false positive associations can result from close relatedness within a mapping population (Aranzana *et al.*, 2005; Mackay and Powell, 2007). Using the markers designed to the CS and PM genomes, the haplotypes of the different cultivars were investigated at the locus between markers 0.3 Mb and 2.6 Mb at the top of chromosome 3. Indeed a high level of relatedness was seen between many of the cultivars, which is not surprising given that they are all common domesticated varieties. The six major IBMP-accumulating varieties Cabernet Franc, Sauvignon blanc, Merlot, Semillon, Ruby Cabernet and Roussanne (Table 3.2) all possessed either the CS haplotype or a heterozygous haplotype in this region. This finding is not unexpected as it is well established that these French cultivars are all closely related (Myles *et al.*, 2011; Boursiquot *et al.*, 2009; Bowers and Meredith, 1997). Similarly 40 cultivars, all of which lacked IBMP, had a haplotype identical to PM in this region of the genome. Over 250 grapevine cultivars are thought to be the direct offspring of Pinot noir (This *et al.*, 2006), which likely explains the high occurrence of the PM haplotype seen at this locus.

All of the 60 cultivars displaying non-unique haplotypes showed the expected phenotype of IBMP-accumulation in berries given their allelic origin at marker 2.20 Mb. Therefore, the removal of these cultivars from the statistical analysis did not positively enhance the associations seen at this locus. However, the removal of these cultivars did allow non-significant associations flanking this marker to be revealed, which would otherwise have been masked if the highly related cultivars were included in the analysis. After relatedness was accounted for, a strong association (p = 0.005) was seen between IBMP-accumulation and the 2.20 Mb marker located in the *VvOMT3* gene (Fig. 3.6). The marker 2.19 Mb, located within the *VvOMT4* coding region, did not show a significant association

with IBMP-accumulation in the berries at the 0.05 level, with a *p* value of 0.093, while markers either side of this region also showed no association with the trait (Fig. 3.6). Despite the significant level of associations seen between the 2.20 Mb marker and IBMP-accumulation in the berries, not all of the cultivars showed the expected phenotype given their genetic origin. The cultivar Tinta amarella contained IBMP yet was homozygous for the PM marker at 2.20 Mb. Conversely five cultivars, Terret noir, Souzao, Raboso Piave, Crouchen and Nebbiolo, were all heterozygous at the 2.20 Mb marker, yet did not contain any detectable levels of IBMP. The reason why these varieties do not show the expected association is possibly because the genetic markers used are only based on the CS and PM genomes and are not indicative of the total variation existing in the cultivar collection. While this marker allows us to differentiate between the CS and PM alleles of the *VvOMT3* gene, it may not represent the exact genetic basis for the phenotype. Hence the functional genetic variation ultimately responsible for IBMP-accumulation remains unknown but is tightly linked to the marker 2.20 Mb. It is also unclear at what stage in the evolution of the cultivars the polymorphisms occurred. If the functional mutation responsible for the IBMP-accumulation trait arose in the PM ancestor allele before the SNP that is utilized at marker 2.20 Mb then varieties that diverged from PM ancestor between these two events would also contain the functional mutation but display the CS allele at this marker. This scenario could explain why the five varieties, Terret noir, Nebbiolo, Crouchen, Souzao and Raboso Piave, are heterozygous at marker 2.20 Mb yet do not accumulate IBMP. Complete sequencing of *VvOMT3* and surrounding regions within these cultivars should ultimately result in the identification of the specific mutation responsible for IBMP-accumulation in berries.

It is also unclear whether the wild-type phenotype and allele is represented by CS or PM. Recessive alleles are usually indicative of a loss of function mutation, which hints that PM allele may have arisen from CS. However, gain of function mutations can also occur, albeit less frequently, and given that the majority of the cultivars investigated are homozygous for the PM allele it also seems plausible that the CS allele mutated from PM. It must also be remembered that the cultivars examined in this study are the result of human selection, therefore it would be interesting to examine wild grapevine species to investigate the ancient origins of the phenotype and its genetic basis.

The work carried out in this chapter has efficiently demonstrated the major advantages of the PM dwarf as a tool for the rapid generation of an F2 mapping population. Genetic mapping in this segregation population allowed the identification of a locus responsible for the majority of the IBMP-accumulation trait in grape berries. This study also validates the applicability of directed association mapping in a collection of grapevine cultivars, which resulted in the detection of strong associations between *VvOMT3* and IBMP-accumulation. These results also demonstrate the advantages of performing both linkage and association mapping to complement each other. In the next chapter the potential role of *VvOMT3* and *VvOMT4* in the biosynthesis of IBMP are further investigated by characterizing the activity of the enzymes encoded by these genes and by studying gene expression patterns in a range of samples differing in IBMP concentrations.

Chapter 4 - Characterisation of two novel *O*-methyltransferases found in the locus linked to methoxypyrazine accumulation in grape berries

4.1 Introduction

The results presented in Chapter 2, together with previous work by Hashizume *et al.* (2001b), had suggested that *VvOMT1* is responsible for the methyltransferase activity involved in IBMP biosynthesis. However, in the previous chapter it was shown that within an F2 population generated from a cross between CS and the PM dwarf, the trait of berry IBMP-accumulation segregates with a 2.3 Mb region atop chromosome 3, in which two putative *O*-methyltransferase genes, *VvOMT3* and *VvOMT4* reside. It was also shown that within 91 different grapevine cultivars there is strong association between IBMP-accumulation and this locus, with the greatest association seen with a marker located within *VvOMT3*. In light of these mapping results it appears that either *VvOMT3* or *VvOMT4* is likely to be the major gene responsible for IBMP accumulation in grape berries.

This chapter describes the characterisation of *VvOMT3* and *VvOMT4* to gain a better understanding of their potential roles in IBMP biosynthesis in grapevines. The methods for characterising *VvOMT3* and *VvOMT4* were essentially the same as those used for *VvOMT1* and *VvOMT2* in Chapter 2, namely functional analysis of recombinant proteins and analysis of gene expression patterns in various grapevine tissues. The genetic basis of the difference in IBMP accumulation in Pinot varieties compared to Cabernet Sauvignon was also investigated. A new method for the quantification of HPs was developed to enable the determination

of HP levels in various grape and vine samples to identify the important variables that influence MP biosynthesis.

4.2 Materials and Methods

4.2.1 Chemicals

Deuterated (D_2)-IBHP was chemically synthesized for use as an internal standard for HP quantification. The method of D_2-IBHP synthesis was the same for IBHP as described in section 2.2.1, but D_2-glyoxal was used in the reaction instead of the non-deuterated substrate. For the synthesis of D_2-glyoxal a 2.5 mmol solution of lithium aluminum deuteride (Sigma) in 40 ml ether was cooled to 0°C. A 10 mmol solution of diethyl oxalate (Sigma) in 40 ml ether was added over a period of 30 min and mixed at 0°C for a further 30 min. The reaction was allowed to warm to room temperature and then 100 ml of water was added. The aqueous layer was then separated and twice washed with 40 ml of ether, to yield a solution of D_2-glyoxal. The identity and purity of synthesized D_2-glyoxal and subsequent D_2-IBHP was confirmed by ^{1}H and ^{13}C nuclear magnetic resonance.

4.2.2 Plant material

CS and PN berry samples were harvested during the 2007/08 season from the Harvey family's Slate Creek vineyard in Willunga, South Australia (latitude 35° 15' S, longitude 138° 33' E). Samples were taken in triplicate from three areas of the vineyard at 2, 4, 5, 6, 7, 8, 9, 10, 12 and 14 wpf (flowering defined as 50% cap fall). Samples for RNA extraction were deseeded to eliminate the high level of *VvOMT1* expression present in berry seeds from the gene expression analyses. Leaves, tendrils, and flowers were all sampled at the time of flowering while the

rachis (bunch stem) sample was taken at 7 wpf. Root material was obtained from grapevine plantlets grown in tissue culture.

4.2.3 RNA extraction and Real-time PCR analysis

Isolation of total mRNA from grape samples and subsequent cDNA synthesis were performed as described in section 2.2.3. The procedure and conditions of Real-time PCR analysis of mRNA levels were the same as described in section 2.2.10. Gene specific primers for the expression analysis of *VvOMT3* and *VvOMT4* are listed in Appendix A.

4.2.4 Isolation of full length VvOMT3 and VvOMT4 cDNAs

Full length coding sequences of *VvOMT3* and *VvOMT4* were amplified from cDNA derived from 4 wpf CS berries using the primers MT3F1/MT3R1 and MT4F1/MT4R1 listed in Appendix A. The coding sequence of *VvOMT4* was also amplified from PN 4 wpf berry cDNA, while PN root cDNA was used to amplify the *VvOMT3* coding sequence as no product could be amplified from PN 4 wpf berry cDNA. All PCR products were cloned into the vector pDRIVE (Qiagen) and the constructs sequenced with the M13 forward and M13 reverse primers (Appendix A) using the BigDye Terminator® v3.1 Cycle Sequencing Kit (Applied Biosystems, Victoria, Australia). Amino acid sequence alignments and phylogenetic analysis were conducted as described in section 2.2.5

4.2.5 Recombinant protein production and enzyme assays

Recombinant VvOMT3 and VvOMT4 proteins were produced using the expression vector pET30a as described in section 2.2.6. Gene specific primers used to clone *VvOMT3* and *VvOMT4* coding sequences into the protein expression

vector pET30a are listed in Appendix A. SDS-PAGE and western blot analyses of recombinant proteins was conducted in the same way as described in section 2.2.7. Subsequent enzyme assays to determine kinetic values of recombinant VvOMT3 and VvOMT4 against HP substrates were performed as described in section 2.2.8.

4.2.6 SPME-GC-MS quantification of MPs

SPME-GC-MS quantification of IBMP in grape samples and enzyme assays was performed the same as detailed in section 2.2.9.

4.2.7 LC-MS quantification of HP substrates

IBHP and IPHP substrates were quantified using a novel LC-MS stable isotope dilution assay (SIDA) modified from the procedures of both Ryona *et al.* (2010) and Hashizume *et al.* (2001). To 1 g of ground frozen grape tissue, 15 ml of boiling water was added and the sample immediately spiked with 25 ng of D_2-IBHP internal standard. The mixtures were boiled for 10 min and then cooled on ice for 20 min before centrifugation at 12,000 × *g* for 10 min. The supernatant was collected and the pellet resuspended in 15 ml water and again centrifuged at 12,000 × *g* for 10 min. The supernatants were pooled and adjusted to pH 2.5 with 6M HCl. Samples were then extracted 5 times with 25 ml of dichloromethane. Pooled dichloromethane extracts were dried over $NaSO_4$, concentrated to 1 ml and transferred to high recovery glass vials. Samples were then left to dry overnight in a fume hood. The dried extract was dissolved in 50 µl of 25% (v/v) methanol and sonicated for 3 min.

Separation and detection of HP compounds was carried out using a 1200 series HPLC coupled to a 6410 triple quad mass spectrometer (Agilent, Santa

Clara, CA, USA). The injected sample (30 µl) was separated on a 2.1 x 30 mm x 3.5 µm Zorbax SB-C18 Rapid Resolution column (Agilent) held at 20°C, using an isocratic gradient of 25% methanol and 0.1% acetic acid (v/v) at a flow rate of 0.2 $ml.min^{-1}$ for 8 min. The effluent was introduced into the ESI ion source with a dissolvation gas temperature of 350°C, the nebulizer pressure at 50 psi, a gas flow rate of 8.0 $ml.min^{-1}$ and a capillary voltage of 1.5 kV. The detection was performed by multiple reaction monitoring (MRM) in positive ion mode with 400 EMV. The optimization of fragmentation was done with chemically synthesised IBHP and IPHP and D2-IBHP standards using the Agilent MassHunter Optimizer software. The collision energy ranged between 12 and 52 eV. The following ions were the major fragments used for quantification, D_2-IBHP *m/z* 155.1 – 113, IBHP *m/z* 153.1 -111, IPHP *m/z* 139.1- 69.1.

4.3 Results

4.3.1 CS and PN as tools to investigate IBMP biosynthesis

Previous findings have suggested that Pinot varieties do not accumulate MPs in the berries (Hashizume *et al.*, 2001a). To confirm this observation PN and CS berries were sampled throughout berry development from adjacent vineyard plots. Analysis of MPs by GC-MS revealed the expected pre-véraison accumulation patterns in CS with IBMP levels increasing from 26 $pg.g^{-1}$ at 2 wpf to a peak of 186 $pg.g^{-1}$ at 7 wpf followed by a steady decrease to 31 $pg.g^{-1}$ at 14 wpf (Fig. 4.1A). IPMP was only detectable at low levels (13-40 $pg.g^{-1}$) between 4-8 wpf in CS (Fig. 4.1B). Conversely, PN berries did not contain detectable levels of either IBMP or IPMP at any point throughout berry development (Fig. 4.1), consistent with results seen in the PM dwarf (Chapter 3). Thus, comparisons between CS and PN are useful to help understand the mechanisms of MP

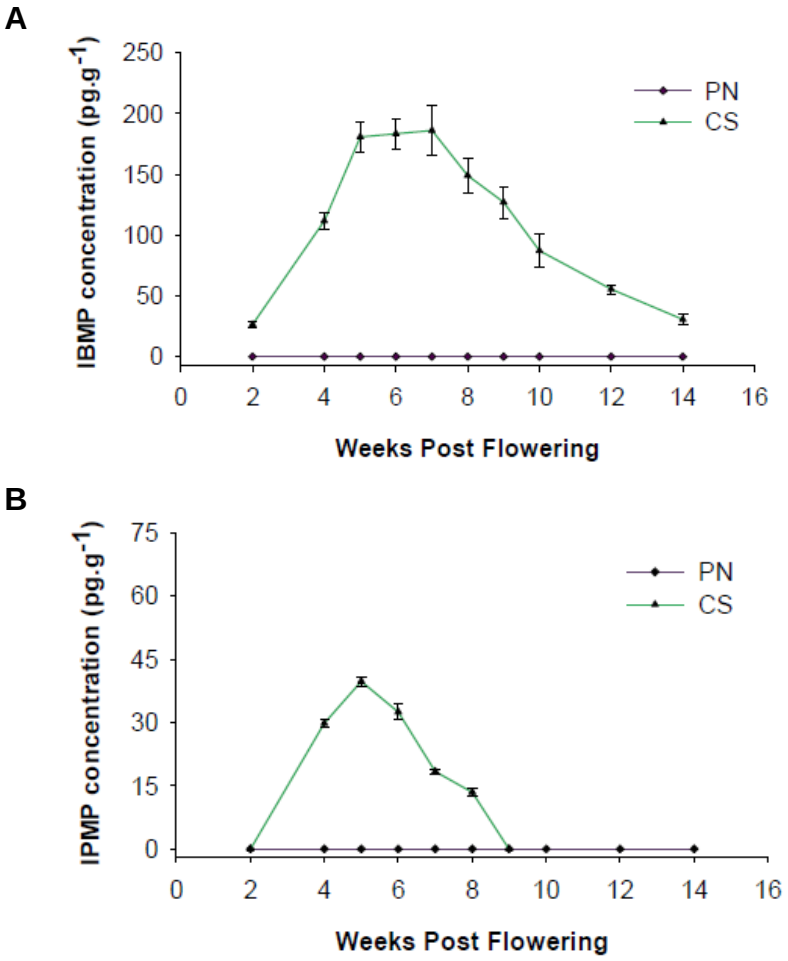


Figure 4.1 - Comparison of IBMP (**A**) and IPMP (**B**) concentrations during the development of CS and PN berries. nd indicates that the compound was below the limit of detection.

biosynthesis in grapes and these comparisons are described in the following sections of this chapter.

4.3.2 Sequence and phylogenetic analysis of VvOMT3 and VvOMT4 encoded proteins

Using the online annotated grapevine genome sequence (Jaillon *et al.*, 2007), a manual search of the 2.3 Mb genomic locus that associates with IBMP-accumulation identified two *O*-methyltransferases, *VvOMT3* and *VvOMT4,* in this region. The annotated genomic sequence has the two genes placed approximately 2 kb apart, both in the negative orientation (Fig. 4.2). Cloning and sequencing of CS gene sequences from both DNA and cDNA revealed that *VvOMT3* contains an intron of 219 bp and *VvOMT4* contains a larger intron of 777 bp, confirming the online genomic sequence. The predicted proteins encoded by *VvOMT3* and *VvOMT4* are 347 and 359 amino acids in length respectively and show 75% sequence identity with each other.

Sequence alignment of VvOMT3 and VvOMT4 with other plant OMTs (Fig. 4.3) showed that the proteins predicted to be encoded by *VvOMT3* and *VvOMT4* contain the five conserved domains characteristic of plant OMTs (Ibrahim *et al.*, 1998). Phylogenetic analysis of the encoded protein products of *VvOMT3* and *VvOMT4* with other plant OMTs suggests that both proteins are class II OMTs. Further phylogenetic analysis with previously characterised members of the class II OMT family (Fig 4.4) reveals that the two proteins show closest homology with VvOMT1 and VvOMT2. Sequence alignment revealed that VvOMT3 has 39% and 40% amino acid identity with VvOMT1 and VvOMT2 respectively, while VvOMT4 has slightly higher identity with these OMTs, being 44% and 45% respectively.

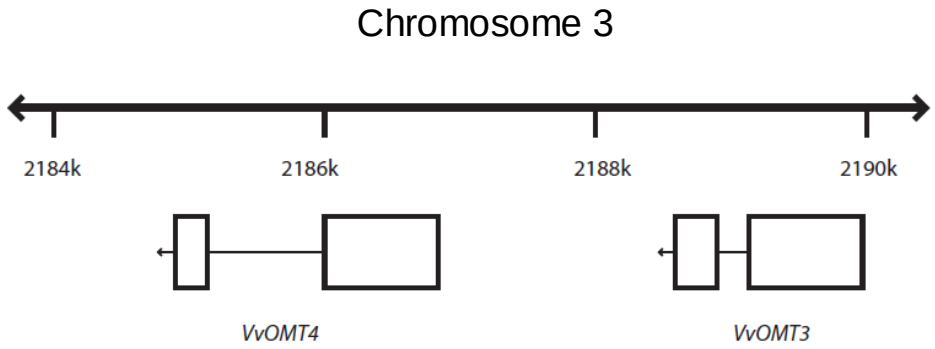


Figure 4.2 Schematic diagram showing the location of the *VvOMT3* and *VvOMT4* genes less than 2 kb apart on chromosome 3. Boxes represent coding sequences and arrows represent putative non-coding sequences of the genes.

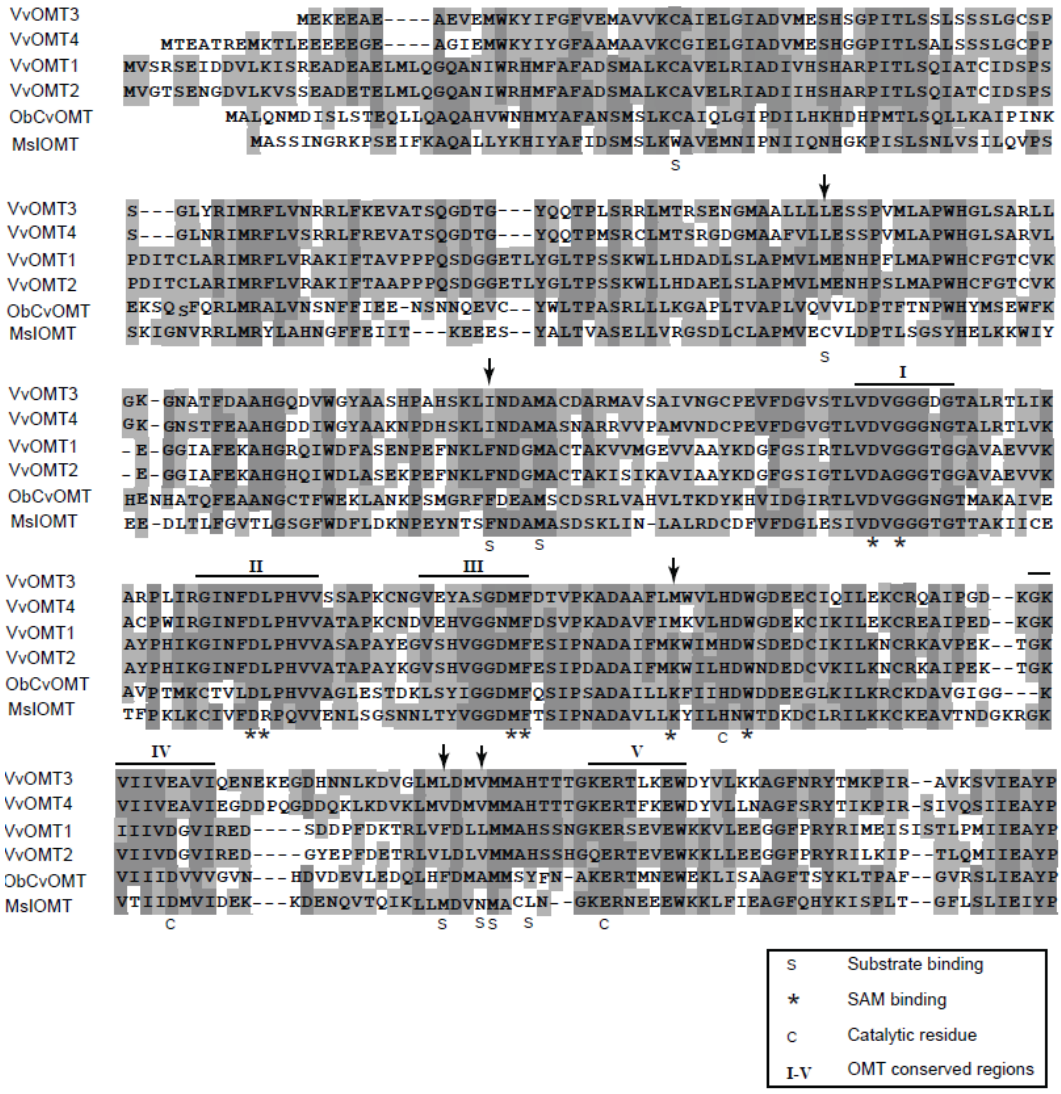


Figure 4.3 - Alignment of the predicted amino acid sequence of the proteins encoded by *VvOMT3* and *VvOMT4* with VvOMT1 and VvOMT2 and two plant OMTs previously analysed for important residues relating to OMT function. Regions I-V are domains conserved among plant OMTs (Gang *et al.*, 2002; Zubieta *et al.*, 2001). C, signifies catalytic residues; *, SAM binding residues; and S, substrate binding residues of MsIOMT as determined by Zubieta *et al.* (2001). ↓ highlights amino acid differences between the VvOMT sequences at putative substrate binding residues. Black and grey shading indicate identical and similar amino acids respectively. ObCVOMT is an *Ocimum basilicum* chavicol OMT (AF435007) and MsIOMT is a *Medicago sativa* isoflavanoid OMT (AF023481).

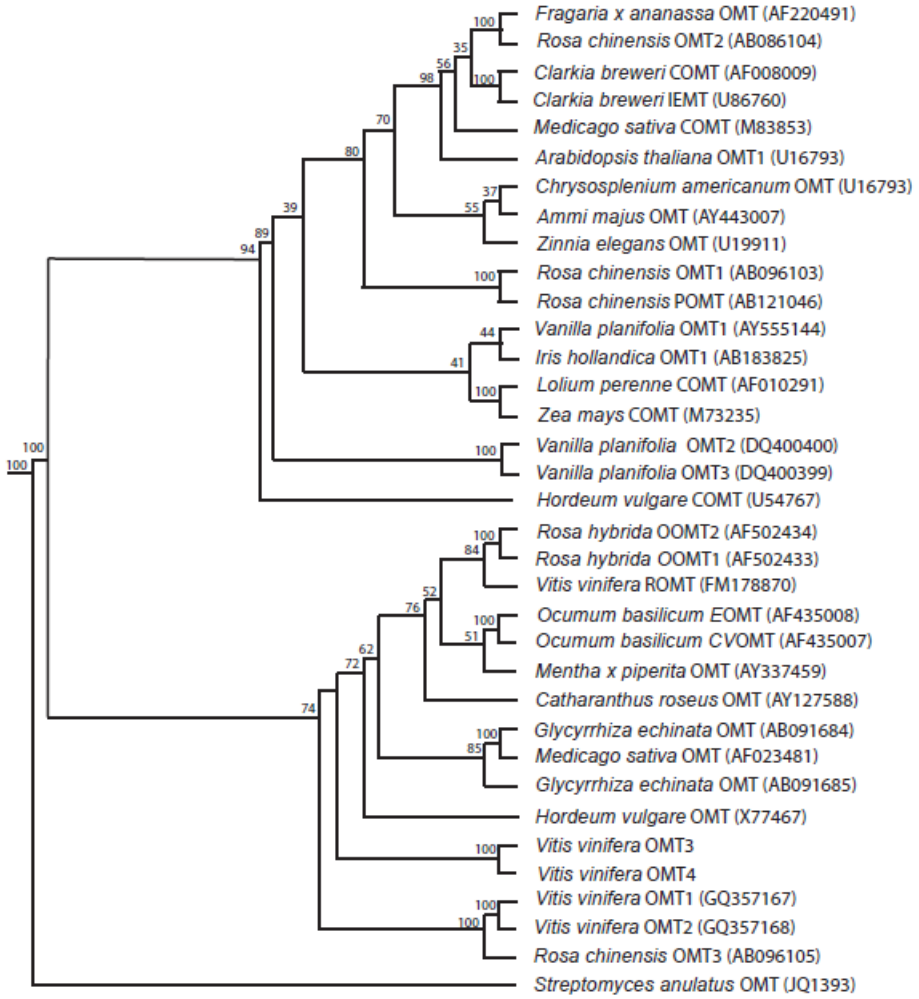


Figure 4.4 - Phylogenetic tree of VvOMT3 and VvOMT4 and other previously characterised plant class II OMTs. VvOMT3 and VvOMT4 are most closely related to VvOMT1 and VvOMT2, which are described in Chapter 2. Numbers at branch points are bootstrap values representing the confidence level as a percentage based on 1,000 repeats. The *Streptomyces anulatus* OMT was used as an outgroup.

4.3.3 Kinetic analysis of recombinant VvOMT3 and VvOMT4

To investigate whether the protein products encoded by *VvOMT3* and *VvOMT4* have methylating activity against HP substrates, recombinant His-tagged fusion proteins were produced in *E. coli* (Fig. 4.5). Functional activity tests revealed that the purified recombinant proteins of *VvOMT3* and *VvOMT4* each possess activity against both IBHP and IPHP. Analysis of the pH optima for the enzymes showed that both VvOMT3 and VvOMT4 have greatest activity at pH 8.0 against both IPHP and IBHP (Fig. 4.6).

To determine kinetic parameters of the recombinant enzymes against HPs, assays were performed at various substrate concentrations and Michaelis-Menten curves produced (Fig 4.7). The resulting kinetic data reveal that VvOMT3 has a strong affinity for both HP substrates with K_m values of 380 nM and 276 nM against IBHP and IPHP respectively. These K_m values are approximately 500 fold lower than the K_m values calculated for VvOMT1, suggesting that VvOMT3 has a much stronger binding affinity for HP substrates. Calculations reveal that VvOMT3 has almost identical k_{cat} values (turnover rate) against IBHP and IPHP that are also similar in range to VvOMT1, which suggests the two enzymes have a similar reaction rate when completely saturated with HP substrate. However, the specificity constant (k_{cat}/K_m) is a better indicator of catalytic efficiency at biologically relevant substrate concentrations. This measure suggests that VvOMT3 has a slightly higher catalytic efficiency against IPHP (94.5 $\mu M^{-1}.S^{-1}$) than IBHP (66.9 $\mu M^{-1}.S^{-1}$), but these values are approximately 600 and 1,400 fold greater than those calculated for VvOMT1, which has lower catalytic efficiencies of 0.109 $\mu M^{-1}.S^{-1}$ and 0.066 $\mu M^{-1}.S^{-1}$ against IPHP and IBHP respectively.

Unlike VvOMT3, VvOMT4 had weak affinity towards HPs with K_m values of 27 µM and 210 µM against IPHP and IBHP respectively (Fig. 4.8).

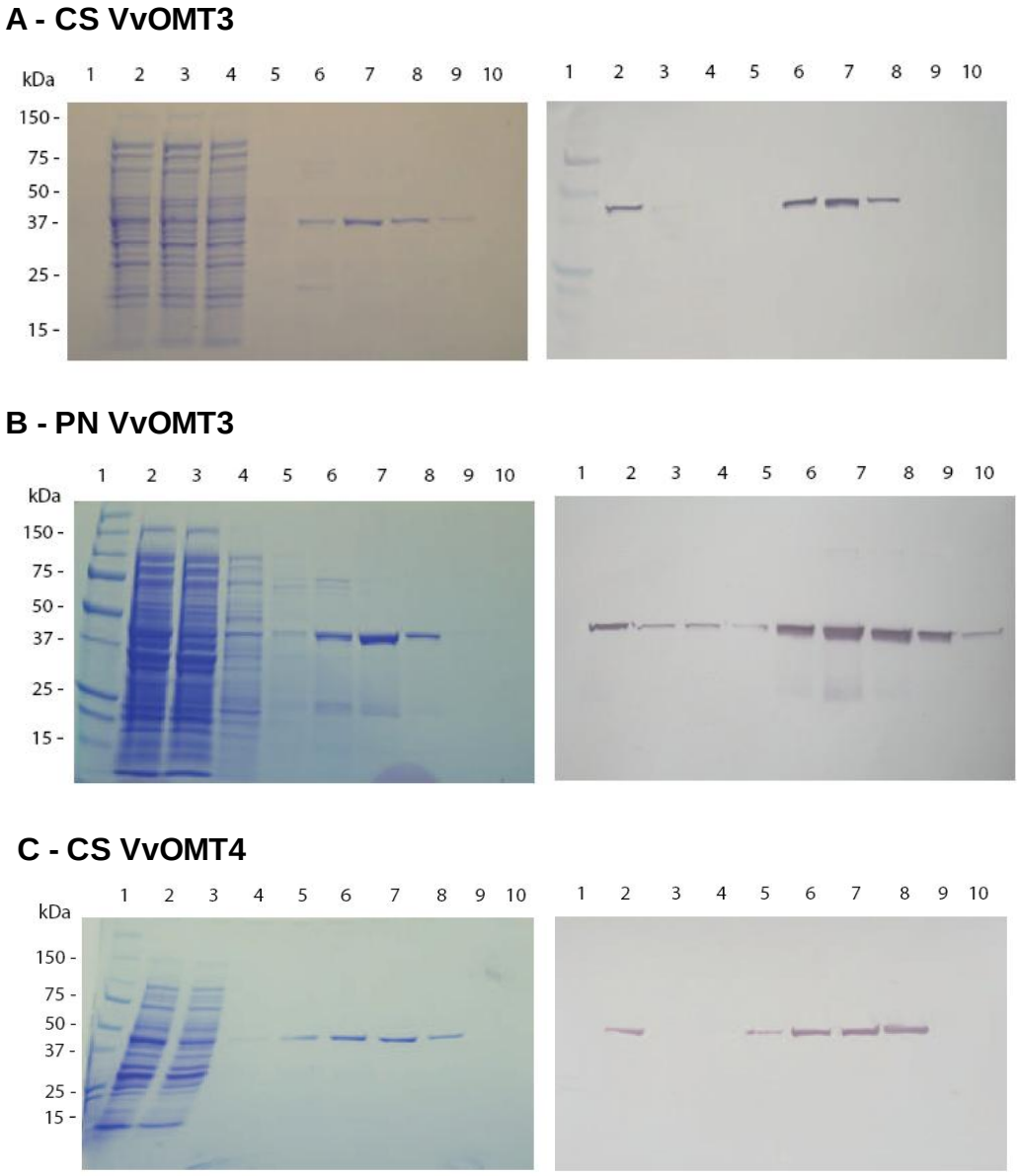


Figure 4.5 - SDS-Page gel (left) and corresponding western blot (right) of samples collected during the HIS-trap column purification of recombinant CS encoded VvOMT3 (**A**), PN encoded VvOMT3 (**B**) and VvOMT4 from CS (**C**). Lane (1) Protein size marker; (2) crude protein extract before purification; (3) flow through after addition of crude extract to HIS column; eluates after the addition of elution buffer containing: (4) 20mM imidazole; (5) 50mM imidazole; (6) 100mM imidazole; (7) 200mM imidazole; (8) 300mM imidazole; (9) 400mM imidazole and (10) 500mM imidazole.

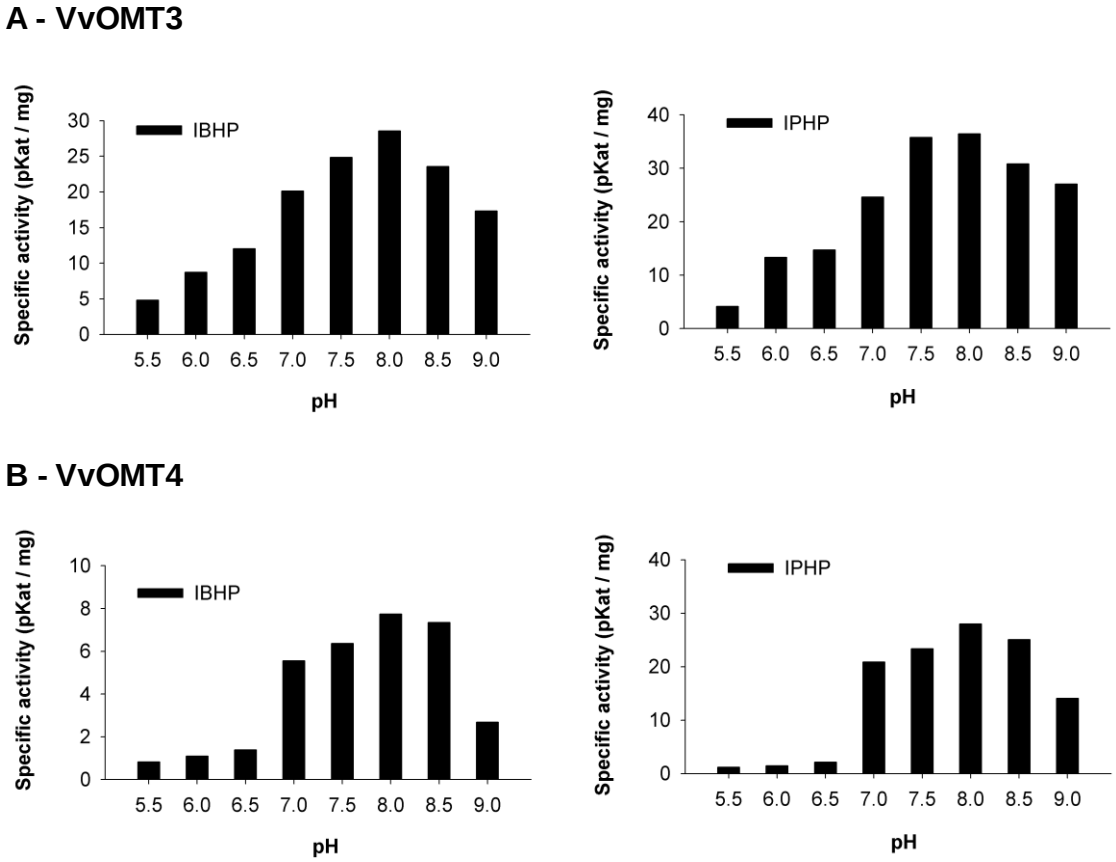
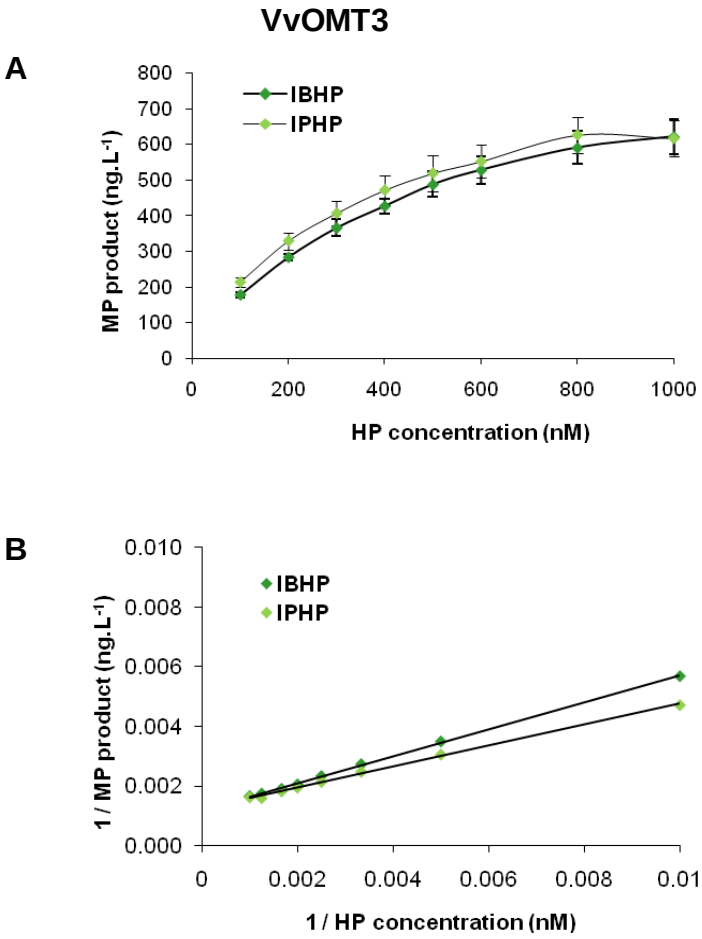
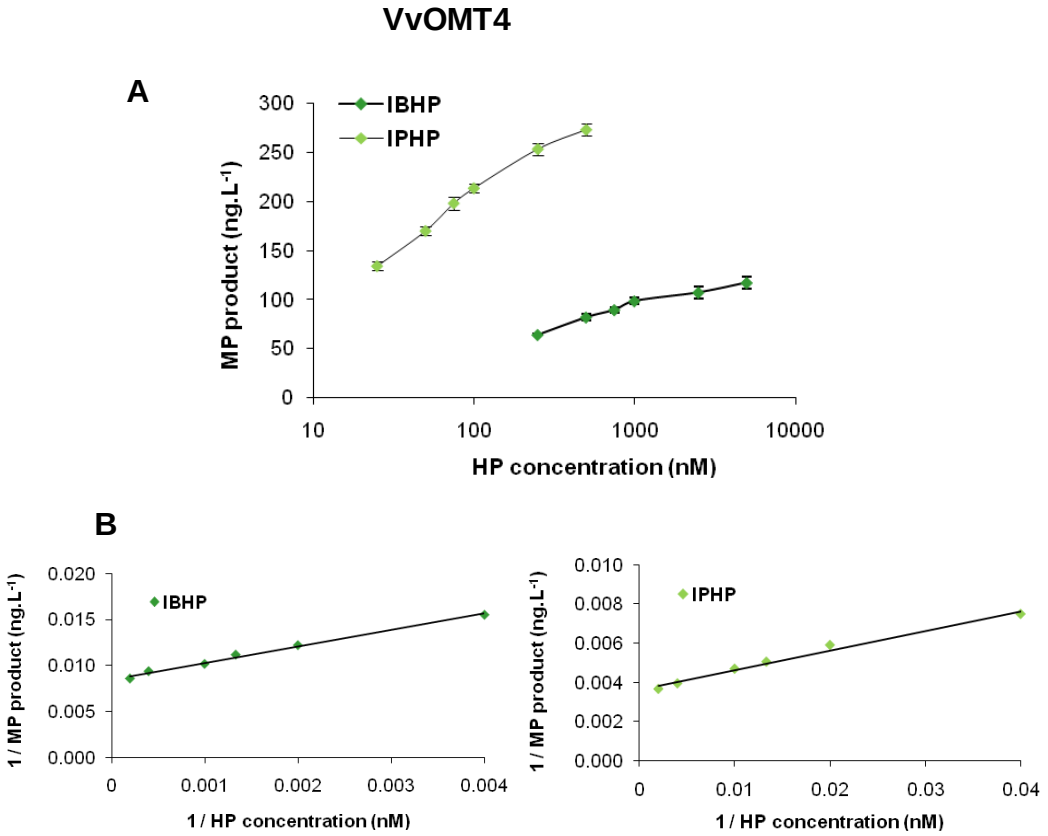


Figure 4.6 - Determination of pH optima for VvOMT3 (**A**) and VvOMT4 (**B**) activity against IBHP (left) and IPHP (right). The buffer MES was used for pH ranges 5.5-6.5, HEPES used for 7.0-7.5 and Tris-HCl used for 8.0-9.0.



Substrate	r^2	Intercept	Slope	K_m (nM)	k_{cat} x 10^{-6} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
IBHP	0.9994	0.0119	4.526	380 ± 0.03	25.4 ± 2.5	66.9 ± 6.6
IPHP	0.9977	0.0127	3.497	276 ± 0.02	26.1 ± 2.9	94.5 ± 10.6

Figure 4.7 - Calculation of the kinetic values of VvOMT3 against HP substrates. (**A**) Michaelis-Menton curve displaying changes in MP formation with various HP substrate concentrations and the same data presented as a Lineweaver-Burk plot (**B**) used to calculate kinetic values. V_{max} is equal to 1 / intercept and indicates the maximum velocity of the enzymatic reaction. K_m is equal to V_{max} / slope and is the concentration of substrate at which reaction velocity is half its maximum. k_{cat} equals V_{max} / moles of enzyme per assay × 40 min × 60 sec and calculates to the number of substrate molecules converted to product per enzyme site per second. k_{cat} / K_m is also called the specificity or rate constant and gives a measure of total catalytic efficiency.



Substrate	r^2	Intercept	Slope	K_m (nM)	k_{cat} x 10^{-6} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
IBHP	0.9924	0.0851	17.89	210,000 ± 28,000	6.0 ± 0.4	0.029 ± 0.002
IPHP	0.9857	0.0363	1.001	27,000 ± 1,400	15.3 ± 0.2	0.554 ± 0.01

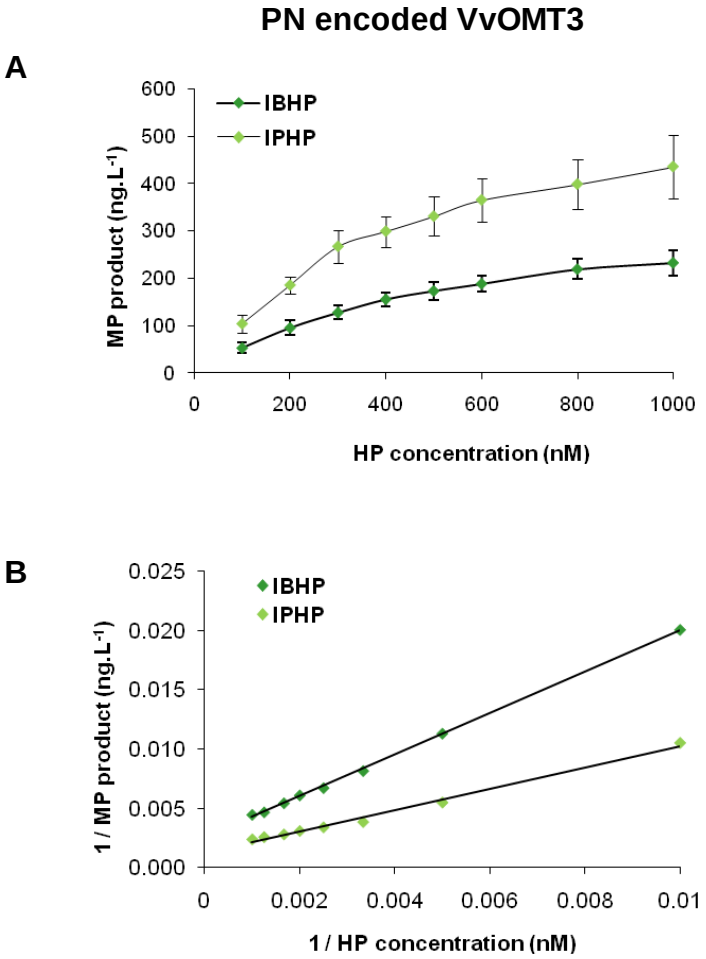
Figure 4.8 - Calculation of the kinetic values of VvOMT4 against HP substrates. (**A**) Michaelis-Menton curve displaying changes in MP formation with various HP substrate concentrations and the same data presented as a Lineweaver-Burk plot (**B**) used to calculate kinetic values. V_{max} is equal to 1 / intercept and indicates the maximum velocity of the enzymatic reaction. K_m is equal to V_{max} / slope and is the concentration of substrate at which reaction velocity is half its maximum. k_{cat} equals V_{max} / moles of enzyme per assay × 40 min × 60 sec and calculates to the number of substrate molecules converted to product per enzyme site per second. k_{cat} / K_m is also called the specificity or rate constant and gives a measure of total catalytic efficiency.

VvOMT4 also had lower k_{cat} values resulting in low catalytic efficiency values of 0.029 $\mu M^{-1}.S^{-1}$ and 0.554 $\mu M^{-1}.S^{-1}$ against IBHP and IPHP respectively, comparable to those of VvOMT1 and VvOMT2.

4.3.4 Kinetic analysis of PN encoded VvOMT3

To determine if Pinot varieties do not accumulate IBMP in the berries because of a mutation in the sequence of *VvOMT3*, the complimentary sequences were amplified from both PN DNA and cDNA. An alignment of the PN *VvOMT3* sequences with those amplified from CS revealed 13 SNPs and a three base deletion in the PN allele of *VvOMT3*. These nucleotide polymorphisms result in seven predicted amino acid changes and one amino acid deletion between the encoded proteins.

Recombinant protein of the PN encoded VvOMT3 was produced and kinetic analyses performed to determine the effect of these amino acid changes on the HP methylating activity of this enzyme (Fig. 4.9). Interestingly only minor differences in kinetic values were seen between the recombinant VvOMT3 protein encoded by the PN and CS alleles (Table 4.1). PN VvOMT3 showed K_m values of 625 nM and 473 nM against IBHP and IPHP respectively. PN VvOMT3 showed double the turnover rate (k_{cat}) against IPHP (34.0 $\times 10^{-6}$ s^{-1}) compared to IBHP (17.9 $\times 10^{-6}$ s^{-1}), whereas the turnover rates for the CS VvOMT3 with the two HP substrates are almost identical (25.4 and 26.1 $\times 10^{-6}$ s^{-1}, respectively). Together these results imply that PN VvOMT3 has a slightly weaker catalytic efficiency against IPHP (72.0 $\mu M^{-1}.S^{-1}$) and IBHP (28.7 $\mu M^{-1}.S^{-1}$) compared to the CS encoded VvOMT3 with values of 94.5 $\mu M^{-1}.S^{-1}$ and 66.9 $\mu M^{-1}.S^{-1}$ against IPHP and IBHP respectively. Yet this small reduction in the activity of the PN enzyme



Substrate	r^2	Intercept	Slope	K_m (nM)	k_{cat} x 10^{-6} (s^{-1})	k_{cat}/K_m ($M^{-1}.s^{-1}$)
IBHP	0.9994	0.0250	17.54	625 ± 0.07	17.9 ± 0.7	28.7 ± 1.2
IPHP	0.9921	0.0851	8.996	473 ± 0.08	34.0 ± 4.4	72.0 ± 9.3

Figure 4.9 - Calculation of the kinetic values of PN encoded VvOMT3 against HP substrates. (**A**) Michaelis-Menton curve displaying changes in MP formation with various HP substrate concentrations and the same data presented as a Lineweaver-Burk plot (**B**) used to calculate kinetic values. V_{max} is equal to 1 / intercept and indicates the maximum velocity of the enzymatic reaction. K_m is equal to V_{max} / slope and is the concentration of substrate at which reaction velocity is half its maximum. k_{cat} equals V_{max} / moles of enzyme per assay × 40 min × 60 sec and calculates to the number of substrate molecules converted to product per enzyme site per second. k_{cat} / K_m is also called the specificity or rate constant and gives a measure of total catalytic efficiency.

Table 4.1 - Comparison of kinetic parameters determined for the recombinant VvOMTs produced in this study against HP substrates.

Enzyme	Substrate	Apparent K_m (nM)	k_{cat} x 10^{-6} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}.s^{-1}$)
VvOMT3	IBHP	380 ± 0.03	25.4 ± 2.5	66.9 ± 6.6
	IPHP	276 ± 0.02	26.1 ± 2.9	94.5 ± 10.6
PN VvOMT3	IBHP	625 ± 0.07	17.9 ± 0.7	28.7 ± 1.2
	IPHP	473 ± 0.08	34.0 ± 4.4	72.0 ± 9.3
VvOMT4	IBHP	210,000 ± 28,000	6.0 ± 0.4	0.029 ± 0.002
	IPHP	27,000 ± 1,400	15.3 ± 0.2	0.554 ± 0.010
VvOMT1	IBHP	539,000 ± 31,000	58.6 ± 2.8	0.109 ± 0.005
	IPHP	493,000 ± 28,000	32.4 ± 1.4	0.066 ± 0.003
VvOMT2	IBHP	628,000 ± 29,000	7.5 ± 0.2	0.012 ± 0.0002
	IPHP	1,264,000 ± 120,000	16.9 ± 1.1	0.013 ± 0.001

against HP substrates compared to the CS enzyme seems unlikely to be the cause of the absence of MP accumulation in PN berries.

4.3.5 Expression analysis of VvOMT3 *in CS and PN berries*

Given that the enzymes encoded by CS and PN alleles of *VvOMT3* were both able to catalyse the final step in MP biosynthesis, it was thought that differences in the transcriptional control of this gene may be the cause of the absence of MP accumulation in PN berries. To investigate this hypothesis the expression of *VvOMT3* was compared throughout the development of CS and PN berries. RNA was extracted from deseeded berry samples and real-time PCR performed on the corresponding cDNA samples. Indeed, major differences in the expression of *VvOMT3* were seen between CS and PN throughout berry development (Fig. 4.10A). In CS, *VvOMT3* expression sharply increases from 2 wpf to a peak at 4 wpf, approximately 18-fold higher than the expression seen at 2 wpf, and this expression then decreases rapidly to low, almost undetectable levels by 7 wpf. The expression of *VvOMT3* in CS coincides with the periods of greatest MP accumulation (Fig. 4.1), supporting the hypothesis that *VvOMT3* is the major OMT involved in MP biosynthesis in grape berries. Furthermore, in PN berries, the expression of *VvOMT3* was not detectable at any time during berry development, suggesting that the lack of MP accumulation in PN berries is caused by an absence of *VvOMT3* expression.

Interestingly, *VvOMT4* had a similar pattern of expression to *VvOMT3* with a major peak at 4wpf in CS (Fig. 4.10B). However, a minor peak of *VvOMT4* expression was also observed at 4 wpf in the PN berry cDNA samples. Analysis of *VvOMT1* expression in these same samples showed that there was more expression in pre-véraison PN berries than CS berries at the same stage of

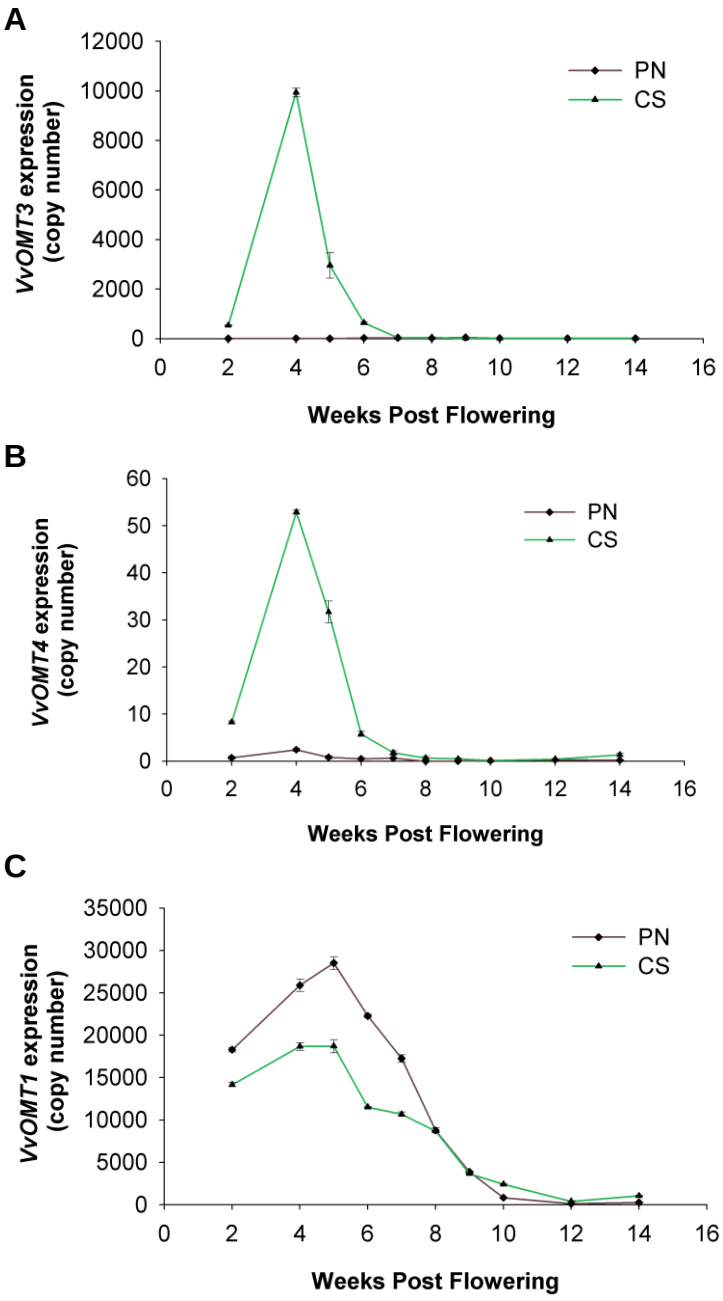


Figure 4.10 - The expression profiles of *VvOMT3* (**A**), *VvOMT4* (**B**) and *VvOMT1* (**C**) throughout the development of CS and PN berries.

development (Fig. 4.10C). This is further evidence that *VvOMT1* is not the major *OMT* gene responsible for IBMP accumulation in grape berries. *VvOMT2* expression was not detectable at any stage in the deseeded CS or PN berries, consistent with the results presented in section 2.3.4, which show that *VvOMT2* is only expressed in the seeds.

4.3.6 HP concentrations in CS and PN berries

Another possible cause of the differences in MP accumulation between CS and PN berries would be a difference in the concentration of HP substrates in the berries of these two varieties. With this in mind, HP substrate concentrations were quantified throughout berry development in both varieties using a newly developed LCMS method incorporating the addition of a deuterium labeled IBHP internal standard (Section 4.2.7). In both CS and PN berries, IBHP concentrations increase after 2 wpf to reach a peak at 6 wpf followed by a steady decrease through until harvest at 14 wpf (Fig. 4.11A). IBHP concentrations were consistently higher in CS berries, which had a peak of 8,110 $pg.g^{-1}$ at 6wpf compared to a maximum of 3,560 $pg.g^{-1}$ measured in PN berries at the same stage of development. IPHP was also detectable in both varieties but at concentrations approximately 10-20 fold lower (26-358 $pg.g^{-1}$) than that of IBHP (Fig. 4.11B). These low levels bordered on the limit of detection for the method and therefore the reproducibility of results was poor, meaning that distinct patterns of IPHP concentration changes during berry development were not observed.

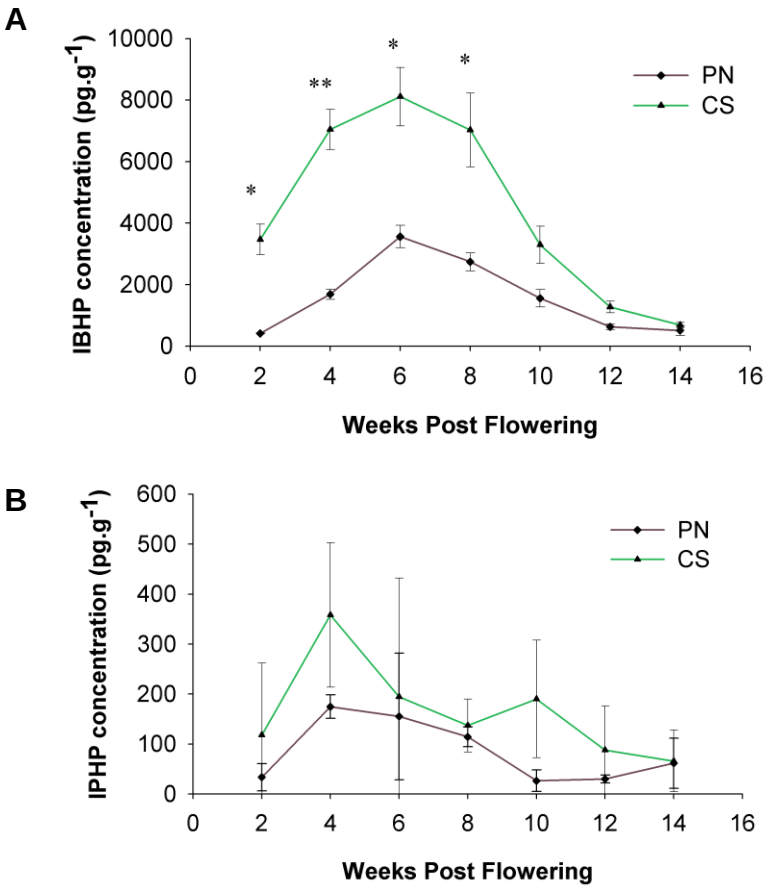


Figure 4.11 - IBHP (**A**) and IPHP (**B**) concentrations in CS and PN berries throughout development. Samples with a statistical significance difference between CS and PN are indicated by * = $p \leq 0.05$, ** = $p \leq 0.01$.

4.3.7 IBMP levels, VvOMT3 *expression and IBHP concentration in the PM dwarf × CS F2 progeny*

The results above suggest that *VvOMT3* expression is a key factor in IBMP accumulation in grape berries. To test this further, *VvOMT3* transcript levels were analyzed in the PM dwarf × CS F2 population segregating for IBMP-accumulation in the berries. Berries were sampled at 4 wpf, corresponding to the peak in *VvOMT3* expression seen in CS during the field trial reported in section 4.3.5. At the time of sampling only 18 of the 62 fruiting F2 plants had fruit at the 4 wpf stage. Of the 18 plants sampled 11 were heterozygous or homozygous for the CS allele of *VvOMT3* and accumulated IBMP at 7 wpf, while 6 plants were homozygous for the PM allele and lacked detectable levels of IBMP. Also included was plant #42, one of the two F2 plants which were homozygous for the PM allele of *VvOMT3* yet accumulate low levels of IBMP at 7 wpf.

Real time PCR analysis of gene expression confirmed that all 11 plants that contained the CS allele of *VvOMT3* and accumulated IBMP also contained high levels of *VvOMT3* expression (Fig. 4.12). Furthermore, the 7 plants homozygous for the PM allele and lacking IBMP also showed relatively low levels of *VvOMT3* expression. Plant #42 which is homozygous for the PM allele but does accumulate a small concentration of IBMP did not possess any significant levels of *VvOMT3* transcript. Therefore, it seems likely that a methyltransferase gene other than *VvOMT3* is responsible for the small amount of IBMP that accumulated in the berries of plant #42. Nevertheless, within this subset of the PM dwarf × CS F2 population there appears to be a relationship between the level of *VvOMT3* expression at 4 wpf and the amount of IBMP in the berries at 7wpf. When *VvOMT3* expression was plotted against IBMP concentration (Fig. 4.12C) the regression line of this plot reveals an r^2 value of

0.536. This indicates that approximately 53.6% of the variation in IBMP concentrations in these samples can be explained by *VvOMT3* expression.

To investigate if HP substrate concentrations also have an effect on the amount of IBMP in berries, IBHP levels were determined in the same plants at 7 wpf (Fig. 4.13). When IBHP is plotted against IBMP the graph reveals an r^2 value of 0.444, implying that IBHP levels explain approximately 44.4% of the variation in IBMP concentrations (Figure 4.13C).

As the function of the VvOMT3 enzyme and IBHP substrate are interdependent in the formation of IBMP, via the formation of an enzyme substrate complex, it seems logical that a combination of these two factors should have an accumulative effect in predicting the extent of IBMP accumulation. To test this hypothesis, *VvOMT3* expression was multiplied with IBHP concentrations for each plant and then plotted against IBMP concentration (Fig. 4.14). Indeed the regression line of this combined plot displays an r^2 value of 0.926 (Fig. 4.14C). Thus the combination of *VvOMT3* expression and IBHP concentration explains approximately 92.6% of the variation in IBMP concentrations at 7 wpf within the sub-population sampled from the segregating F2 progeny.

4.3.8 Analysis of MP and HP concentrations, and VvOMT3 *expression in grapevine tissues*

The level of *VvOMT3* expression and HP concentrations were measured in other grapevine tissues to determine the relationship between these parameters and the resulting MP levels. Rachises, flowers, tendrils, leaves and roots were sampled from both CS and PN, and both MP and HP concentrations and *VvOMT* expression levels determined. Interestingly, despite the lack of MP in PN berries

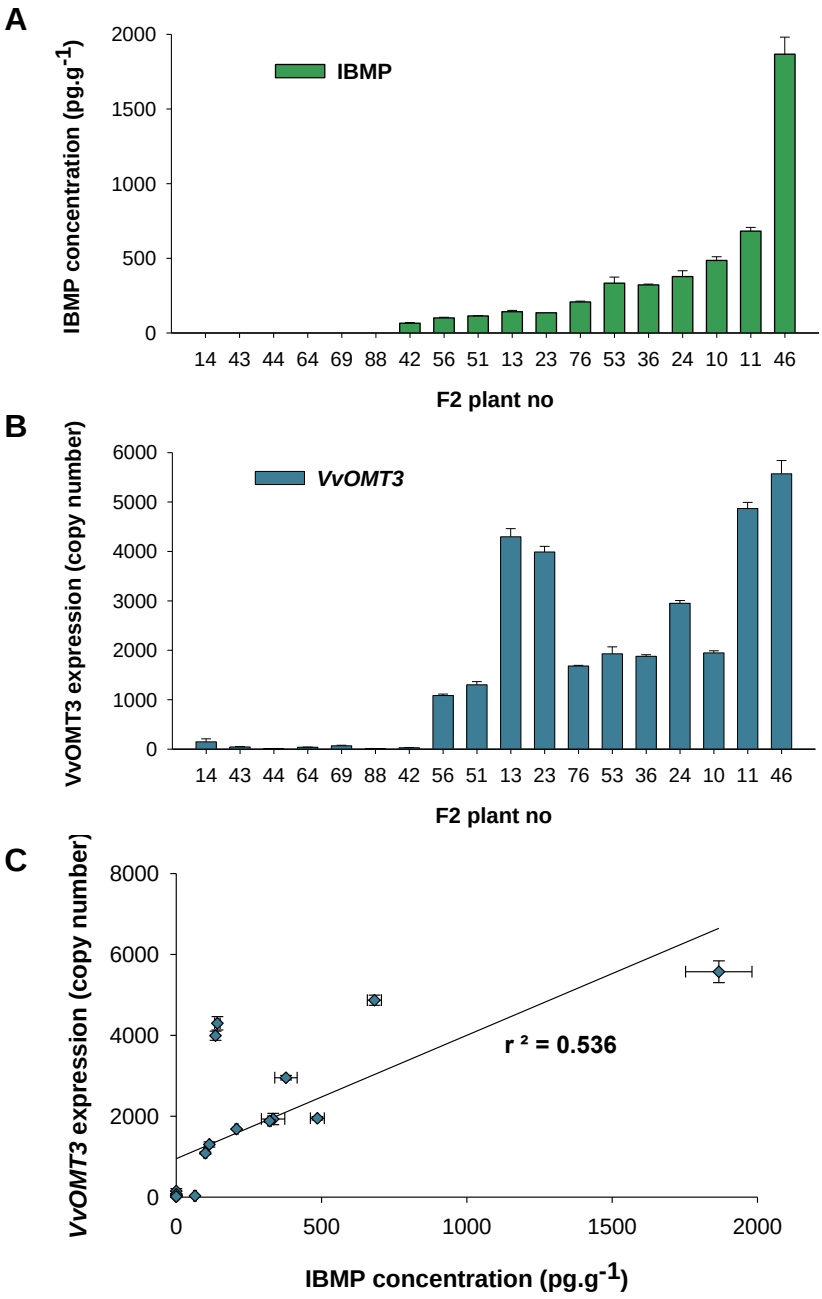


Figure 4.12 - IBMP concentrations at 7 wpf (**A**) and the level of *VvOMT3* expression at 4 wpf (**B**) in a subset of F2 progeny from the CS × PM cross. (**C**) A plot of *VvOMT3* expression against IBMP concentration reveals a positive correlation with an r^2 value of 0.536.

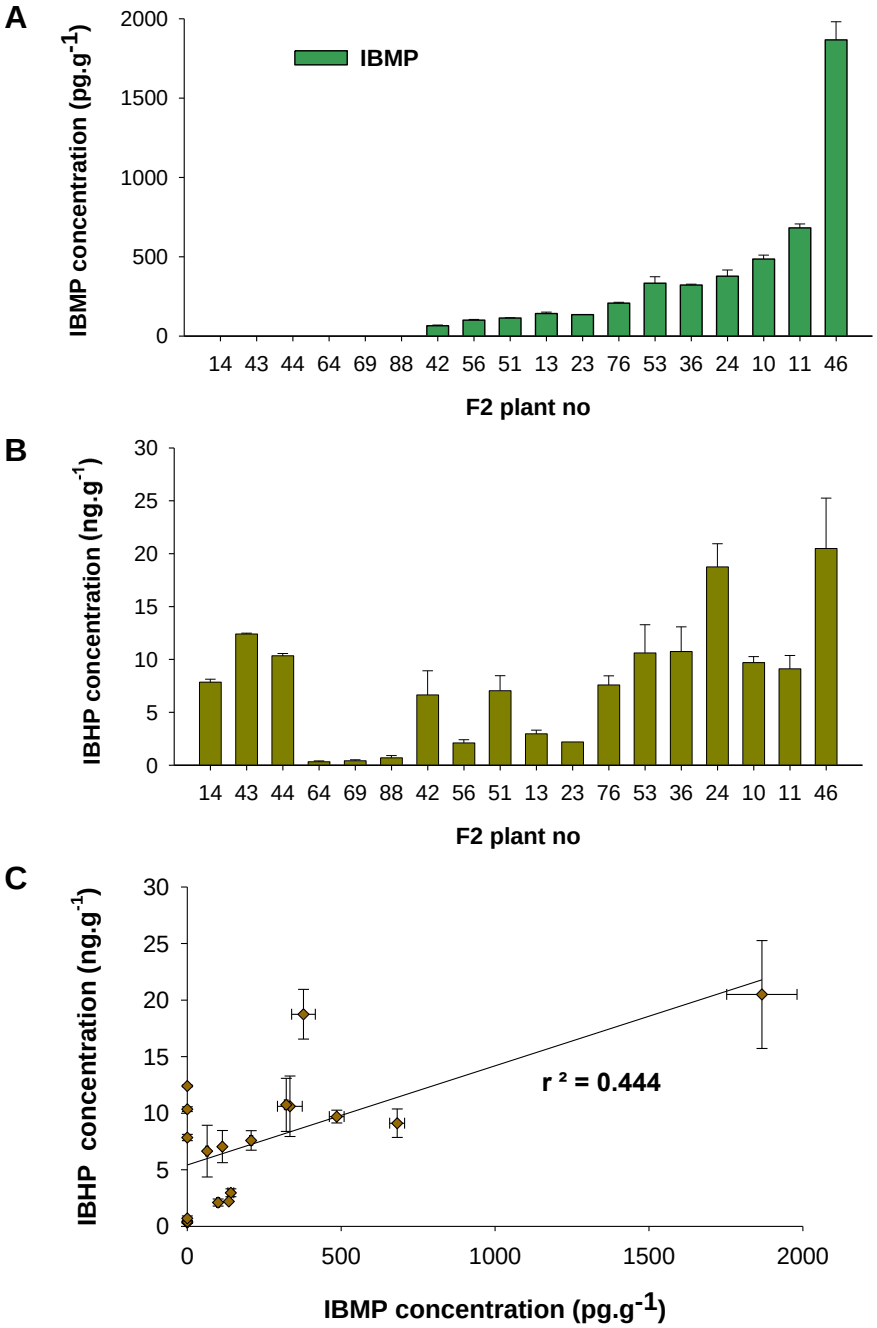


Figure 4.13 - IBMP (**A**) and IBHP (**B**) concentrations in a subset of F2 progeny from the CS × PM cross at 7wpf. (**C**) A plot of IBHP concentration against IBMP concentration reveals a positive correlation with an r^2 value of 0.444.

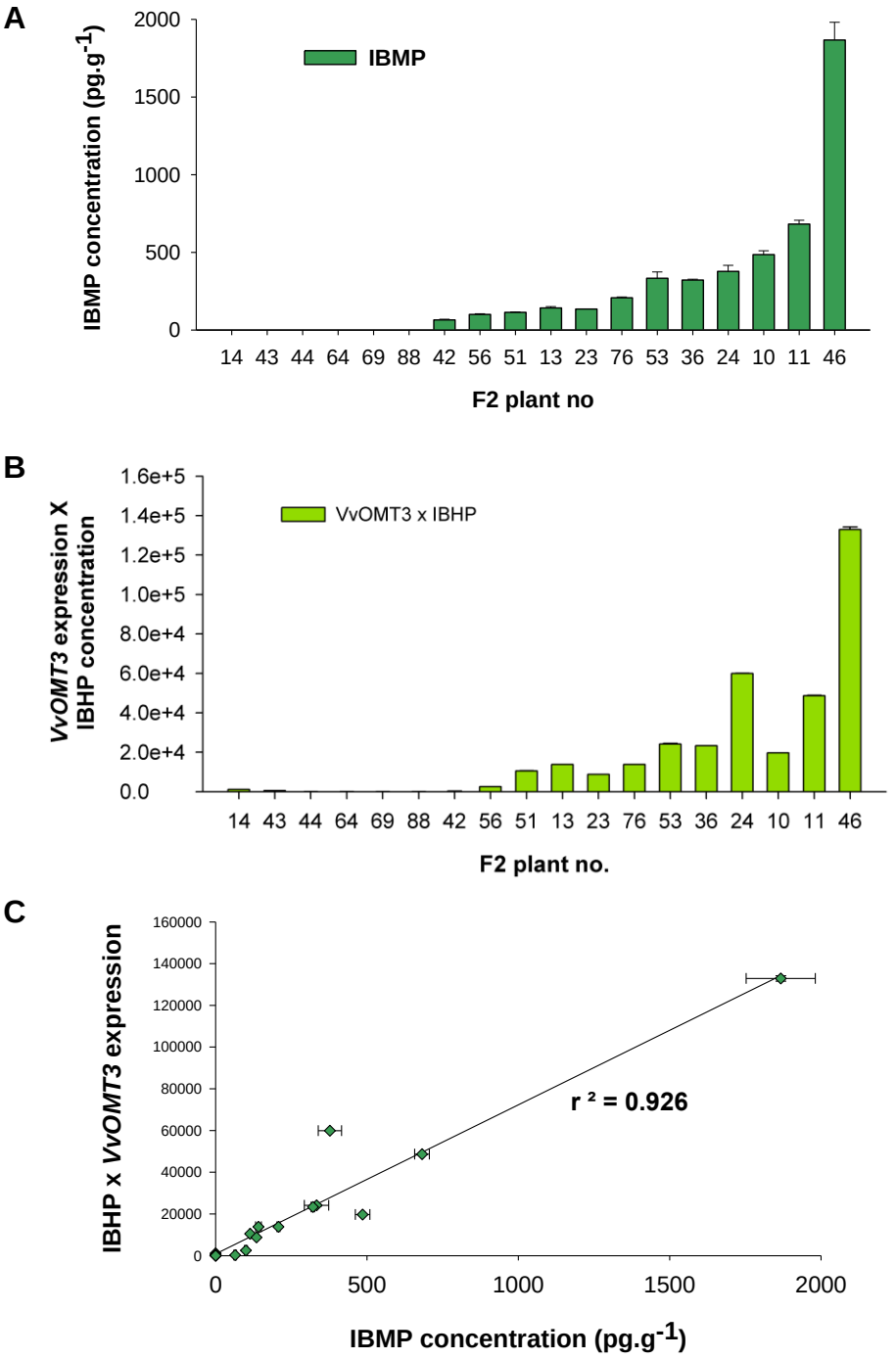


Figure 4.14 - (**A**) IBMP concentrations in a subset of F2 progeny from the CS × PM cross at 7wpf. (**B**) The product of the multiplication of IBHP concentration at 7wpf with *VvOMT3* expression at 4 wpf in the same subset of the F2 progeny. (**C**) A plot of IBHP concentration multiplied by *VvOMT3* expression against IBMP concentrations at 7 wpf. This plot reveals that the combination of *VvOMT3* expression and IBHP concentration is strongly correlated (r^2 = 0.926) with the resulting IBMP concentration.

(Fig. 4.1), PN had a similar pattern of MP accumulation to CS in all other tissues examined (Fig. 4.15A&B). In PN, both IPMP and IBMP are found at the highest concentrations in the roots, being measured at 4,916 and 1,119 $pg.g^{-1}$ respectively. Tendrils contained the next highest levels with 170 and 49 $pg.g^{-1}$ of IBMP and IPMP respectively followed by the rachises which contained 71 and 30 $pg.g^{-1}$ respectively. No detectable levels of MP were found in the flowers or leaves of PN.

Only the roots of CS showed significant levels of expression of *VvOMT3* (Fig. 4.15C), which was approximately 25 fold lower than the peak of expression observed in berries at 4 wpf (Fig. 4.1). In all PN tissues and CS tissues other than the roots, the expression levels of *VvOMT3* and *VvOMT4* were extremely low (Fig 4.15C&D), similar to the low expression levels seen in PN berries (Fig 4.10). In these other grapevine tissues, the levels of IBHP were approximately 8-20 fold higher than IPHP (Fig. 4.15E&F), consistent with the ratio seen in berries. In both CS and PN, the highest levels of IBHP were seen in the tendrils (10,065 and 9,840 $pg.g^{-1}$), while PN also contained high levels of IBHP in the rachis sample (6,432 $pg.g^{-1}$), but CS contained lower levels (1,985 $pg.g^{-1}$) in this tissue. In Chapter 2, it was proposed that the high levels of MP accumulation seen in the roots of CS was likely due to similarly high levels of HP substrates in this tissue. However, CS roots were found to contain only moderate levels of HPs comparable to that of berries and other tissues. Furthermore no IBHP or IPHP were detectable in PN roots despite accumulating high levels of the respective MP products.

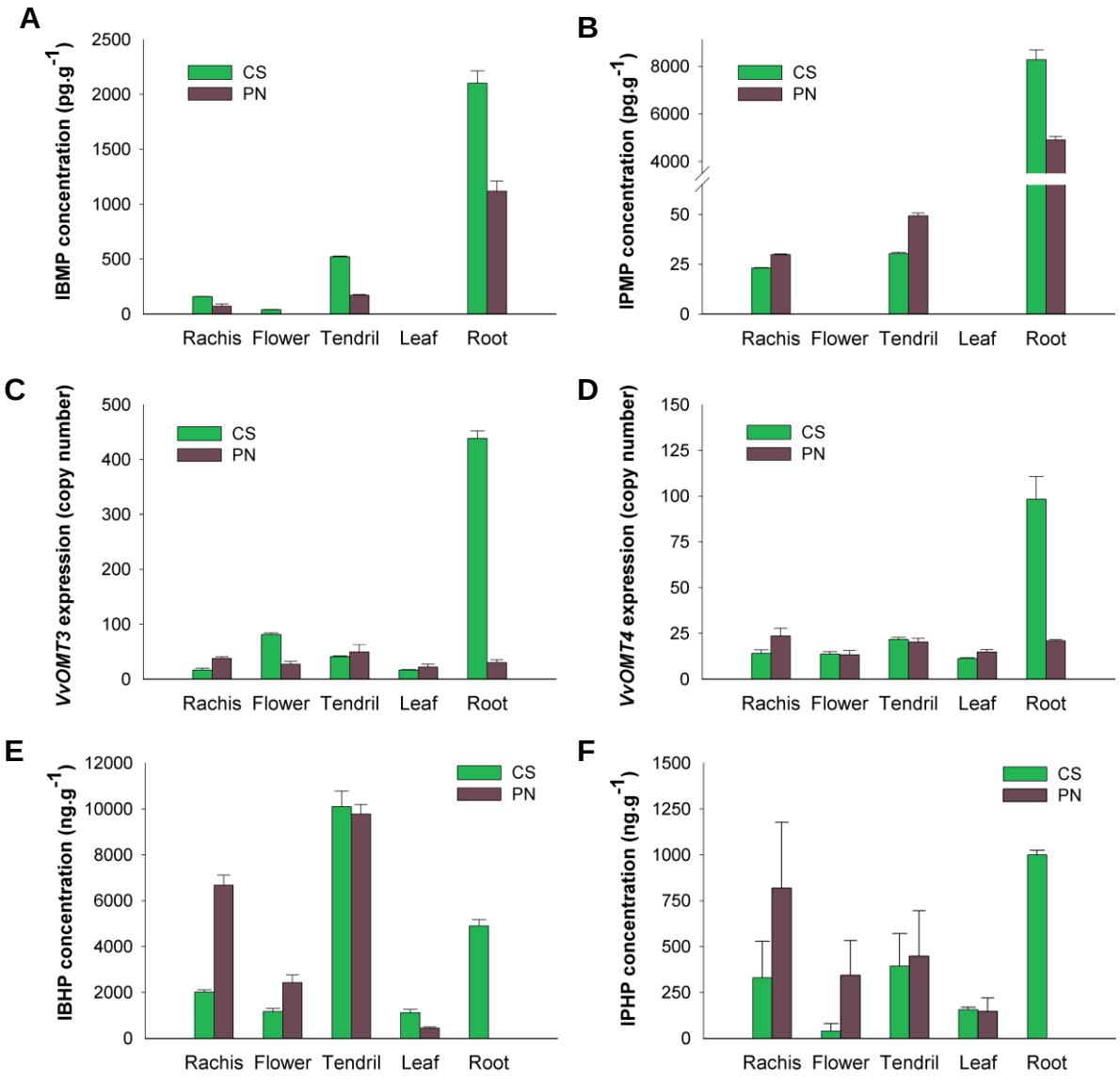


Figure 4.15 - IBMP (**A**) and IPMP (**B**) concentrations in various grapevine tissues from CS and PN. The expression of *VvOMT3* (**C**) and *VvOMT4* (**D**) as well as the concentration of IBHP (**E**) and IPHP (**F**) were also determined in the CS and PN samples.

4.3.9 Comparing the genomic regions surrounding VvOMT3 *and* VvOMT4 *in CS and PN*

The results presented thus far clearly indicate that the expression of *VvOMT3* has an important role in the accumulation of IBMP in CS berries. It is also clear that *VvOMT3* expression as well as *VvOMT4* expression is suppressed in all tissues of PN, and this coincides with a lack of IBMP accumulation in the berries. However, the genetic cause for the apparent suppression of both genes in PN is unclear.

Preliminary investigations were begun to determine if any sequence polymorphisms in the genomic region around the *VvOMT3* and *VvOMT4* genes might be responsible for their repression in PN. For this, two reference sources were used, the Pinot noir genomic BAC contig sequences (Velasco *et al.*, 2007) and the 12× annotated genome sequence based on the genome of PN40024 (Jaillon *et al.*, 2007). PN40024 is a highly homozygous (97%) line derived from nine successive selfings of Pinot noir and an accidental outcross with the variety Helfensteiner sometime within the 1st, 2nd or 3rd generation (Jaillon *et al.*, 2007). Helfensteiner itself is the result of an outcross between Pinot noir and Trollinger (also known as Frankenthal). Jaillon *et al.* (2007) compared 20 SSR markers between PN40024, Pinot noir and Helfensteiner which revealed that 6 of 20 SSR markers in PN40024 are not of Pinot noir origin and are therefore derived from Helfensteiner, making them of Trollinger origin (Jaillon *et al.*, 2007). Based on this limited data it can be estimated that approximately 70% of the PN40024 genome is that of Pinot noir and 30% is that of Trollinger.

The 12× annotated genome sequence of PN40024 has *VvOMT3* located 2 kb upstream of *VvOMT4* and 3.6 kb downstream of a gene of unknown function, all in the same negative orientation (Fig. 4.16). A BLAST (Altschul *et al.*, 1990) of

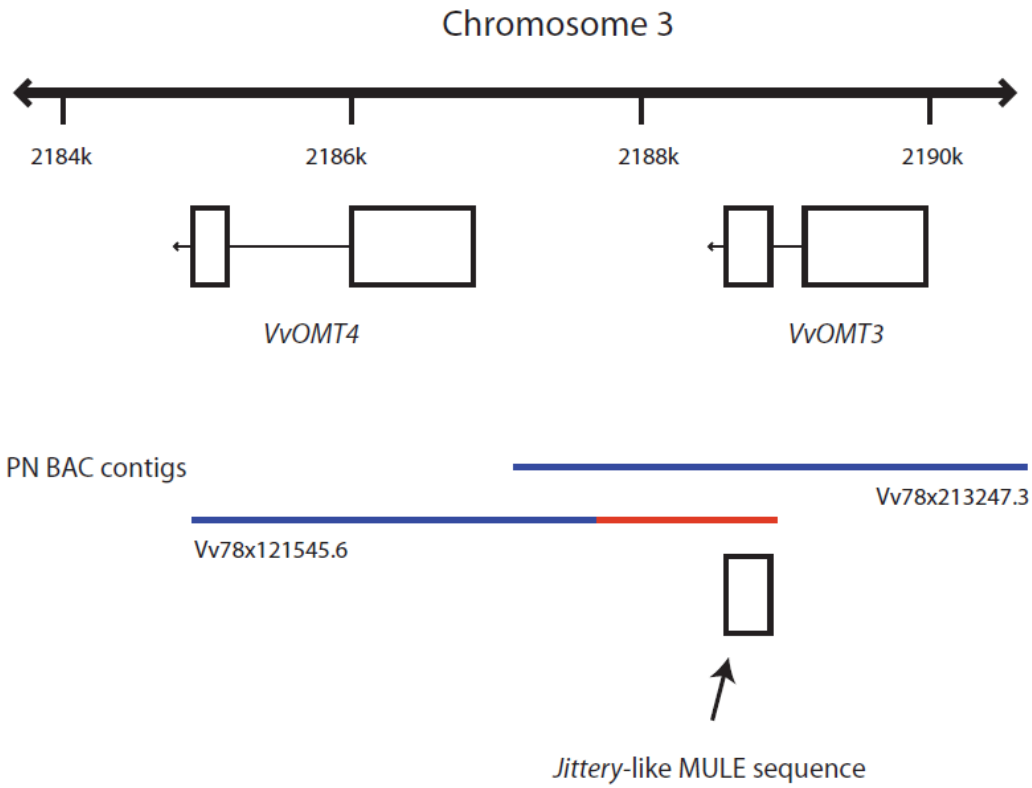


Figure 4.16 - Schematic diagram of the location of the *VvOMT3* and *VvOMT4* genes on chromosome 3 according to the 12× annotated genomic sequence on the NCBI database (**A**). Boxes represent coding sequences and arrows represent putative non-coding regions of the genes. (**B**) Relative positions of the of PN BAC contigs with sequence homology to this region and the position of the *Jittery*-like MULE transposon sequence located between *VvOMT3* and *VvOMT4* found in one of these contigs. Blue contig regions represent sequence that matches that of the 12× annotated sequence and the red region represents a sequence that does not match the latest genome build on the NCBI database.

the PN40024 sequence at this region against a library of BAC contigs from PN revealed some ambiguities between the position of *VvOMT3* and *VvOMT4*. Analysis of the Pinot noir BAC contigs revealed the two genes are each located on different contigs which do not perfectly overlap with each other (Fig. 4.16). The contigs share a near identical sequence at opposing ends except for a 1,134 bp sequence present on the contig containing *VvOMT3* (Fig. 4.16). This 1,134 bp sequence is not present in the 12× annotated sequence of PN4004. A BLAST of this sequence against a library of repetitive elements (Smit *et al.* 1996) revealed that 323 bp at the 5' end of this sequence has close homology to a class II transposable element from maize, named *Jittery* (Xu *et al.*, 2004).

Cloning of these genomic regions around *VvOMT3* and *VvOMT4* from both CS and the PM dwarf is currently underway by Dr. Paul Boss (CSIRO, Plant Industry). Preliminary results using promoter walking confirmed the presence of a transposon downstream of *VvOMT3* in the PM dwarf, but PCR amplification of this allele has thus far been unsuccessful, probably due to the size of the transposable element (Dr Paul Boss pers. comm.). Sequence data obtained from CS suggests that the *Jittery*-like transposon has excised from the genome of CS in this region (Dr Paul Boss pers. comm.).

4.4 Discussion

In the previous chapter, two novel *O*-methyltransferase genes, *VvOMT3* and *VvOMT4*, were shown to be associated with the trait of IBMP-accumulation in both a segregating F2 progeny as well as in a collection of 92 existing grapevine cultivars. The results of the current chapter confirm that the protein encoded by *VvOMT3* has methylating activity against HPs with a strong affinity for these substrates and is likely to be responsible for MP biosynthesis in grape berries.

Kinetic analysis of the recombinant VvOMT3 protein from CS revealed that this enzyme has strong catalytic activity against both IBHP and IPHP substrates, which was approximately 500 - 5,000 fold greater than the activity of VvOMT1, VvOMT2 and VvOMT4 against the same substrates (Table 4.1). Despite 7 amino acid differences, the PN-encoded VvOMT3 enzyme showed similar kinetic values to that of the CS-encoded VvOMT3 (Table 4.1). Therefore, the reason why PN does not accumulate MP in berries is not due to polymorphisms between the CS and PN alleles of *VvOMT3*. However the CS encoded VvOMT3 and VvOMT4 sequences differ from the VvOMT1 and VvOMT2 sequences at 3 amino acid residues important for substrate binding, highlighted in Fig. 4.3. These amino acid differences may be responsible for the differences in catalytic activity of these enzymes, however computer simulated docking studies, as performed by Vallarino *et al.* (2011) on VvOMT1 and VvOMT2, need to be performed for VvOMT3 and VvOMT4 to determine the structural relationships of these enzymes.

Transcript analysis throughout CS berry development revealed that *VvOMT3* is highly expressed pre-véraison (Fig. 4.10A), coinciding with the major period of IBMP accumulation. This is further evidence supporting a role for *VvOMT3* in IBMP biosynthesis in grape berries. Furthermore, in PN, the expression of *VvOMT3* was absent throughout berry development (Fig. 4.10A). Given the lack of IBMP in this variety, this observation again strengthens the hypothesis that *VvOMT3* is responsible for the final step of IBMP biosynthesis in grape berries.

Interestingly, the accumulation patterns of HPs appeared to match that of MPs with a peak in concentration pre-véraison (Fig. 4.11A). The concentrations of IBHP observed here are consistent with those determined by Hashizume *et al.*

(2001a) but are approximately 20 times higher than those recently reported in Cabernet Franc grapes by (Ryona *et al.*, 2010). Ryona and co-workers (2010) also observed that IBHP concentrations continually increased during development, contrary to the accumulation pattern seen here. Given the parent-offspring relationship between these two varieties it seems unlikely that they would have such different patterns of IBHP accumulation. A more probably explanation lies in the method of IBHP quantification used by Ryona *et al.* (2010) in which the compound 2-*sec*-butylphenol was used as an internal standard, rather than a deuterated IBHP analogue. As 2-*sec*-butylphenol has many structural differences to IBHP it is doubtful that it behaves identically to IBHP during the lengthy extraction procedure used, and given the dramatic changes in the chemical matrix of ripening grape berries, the differences in the extractability of the internal standard and the target compound could potentially lead to inaccurate results. Furthermore, the 2-*sec*-butylphenol standard was only added after an initial solid phase extraction step in the extraction protocol, which may also introduce error into the HP measurements (Ryona *et al.* 2010).

The levels of IBHP in pre-véraison CS grapes in this study were found to be between 3,470 and 8,110 $pg.g^{-1}$ (Fig. 4.11C), equating to approximately 23-53 nM. These substrate concentrations are lower than the observed K_m (380 nM) for recombinant VvOMT3, but localized concentrations of IBHP may increase the concentration of the substrate *in planta*. Interestingly, this low K_m value calculated for VvOMT3 against HP substrates is between 30 and 1,000 times lower than that reported for other characterised plant OMTs against their preferred substrates *in vitro* (Gang *et al.*, 2002; Hugueney *et al.*, 2009; Lavid *et al.*, 2002; Schmidlin *et al.*, 2008; Wein *et al.*, 2002; Wu *et al.*, 2003). Due to time constraints in this study, the activity of VvOMT3 against other possible substrates was not

investigated. The low K_m of VvOMT3 against HP compounds implies that they are likely to be preferred substrates. Nevertheless, in the future, activity against other potential substrates should be investigated.

Additional evidence for the importance of *VvOMT3* in MP synthesis was obtained when the trait of IBMP-accumulation in berries was found to be linked with the presence of *VvOMT3* expression in a subset of the segregating PM dwarf × CS F2 progeny (Fig. 4.12). Furthermore, the level of *VvOMT3* expression was correlated with IBMP concentration, accounting for 53.6% of the variation within these samples. Differences in IBHP concentration was found to account for a further 44.4% of the variation in IBMP levels (Fig. 4.13). When the *VvOMT3* expression and IBHP concentration variables were combined, the resulting values accounted for 92.6% of the variation in IBMP accumulation within this population (Fig. 4.14). The remaining variation may be attributed to differences in other variables such as IBMP degradation and volatilization, or the availability of the methyl donor SAM. However, it is also possible that variation arose from random errors such as the timing of the berry sampling which may not have reflected the peak in the concentrations of IBHP and IBMP or the peak in expression of *VvOMT3*. Given the potential genetic differences in the progeny and the effect this would have on berry development, this variation would be very difficult to eliminate in any experimental strategy. However, this genetic variation could also be useful for further studies. In the previous chapter it was suggested that the L1 × CS F1 and F2 populations could be used for QTL mapping to identify genetic factors involved in determining IBMP concentrations in berries. Given that the extent of pre-véraison IBMP accumulation is correlated with both IBHP accumulation and *VvOMT3* expression, QTL mapping could lead to the

identification of the genetic elements involved in IBHP biosynthesis as well as the regulatory elements responsible for *VvOMT3* transcription.

IPHP and IPMP were not able to be quantified in the F2 progeny due to the low concentrations of these compounds in the berries. Therefore, because of a lack of *in planta* evidence, it is prudent not to infer that *VvOMT3* is responsible for IPMP biosynthesis in berries. However, given that the recombinant VvOMT3 enzyme has similar catalytic activity against both IBHP and IPHP (Table 4.1) it is very likely that *VvOMT3* is also responsible for IPMP biosynthesis in berries. Similarly, in the F2 progeny IBMP was only measured at 7 wpf. Therefore, it is unclear if the relationship between IBHP levels and *VvOMT3* expression also correlates with the concentration of IBMP at harvest. A previous study has shown that a strong correlation ($r^2 = 0.936$) exists between pre-véraison IBMP levels and those measured at harvest (Ryona *et al.*, 2008), suggesting that the rate of IBMP decline is not a major variable in determining MP concentrations in ripe berries. However, this study only compared Cabernet Franc berries from different vineyards within a single viticultural region in north-west USA. In order to determine if the decline in IBMP is a passive, constant phenomenon or a process that is variable according to genotype, a future study could be conducted comparing pre-véraison and harvest levels of IBMP within the F2 progeny as well as the 11 existing cultivars that accumulate IBMP. This would provide valuable information about the nature of the mechanism driving the decrease in IBMP levels in post-véraison berries, of which very little is currently known.

In the previous chapter it was noted that two F2 individuals, #3 and #42 were homozygous for the PM allele of *VvOMT3*, yet accumulated small amounts of IBMP. It was proposed that the ability of these plants to accumulate IBMP despite little or no VvOMT3 activity was possibly due to high levels of IBHP

substrate. In this chapter plant #42 was indeed found to contain relatively high levels of IBHP at 7wpf (Fig 4.13B). However, other F2 plants #14, #43 and #44 which do not accumulate IBMP and do not express VvOMT3 expression also contained high levels of IBHP, which may suggest that other postulated OMTs thought to be responsible for the small levels of IBMP biosynthesis in #3 and #42, are segregating in this population. Nevertheless, it is likely that these low concentrations of IBMP present in the 7 wpf berries of plants #3 and #42 would have decreased to concentrations well below the odour detection threshold by harvest and therefore have no sensory impact on wines made from these berries. Again, the study postulated in the previous paragraph would determine if this was indeed the case.

Of the five other grapevine tissues examined only the roots of CS contained any notable levels of *VvOMT3* transcript (Fig. 4.15C), yet the levels in roots were approximately 25 times lower than the expression seen in the berries at 4 wpf (Fig. 4.10A). Given the high concentrations of MPs that accumulate in roots, the low level of *VvOMT3* expression tends to suggest that other OMTs are responsible for this activity in this tissue. Further evidence to support this hypothesis is that the expression of *VvOMT3* appeared to be suppressed in not just berries but all PN tissues (Fig. 4.15C) despite accumulating MPs in the roots, tendrils and rachis comparable to concentrations seen in similar tissues in CS (Fig. 4.15A. Importantly, these results imply that MP biosynthesis is tissue specific and agree with the findings of Koch *et al.* (2010) who recently demonstrated that MPs are not translocated into berries from elsewhere in the vine.

In Chapter 2 it was hypothesised that the reason grapevine roots contained high concentrations of MPs was possibly due to high concentrations of HPs. However, this theory does not appear to hold true, as the roots of CS were found

to contain HP concentrations similar to both berries and other tissues, while in PN roots the HPs were below the limit of detection (Fig. 4.15E&F). This unexpected finding suggests that the OMT responsible for MP biosynthesis in roots has an extremely high catalytic activity against these substrates and/or is highly expressed, resulting in a strong flux from HPs to MPs. It is also possible that the degradation or further metabolism of MPs that is thought to be responsible for the decline in MP concentration in berries, is less active in the roots resulting in greater accumulation. This also implies that any MPs produced may be maintained in tissues long after the substrates have themselves been degraded or metabolized, hence the low concentrations of HPs observed in the roots.

Overall, there is now much evidence that, in grape berries, VvOMT3 is the major enzyme responsible for the final step in the biosynthesis of IBMP. In the berries of both PN and some F2 progeny, the lack of *VvOMT3* expression was associated with a lack of MP accumulation in these plants. Interestingly, *VvOMT4* expression was also absent in these plants (Fig. 4.10B), which may provide a clue as to the mechanism behind the apparent suppression of the expression of these two genes in the PN genome. It is unlikely that two separate mutations would have arisen independently leading to the suppression of each gene. This could be tested by studying the expression of these genes in other varieties to see if there are cases where the expression of one or other of these genes is suppressed independently. However, it is most likely that a single genetic factor is responsible for the down-regulation of both *VvOMT3* and *VvOMT4* in PN and PM. The results reported in Chapter 3 showed that the trait of IBMP-accumulation in berries is strongly associated with the *VvOMT3* locus in the 91 different cultivars examined. While it has yet to be shown that this trait is attributed to *VvOMT3* expression in these cultivars, as the evidence strongly suggests for the PM dwarf × CS F2

progeny, this is most likely the case. This would therefore suggest that the genetic cause for the lack of *VvOMT3* and *VvOMT4* expression is tightly linked to the *VvOMT3* locus and not due to a trans-acting regulatory element, such as a shared transcription factor. Analysis of a PN derived BAC contig sequences (Velasco *et al.*, 2007) from this locus suggest that a *Jittery*-like transposable element is located downstream of *VvOMT3* and this has recently been confirmed in PM by promoter walking, performed by Dr Paul Boss (CSIRO, Plant Industry). The presence of a large transposon at this region seems the most likely scenario for the apparent co-suppression of *VvOMT3* and *VvOMT4* in Pinot varieties.

Jittery, identified in maize, is a 3.9 kb transposon belonging to the *mutator*-like (MULE) super family (Xu *et al.*, 2004). *Jittery* contains terminal inverted repeats of 181 bp, appears to excise autonomously and, unlike most transposable elements, does not leave footprints upon excision or reinsertion in the genome. Since the first identification of *Jittery* in maize, *Jittery*-like transposons have been found in rice and the model legume *Lotus japonicas* (van Leeuwen *et al.*, 2007; Benjak *et al.*, 2009; Holligan *et al.*, 2006). Furthermore 51 complete and 62 incomplete copies of *Jittery*-like transposon sequences have previously been identified in the grapevine genome, ranging in size from 11.9-14.4 kb (Benjak *et al.*, 2009). The presence of a large transposable element in this locus within Pinot varieties is the most likely explanation, based on our current knowledge, for the loss of expression of both *VvOMT3* and *VvOMT4*. Disruption of gene expression as a result of transposon insertions in upstream promoter regions has been widely observed in plants (Xu *et al.*, 2007; Yang *et al.*, 2005). Two examples of altered gene expression due to transposon insertion have also previously been observed in grapevine. For instance, the skin colour of white grapes has been shown to be due to the insertion of a *Gret*1 retrotransposon in the

promoter region of the *VlMybA1* gene (Kobayashi *et al.*, 2004), which regulates anthocyanin biosynthesis (Walker *et al.*, 2007). Another example is the Reiterated reproductive meristem (RRM) variant of the cultivar Carignan, which displays early inflorescence development, delayed anthesis and bunches with altered size, structure and ripening patterns (Fernandez *et al.*, 2010). These authors showed that the insertion of a class II transposon is responsible for the RRM phenotype resulting from the activated expression of a *TERMINAL FLOWER 1* homolog (Fernandez *et al.*, 2010).

In addition to altering gene expression through disruptive insertions, transposable elements can lead to a repression of gene expression through the recruitment of chromatin remodeling machinery, which effectively silences active transposable elements (Lippman *et al.*, 2004). It is thought that eukaryotic genomes have evolved this silencing mechanism to suppress the potentially harmful effects of repetitive mobilization and duplication of transposable elements (Slotkin and Martienssen, 2007). An example of this is found in the Landsberg *erecta* accession of *Arabidopsis* in which the presence of a MULE transposon located in an intron of the *FLOWERING LOCUS C* gene results in repressive chromatic modifications via DNA methylation (Liu *et al.*, 2004). These chromatic changes are mediated by short interfering RNAs generated from homologous MULE transposable elements elsewhere in the genome (Liu *et al.*, 2004; Zhai *et al.*, 2008). It is possible that a similar occurrence results in chromatin remodeling at the *VvOMT3* locus due to the presence of a *Jittery*-like MULE transposon. This scenario would explain why the expression of both *VvOMT3* and *VvOMT4* appears to be repressed in PN. Unfortunately, time did not permit a full investigation into the presence of a transposon between *VvOMT3* and *VvOMT4* in PN, and this should be a strong focus of future work. In addition, the

presence of a transposon at this locus in the cultivars used for the association mapping study should be determined as further evidence for it being the genetic element responsible for the IBMP-accumulation trait in grape berries.

The results of this chapter together with the mapping results reported in the previous chapter provide strong evidence that the *VvOMT3* gene is responsible for the final step in IBMP biosynthesis in grape berries. In the next chapter the expression of *VvOMT3* in response to two different variables (sunlight exposure and yield) is investigated as these factors have previously been shown to affect MP accumulation in grapes.

Chapter 5 - Investigating the effect of sunlight exposure and fruit yield on IBMP biosynthesis in developing grape berries

5.1 Introduction

5.1.1 General introduction

IBMP has an extremely low odor detection threshold which means that minor changes in IBMP concentration can have a major impact on the consumer acceptability of a wine. At low concentrations IBMP adds complexity and vegetative varietal characters, but at high levels IBMP becomes detrimental to wine quality as its strong herbaceous and earthy flavours mask more desirable floral and fruity characters (Parr *et al.*, 2007). Currently there are no known winemaking techniques that can be employed to significantly reduce the vegetative characters imparted by a high IBMP content (Lopez *et al.*, 2001; Perez-Coello *et al.*, 2000; Pickering *et al.*, 2006). Given the lack of a successful method for removing IBMP from wines, the most effective way of producing wines with the most palatable IBMP concentrations is by controlling the abundance of the compound present in the grapes at harvest. Therefore, understanding the factors that influence the accumulation of IBMP in grapes is vital to producing grapes and wines with a suitable IBMP content.

As discussed in Chapter 1, it is well established that the IBMP concentration of grapes is influenced by many variables including maturity, sunlight exposure, temperature, water status, vine vigor and yield (Belancic and Agosin, 2007; Hashizume and Samuta, 1999; Heymann and Noble, 1987; Lacey *et al.*, 1991; Noble *et al.*, 1995; Roujou de Boubee *et al.*, 2000; Ryona *et al.*, 2008; Sala *et al.*, 2005). The majority of these previous studies were performed

from a viticultural perspective and as such sought to determine the affect of a certain environmental stimulus or management practice on IBMP concentrations, and did not seek to gain an understanding of the mechanism through which each variable altered IBMP levels. Furthermore, many of these variables interact and consequently it is difficult to establish whether they are having a direct effect on the concentration of IBMP or whether their influence is exerted through a secondary effect when they alter another variable. However, there is evidence to suggest that two variables, sunlight exposure and grape yield, do have a direct influence on the concentration of IBMP in grape berries, yet the mechanisms by which they do so remains unclear.

Previous studies have shown that increased sunlight exposure to grape bunches generally leads to a decrease in IBMP accumulation (Allen *et al.*, 1996; Marais *et al.*, 1999; Noble *et al.*, 1995). This observation was attributed to increased photo-degradation, as it has been shown that IBMP is degraded by light when in an aqueous solution (Heymann *et al.*, 1986). However, more recent studies have shown that berry shading has a greater influence on IBMP concentrations in pre-véraison berries, when MPs accumulate, than during the ripening phase when IBMP is supposedly photo-degraded (Ryona *et al.*, 2008; Scheiner *et al.*, 2010). These authors suggest that shading may actually increase IBMP accumulation through increased biosynthesis rather than decreased degradation.

An experiment in which CS vines were winter pruned to produce vines of six different yield treatments showed that a significant ($p < 0.001$) negative correlation existed between yield and IBMP concentrations in the subsequent wines (Chapman *et al.*, 2004). In this study the vines of lowest yield (12 buds per vine) produced wines with approximately 2 to 5 times higher concentrations of

IBMP than vines of highest yield (48 buds per vine). The authors noted that because of the pruning method employed the low yielding vines also had fewer shoots and leaves and so the bunches experienced greater sunlight exposure than those grown on the higher yielding vines. Therefore, despite a presumed increase in light exposure, which usually leads to lower IBMP accumulation, wines of low yielding vines still produced significantly greater amounts of IBMP than high yielding vines. These results suggest that fruit yield may have a direct influence on IBMP levels independent of sunlight exposure.

5.1.2 Aims

While sunlight exposure and crop yield each appear to directly influence the concentration of IBMP in grapes, the mechanism by which they do so remains unclear. Both of these variables are easily and commonly manipulated in the vineyard by practices such as winter pruning, leaf plucking and bunch thinning (Nuzzo and Mathews, 2006; Myers *et al.*, 2008; Dokoozlian and Kliewer, 1995). As such these practices potentially provide grape growers with a simple and effective approach to producing grapes of desired IBMP content. However, a clear understanding of how each factor influences IBMP accumulation is needed before attempting to determine the optimum combination of yield and bunch sunlight exposure for different locations and environmental conditions.

The aim of the work presented in this chapter was to determine the effect of sunlight exposure as well as the effect of grape yield on the expression of *VvOMT3* and the concentration of IBHP as possible mechanisms to explain the influence of these variables on IBMP accumulation. The influence of sunlight exposure on the post-véraison degradation of IBMP was also investigated to gain

a greater understanding of the reduction in IBMP concentrations during berry ripening.

5.2 Materials and Methods

5.2.1 Sunlight exposure experiment

The sunlight exposure experiment was conducted during the 2008/2009 growing season using Cabernet Sauvignon vines located at the Harvey family's commercial Slate Creek vineyard in Willunga, South Australia (latitude 35^{o} 15' S, longitude 138^{o} 33' E). Opaque boxes designed to eliminate light, as described by Downey and co-workers (Downey *et al.*, 2004), were applied to individual grape bunches. The boxes were 250 mm in length and 150 mm wide with a depth of 120 mm, and were made from 0.6mm thick white polypropylene sheeting painted black on the inside. The boxes were designed with overlapping semi-opened sides to maximize the airflow in order to minimize changes in temperature and humidity within the boxes compared to the outside environment. The experiment consisted of three boxing treatments and a non-boxed control. In treatment one, boxes were applied to whole bunches at flowering (100% cap-drop) and bunches were then sampled at 2, 4, 6, 8, 10, 12 and 14 wpf. Treatment two also consisted of applying boxes at flowering, but then the boxes were removed at véraison (8 wpf) and samples taken at 10, 12 and 14 wpf. Treatment three is the converse of treatment two with boxes being applied to non-boxed bunches at véraison and sampled at 10, 12 and 14 wpf. All samples were taken in triplicate with 3-6 bunches constituting one triplicate sample. Samples were taken by completely removing the cane of the boxed bunch from the vine with the box still in place. Once in the laboratory and under low light conditions bunches were removed from the boxes and the berries separated from the stems and immediately frozen

in liquid nitrogen and stored at -80°C. For each sample 30 fresh berries were randomly selected for measurement of berry weight and total soluble solids (°Brix).

5.2.2 Yield trial

A vineyard trial aimed at manipulating fruit yield was conducted by Dr Paul Boss during the 05/06 season using Cabernet Sauvignon vines at the Harvey family's Slate Creek vineyard. In this trial inflorescences were removed immediately prior to cap-drop to produce vines of high and low fruit yield, thereby having no secondary effect on vine canopy. Each treatment consisted of 16 vines randomly assigned to either yield category across three rows of the vineyard plot. Samples were taken from each treatment at two week intervals from 1 to 15 wpf. Berries were separated from the stems and immediately frozen in liquid nitrogen and stored at -80°C. For each sample, 30 fresh berries were randomly selected for measurement of berry weight and total soluble solids (°Brix) at each time point.

5.2.3 Analysis of VvOMT transcript levels

RNA extraction, cDNA synthesis and real-time PCR analysis of transcript levels were performed as described in sections 2.2.3 and 2.2.10 respectively. Gene specific primers used for real time PCR analysis are given in Appendix A.

5.2.4 IBMP and IBHP quantification

GC-MS quantification of IBMP was conducted according to the method in section 2.2.9. IBHP quantification by LC-MS was carried out as described in section 4.2.7

5.3 Results

5.3.1 Effect of sunlight exclusion on fruit ripening

A previous study demonstrated the effectiveness of the opaque boxes used in this experiment to exclude sunlight from bunches while causing no significant difference to temperature or humidity within the box compared to that of the natural vine canopy (Downey *et al.*, 2004). That study also showed that the boxes effectively eliminated 99.5-99.9% of natural light (wavelengths of 220-800 nm). Therefore, it is sufficient to conclude that any effects observed as a result of the boxing of bunches in this experiment can be directly attributed to the elimination of sunlight.

The act of boxing grape bunches had no significant affect on the weight of the berries except at 8 wpf where the light excluded berries weighed significantly ($p < 0.01$) less than the control berries (Fig. 5.1A). Similarly, no significant differences were observed in ^{o}Brix measurements throughout berry development with the exception of 12 and 14 wpf (Fig. 5.1B). At 12 wpf the control sample was significantly ($p < 0.01$) different from the boxed and post-véraison boxed samples and at 14 wpf the boxed and post-véraison boxed samples were the only samples not significantly different from each other ($p < 0.001$). Obvious changes could also be seen in the chlorophyll content of the berries at pre-véraison stages with the boxed samples displaying a pale yellow colour compared with the normal green colour of unripe berries (Fig. 5.2). No visible differences were seen in the anthocyanin content of boxed and non-boxed berries post-véraison (data not shown).

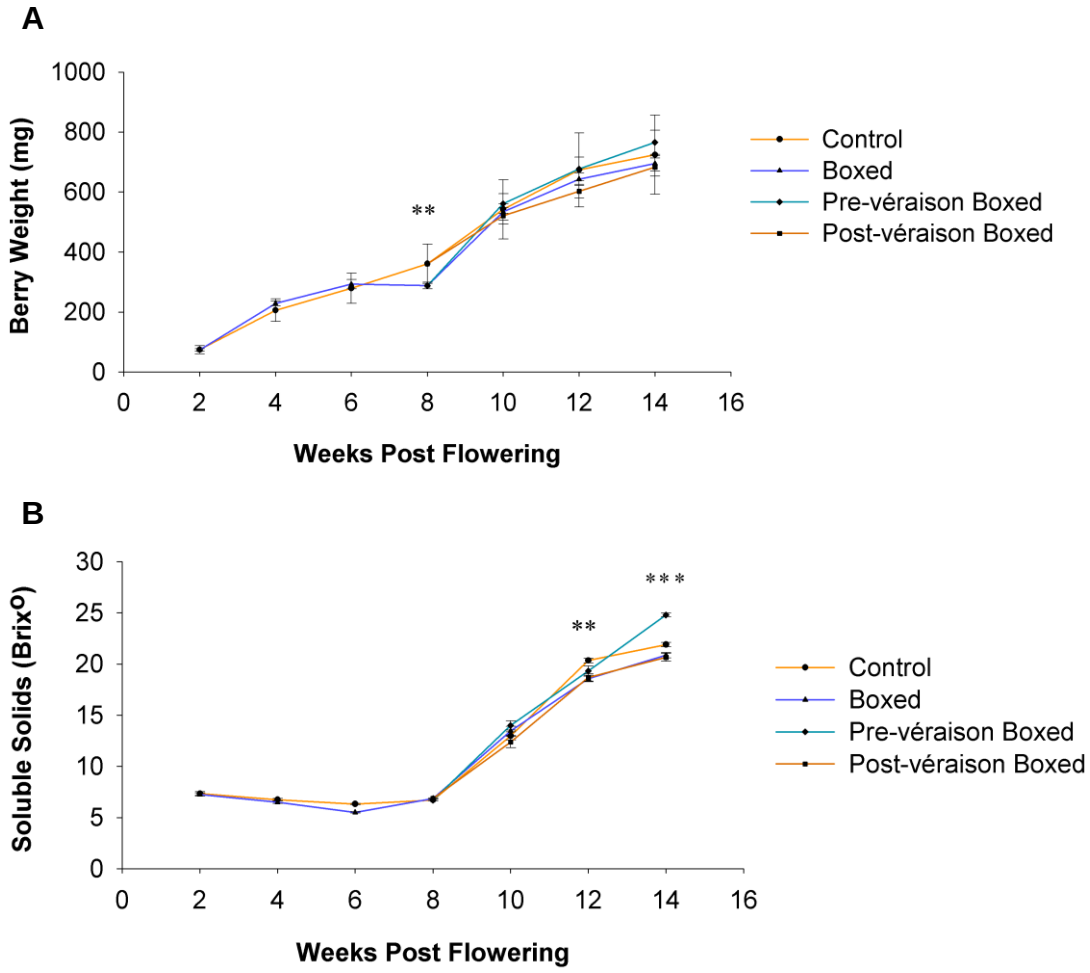


Figure 5.1 - Growth parameters of CS grape berries subjected to different boxing regimes to exclude sunlight during development. (**A**) Average berry weight in mg, and (**B**) average total soluble solids measured as °Brix. Statistically significant differences between at least two of the values are denoted by * = $p \leq 0.05$, ** = $p \leq 0.01$ or *** = $p \leq 0.001$.

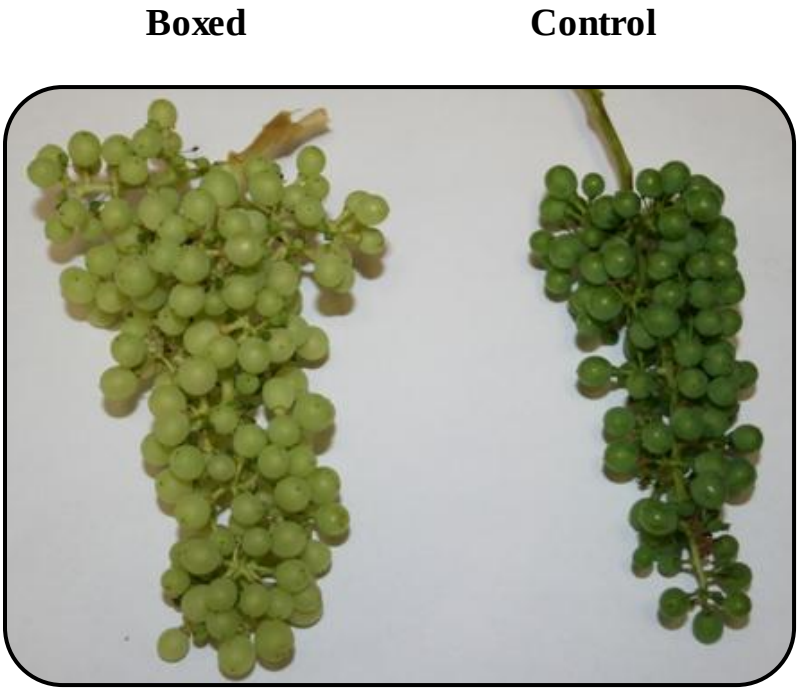


Figure 5.2 - Visible changes in the chlorophyll content of boxed and un-boxed control buches of CS at 6 wpf.

5.3.2 Effect of sunlight exclusion on IBMP accumulation

The primary aim of this sunlight exclusion experiment was to determine first, if the boxing treatment caused changes in IBMP concentrations and second, if these changes were due to the alteration of either *VvOMT3* expression or IBHP concentrations. Furthermore, the timing of the application and removal of boxes was tailored in such a way to determine the effect of sunlight exposure on IBMP concentrations in berries both pre- and post-véraison. The exclusion of sunlight from berries resulted in a significant ($p < 0.05$) increase in the concentration of IBMP compared to the unboxed control throughout berry development (Fig. 5.3). In both the control and boxed treatments, IBMP concentrations were below the level of detection at 2 wpf, but the concentrations in the treatments then diverged as the berries began to accumulate IBMP. The control treatment had a peak of IBMP concentration of 120 $pg.g^{-1}$ at 8 wpf, whereas the boxed samples contained almost three times that amount peaking at 340 $pg.g^{-1}$at the same time point (Fig. 5.3). The level of IBMP then declined in both treatments. At the time of harvest (14 wpf) IBMP could not be detected in the control berries, whereas the berries that had been boxed throughout development had an IBMP concentration of 34 $pg.g^{-1}$.

The application of boxes at véraison also had a significant ($p < 0.01$) effect on IBMP levels at harvest (Fig. 5.4A). This treatment, in which boxes were applied at 8 wpf, resulted in a lesser reduction of IBMP levels in the berries (16.7 $pg.g^{-1}$ at 14 wpf) compared to the control treatment (not detected at 14 wpf). The pre-véraison boxed treatment, in which the boxes were removed at 8 wpf, resulted in a greater decrease in IBMP concentration in the berries (27.8 $pg.g^{-1}$ at 14 wpf) compared to the boxed treatment (33.6 2 $pg.g^{-1}$ at 14 wpf), but these differences were not statistically significant (Fig. 5.4B).

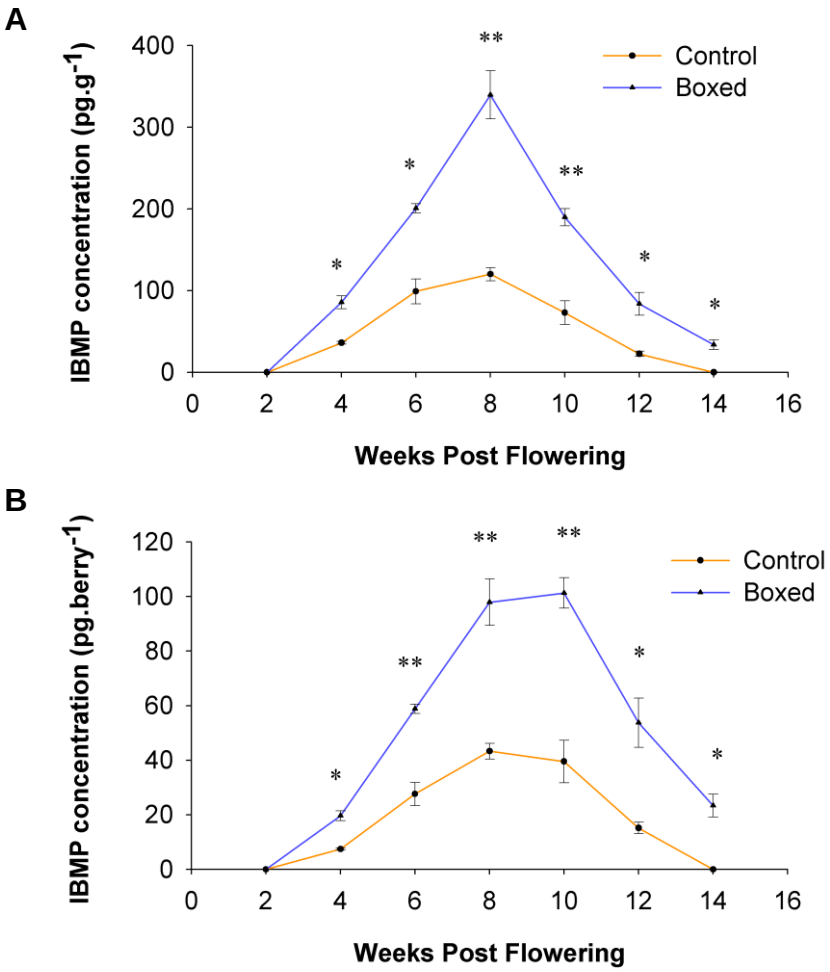


Figure 5.3 - IBMP accumulation during the development of CS grape berries subjected to different boxing regimes. IBMP concentrations are expressed as either pg.g^{-1}of fresh weight of berry (**A**) or as pg.berry^{-1} (**B**). Statistically significant differences are denoted by * = $p \leq 0.05$, ** = $p \leq 0.01$ or *** = $p \leq 0.001$.

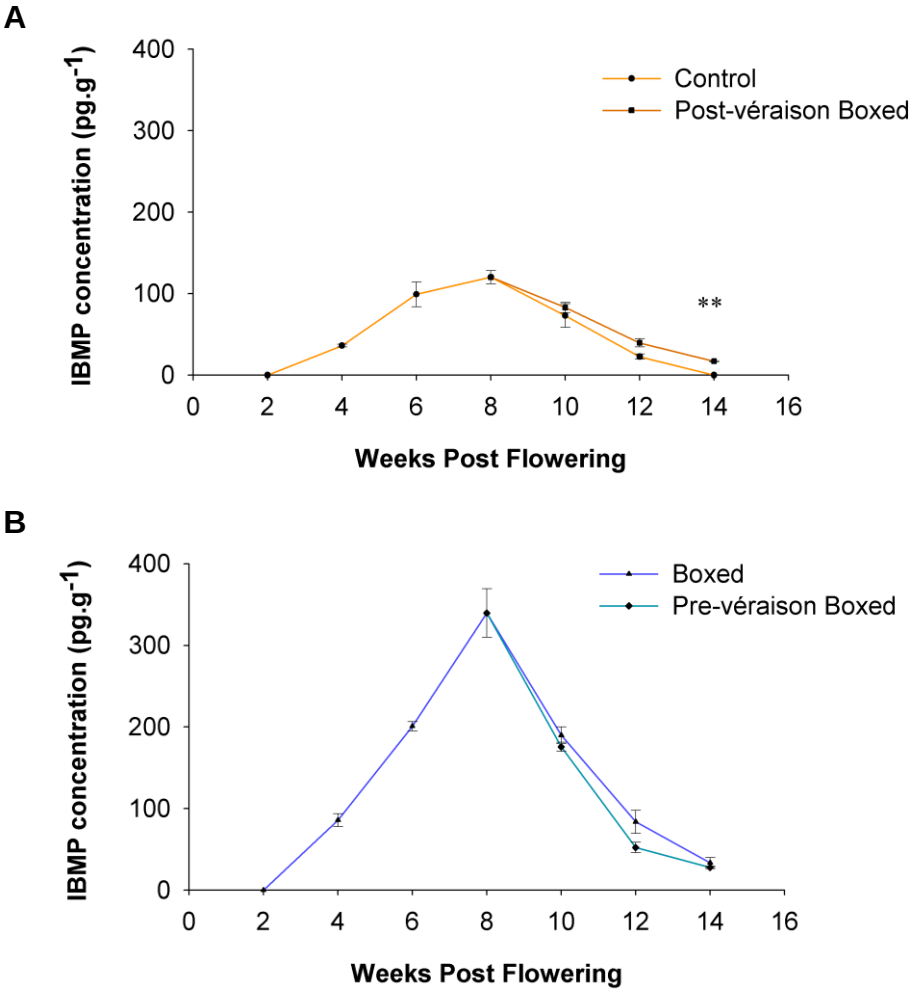


Figure 5.4 - Changes in post-véraison IBMP concentrations of developing CS grape berries subjected to **(A)** the addition of boxes at véraison and **(B)** the removal of boxes at véraison. Statistically significant differences are denoted by * = $p \leq 0.05$, ** = $p \leq 0.01$ or *** = $p \leq 0.001$.

5.3.3 Effect of sunlight exclusion on VvOMT3 expression

The expression of *VvOMT3* was analysed to determine if the increase in IBMP accumulation due to sunlight exclusion was related to changes in the amount of the *VvOMT3* gene transcript present in the berries. Indeed this was the case with significantly higher ($p < 0.05$) levels of *VvOMT3* expression in the boxed samples compared to the controls (Fig. 5.5A). In both the control and boxed samples the expression of *VvOMT3* was greatest at 4 and 6 wpf and in the boxed samples the expression at these stages was approximately 75% higher than of that in the control samples. From 10 wpf onwards, *VvOMT3* expression was not detected in any treatment including the pre- and post-véraison boxed treatments.

Surprisingly the expression of *VvOMT4* showed the converse trend to *VvOMT3* with a 40% greater level of expression of *VvOMT4* at 6 wpf in the control sample than in the boxed sample (Fig. 5.5B). No significant levels of *VvOMT4* expression were detected following this peak at 6 wpf in berries from any of the treatments. No differences were seen in VvOMT1 expression between the two boxing treatments (Fig. 5.5C).

5.3.4 Effect of sunlight exclusion on IBHP accumulation

IBHP was quantified in the various boxing treatments to investigate whether changes in substrate availability may play a role in the differences seen in IBMP concentrations between the different light exclusion treatments. Interestingly, at all time points, IBHP levels were greater in the boxed berries than in the control berries. However, these differences were only significant ($p < 0.05$) at 8 and 10 wpf (Fig. 5.6A). At 2 wpf the boxed grapes (3.6 $ng.g^{-1}$) already contained twice the amount of IBHP than was observed in the control grapes

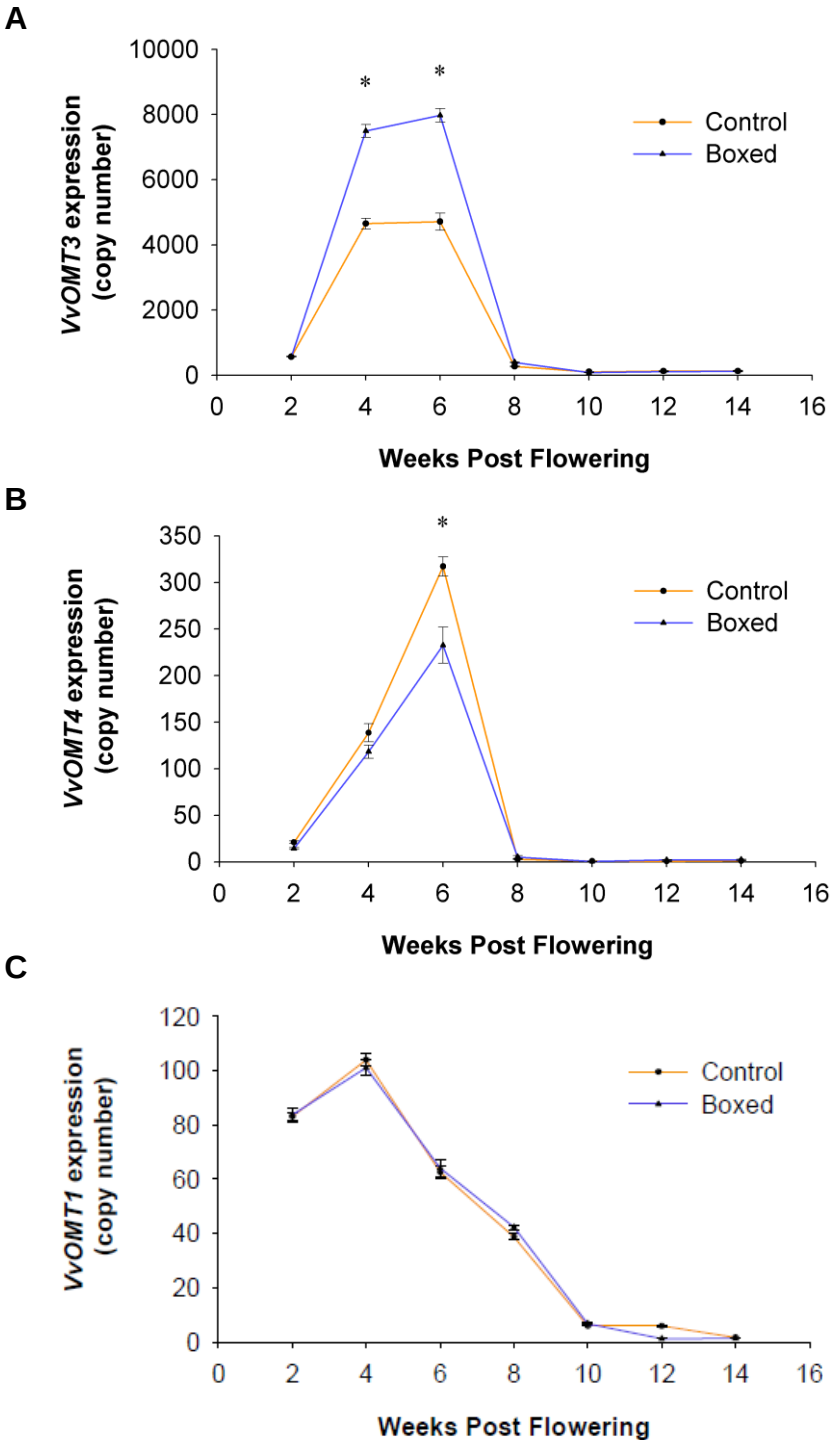


Figure 5.5 - Analysis of the expression of **A**, *VvOMT3*, **B**, *VvOMT4* and **C** *VvOMT1* during the development of CS grape berries subjected to different boxing regimes. Statistically significant differences are denoted by * = $p \leq 0.05$, ** = $p \leq 0.01$ or *** = $p \leq 0.001$.

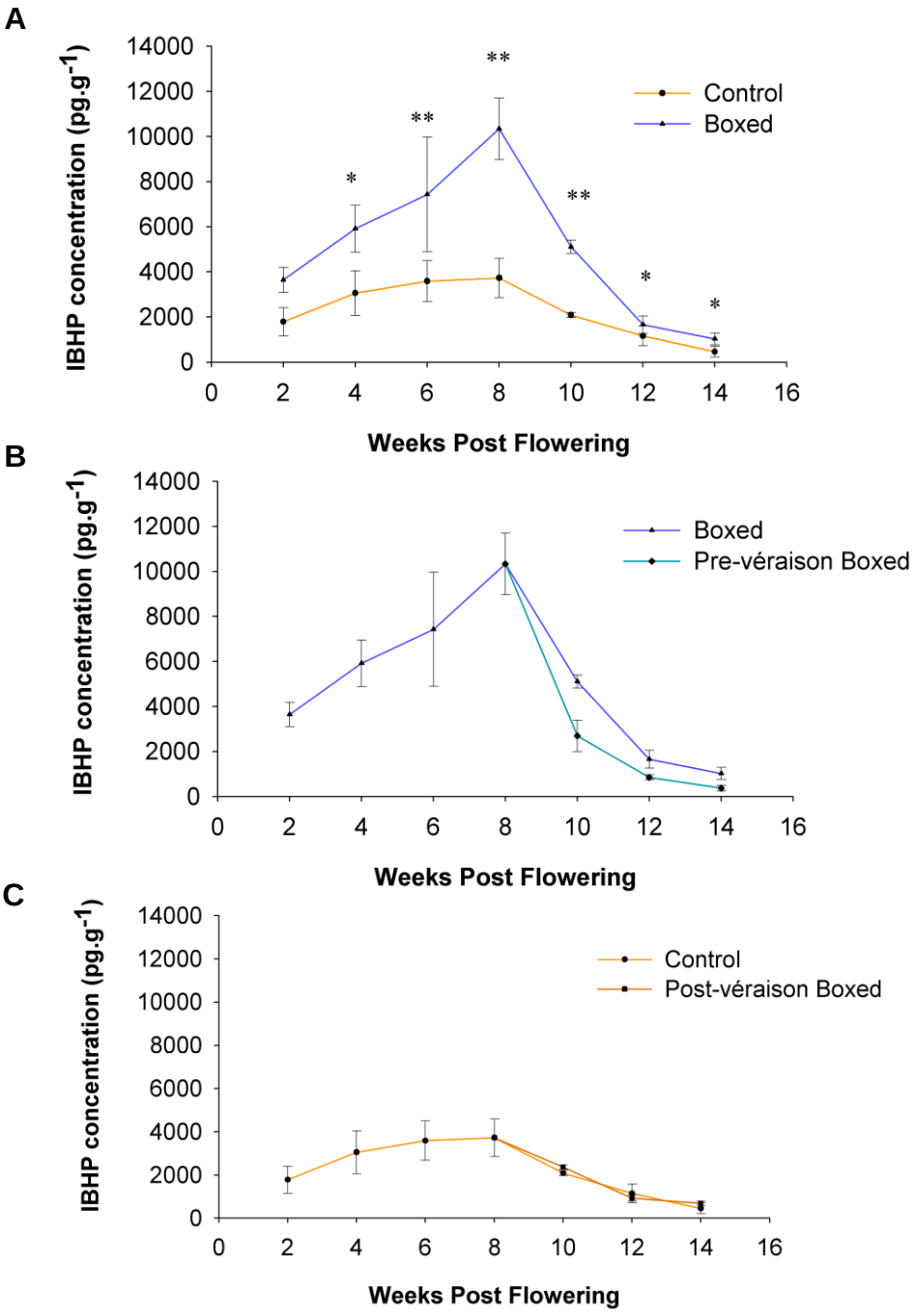


Figure 5.6 - IBHP concentrations measured in berry samples taken during the development of CS grape berries subjected to different boxing regimes. Statistically significant differences are denoted by * = $p \leq 0.05$, ** = $p \leq 0.01$ or *** = $p \leq 0.001$.

(1.8 ng.g^{-1}). By 8 wpf the concentration of IBHP in the boxed sample (10.3 ng.g^{-1}) was approximately 2.5 fold greater than the IBHP concentration in the control sample (3.7 ng.g^{-1}). In the subsequent samples the level of IBHP declined in both treatments, but by 14 wpf the boxed sample (1.0 ng.g^{-1}) still contained twice the concentration of IBHP compared with the control (0.5 ng.g^{-1}).

The removal of boxes at véraison resulted in a lower mean IBHP concentration in berries at harvest (0.4 ng.g^{-1} at 14 wpf) compared to those berries boxed throughout development (1.0 ng.g^{-1} at 14 wpf). However, these differences were not statistically significant (Fig. 5.6B). The addition of boxes at véraison did not have any effect on the concentration of IBHP compared to the un-boxed control treatment (Fig. 5.6C).

5.3.5 Effect of yield on fruit ripening characteristics

The removal of inflorescences at flowering resulted in vines with an average of 35 bunches per vine (low yield) compared to the control vines which had an average of 127 bunches per vine (high yield). The average weight of berries from low yielding vines was significantly ($p < 0.01$) greater than the weight of the berries high yielding vines at most time points, and at harvest were approximately 27% heavier than the berries from the control vines (Fig. 5.7A). Manipulation of crop yield also resulted in a significant change in °Brix measurements with the berries of low yielding vines approximately 5-18% higher in °Brix during the ripening period (Fig. 5.7B). However, by harvest the high yield berries had caught up to the low yield berries with similar °Brix measurements of 23.2 and 22.1 respectively.

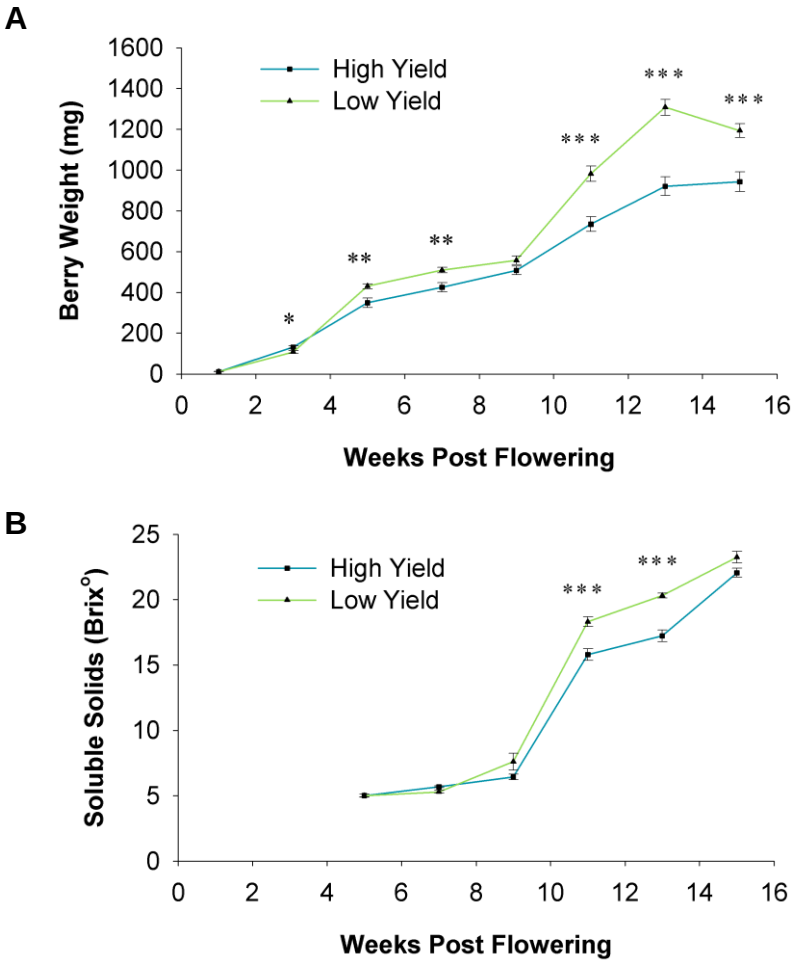


Figure 5.7 - Growth parameters of developing CS grapes on vines with differing crop loads. High yield vines contained an average of 127 bunches and low yield vines contained an average of 35 bunches. (**A**) Berry weight in mg. (**B**) Total soluble solids measured as °Brix. Statistically significant differences are denoted by * = $p \leq 0.05$, ** = $p \leq 0.01$ or *** = $p \leq 0.001$.

5.3.6 Effect of yield on IBMP accumulation

The change in crop load on the Cabernet Sauvignon vines had a significant influence on IBMP concentrations. Accumulation of IBMP peaked at 7 wpf in both treatments with the fruit on the low yielding vines containing 300 $pg.g^{-1}$ compared with 242 $pg.g^{-1}$ in the berries on the high yielding vines (Fig.5.8A). As the grapes ripened the differences between the two treatments diminished with equal concentrations of IBMP at 10 wpf. However, at harvest the grapes of low yield again contained approximately 40% more IBMP than the high yield grapes (36.0 $pg.g^{-1}$ and 25.6 $pg.g^{-1}$ respectively). Because of the large differences in the berry weights of the two treatments, when IBMP content is expressed on a per berry basis the differences between the two treatments is much greater. From 5 wpf through until harvest, the fruit from the low yielding vines contained approximately 19 - 82% more IBMP per berry than those from the high yielding vines (Fig. 5.8B).

5.3.7 Effect of yield on VvOMT *gene expression*

The expression of *VvOMT3* was investigated in the berry samples to determine if changes in the transcript level were responsible for the differences in IBMP concentrations. *VvOMT3* expression was slightly higher in the grapes of high yielding vines although the differences were not significant (Fig. 5.9A). There were no significant differences in the expression of *VvOMT4* between the berries from the high and low crop load treatments (Fig. 5.9B).

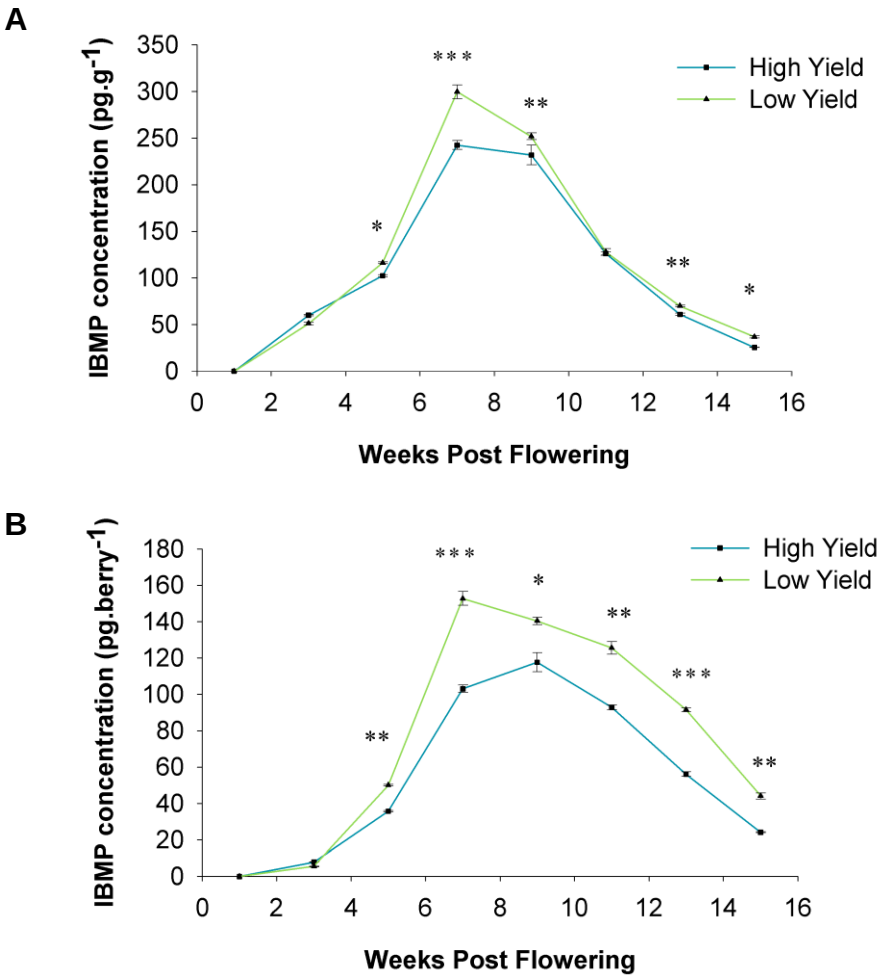


Figure 5.8 - IBMP concentrations in CS grape berries from vines with high or low crop loads. IBMP concentrations are expressed as either pg.g^{-1}of fresh weight (**A**) or pg.berry^{-1} (**B**). Statistically significant differences are denoted by * = $p \leq 0.05$, ** = $p \leq 0.01$ or *** = $p \leq 0.001$.

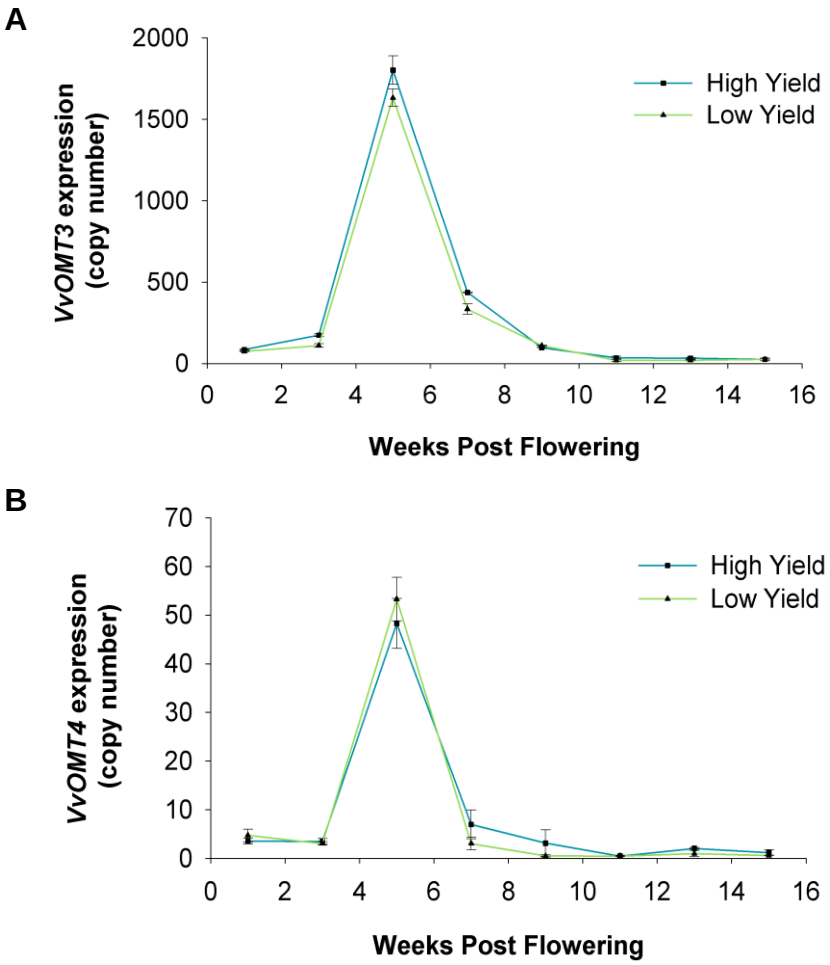


Figure 5.9 - Analysis of the expression of (**A**) *VvOMT3* and (**B**) *VvOMT4* during the development of CS grape berries from vines with high or low crop loads. No statistically significant differences were seen between the two treatments.

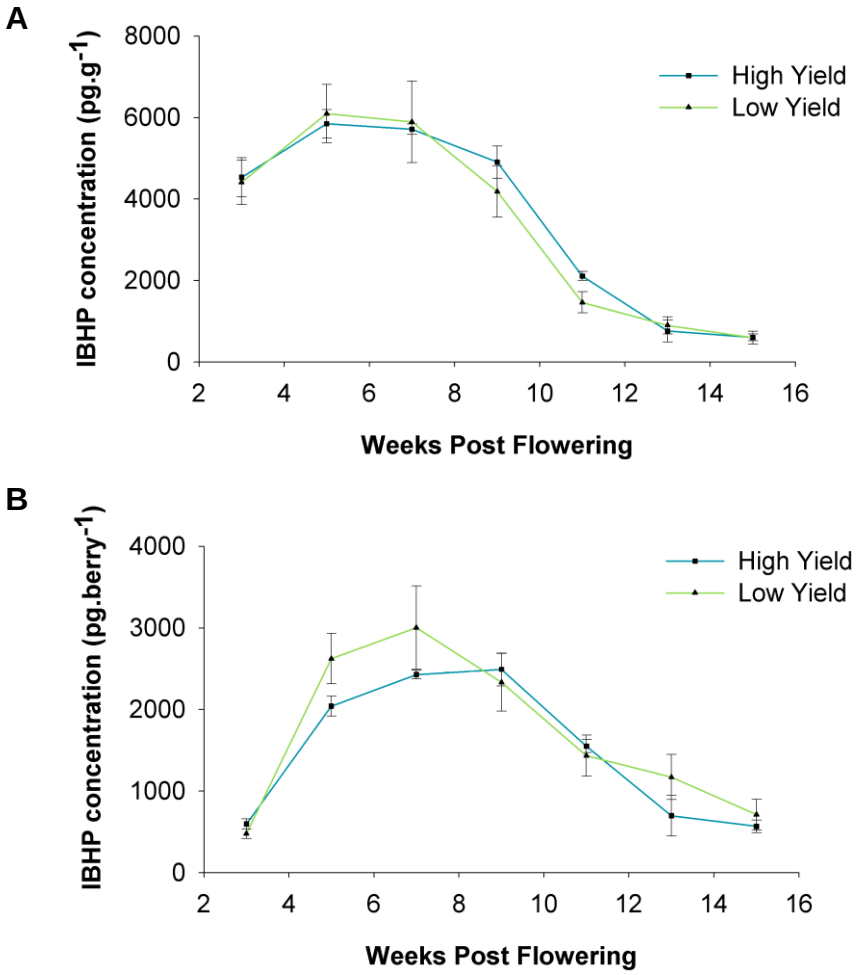


Figure 5.10 - IBHP concentrations in CS grape berries throughout development from vines with high or low crop loads. IBHP concentrations are expressed as (**A**) $ng.g^{-1}$ of fresh weight or (**B**) $ng.berry^{-1}$. No significant differences were observed between the two yield treatments at any time point.

5.3.8 Effect of yield on IBHP accumulation

IBHP levels were measured in berries from each of the crop load treatments to investigate whether differences in substrate levels could account for the increased concentration of IBMP in berries from low yielding vines. However, no significant differences in IBHP concentration were seen between the two treatments at any stage throughout berry development (Fig. 5.10A). When expressed on a per berry basis, the grapes of low yielding vines did appear to have a greater amount of IBHP at 5 and 7 wpf (2.6 and 3.0 ng.berry^{-1} respectively) than berries from the high yielding vines (2.0 and 2.4 ng.berry^{-1} respectively). However, the differences were not significant (Fig. 5.10B).

5.3.9 Expression analysis of VvOMT3 *in the skin, flesh and seeds of CS berries*

In Chapter 2 it was shown that the spatial accumulation of IBMP varied within the different tissues of grape berries, and that *VvOMT1*, which at the time was thought to be responsible for IBMP production, showed a similar pattern of expression to IBMP concentrations in the skin and the flesh during berry development. However, subsequent work suggested that *VvOMT3* and not *VvOMT1* is responsible for IBMP accumulation in grape berries. Therefore, the expression of *VvOMT3* was investigated in the different berry tissues to see if it matches MP accumulation and to see if this expression may explain some of the observations made in the field trials.

As was shown in Chapter 2, the skins contain between 2 and 12 times more IBMP than the flesh, with minimal amounts found in the seeds (Fig. 5.11A). The proportionate weights that each tissue contributes to a whole berry were used to calculate the amount of IBMP located in each tissue of an average berry

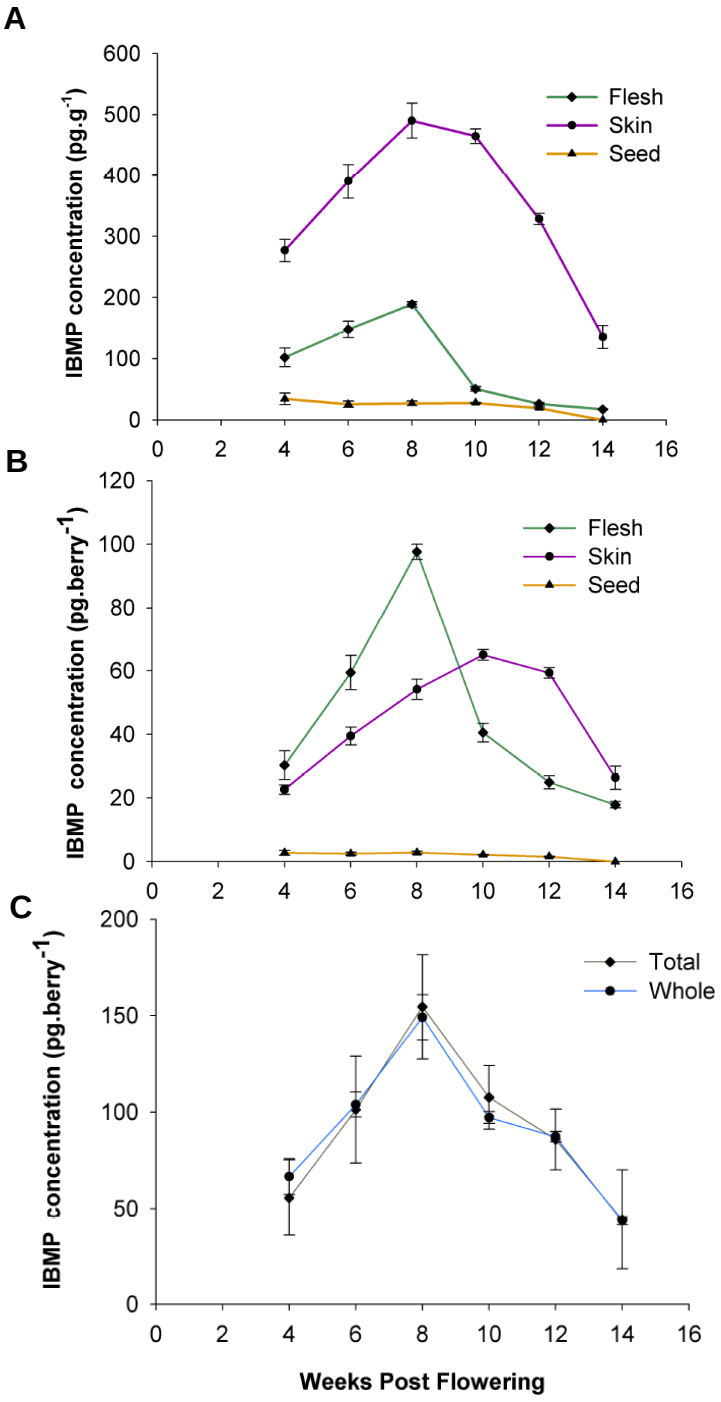


Figure 5.11 - IBMP concentrations in the skin, flesh and seed of developing CS grape berries. IBMP concentrations are expressed as (**A**) $pg.g^{-1}$ of fresh weight or (**B**) $pg.berry^{-1}$. **C** shows the total IBMP in the skin, flesh and seed as calculated using the values from B (Total), compared to the values obtained from a direct analysis of the IBMP concentration in whole Cabernet Sauvignon berries at each time point (Whole).

(Fig. 5.11B). When expressed on a per berry basis, the flesh actually contributes the majority (58-64%) of IBMP pre-véraison, between 4 and 8 wpf. Conversely, during the post-véraison stage of berry development (10-14 wpf) the skin contributes the majority (61-68%) of the total IBMP in the berry. The validity of this data is confirmed when the sum total IBMP content of the skin, flesh and seed is compared with the IBMP content measured using whole berries from the same samples (Fig. 5.11C).

The expression patterns of *VvOMT3* and *VvOMT4* did not match the IBMP concentrations in the different berry tissues with approximately 5 times more expression of both genes in the flesh than the skins and seeds at 4 wpf (Fig 5.12). By 6 wpf the skin and seeds contained no detectable levels of *VvOMT3* or *VvOMT4* while significant levels were found in the flesh. From 8 wpf onwards all tissues lacked expression of either *VvOMT3* or *VvOMT4*.

5.3.10 Analysis of IBHP levels in the skin, flesh and seeds of CS berries

IBHP concentrations were also quantified in the skin, flesh and seed samples to determine if the concentration of IBHP substrate was controlling the pattern of IBMP accumulation in the berry tissues. This did not appear to be the case, as IBHP concentrations were found to be highest in the flesh measured at 5,600 and 5,700 $pg.g^{-1}$ at 4 and 6 wpf respectively (Fig. 5.13A). The concentration of IBHP in the skin was approximately half that of the flesh at these time points (2,200 and 3,170 $pg.g^{-1}$ respectively). By 8 and 10 wpf the concentrations of IBHP were similar in the skin (3,100 and 1,200 $pg.g^{-1}$ respectively) and flesh (2,700 and 1,200 $pg.g^{-1}$). At all time points the seed contained concentrations of IBHP that were below the limit of detection. When expressed on a per berry basis the differences between skin and flesh are compounded, with the flesh containing

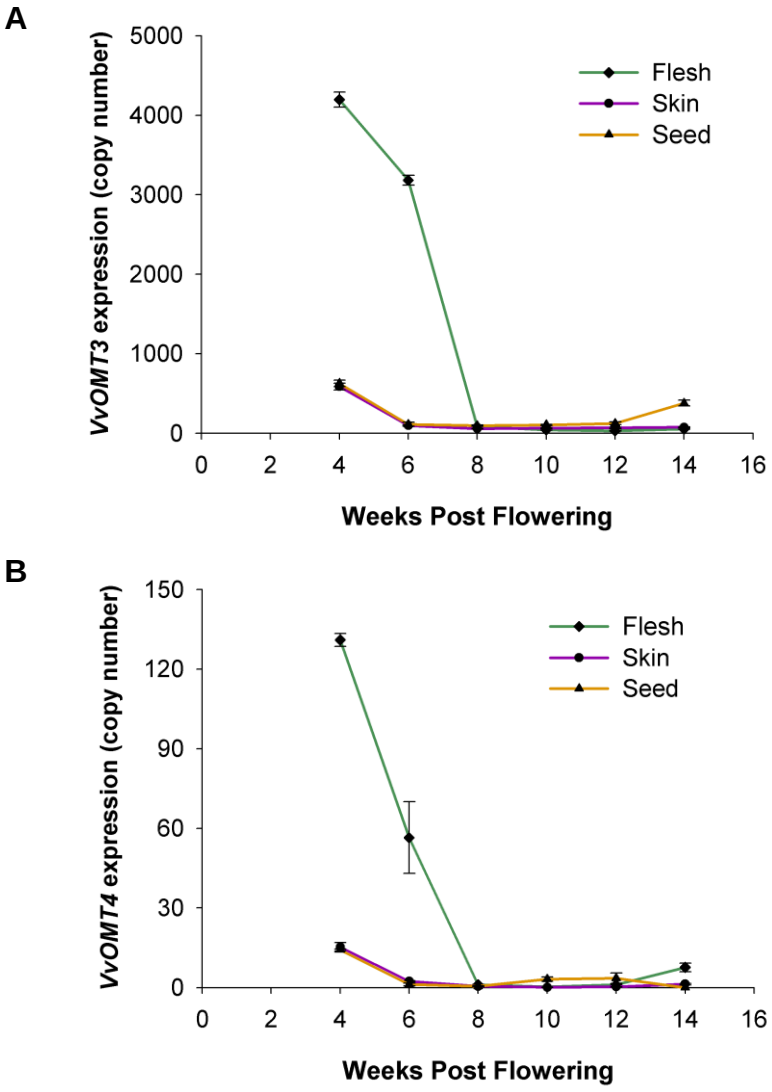


Figure 5.12. The expression of (**A**) *VvOMT3* and (**B**) *VvOMT4* in the skin, flesh and seed of CS grape berries throughout development.

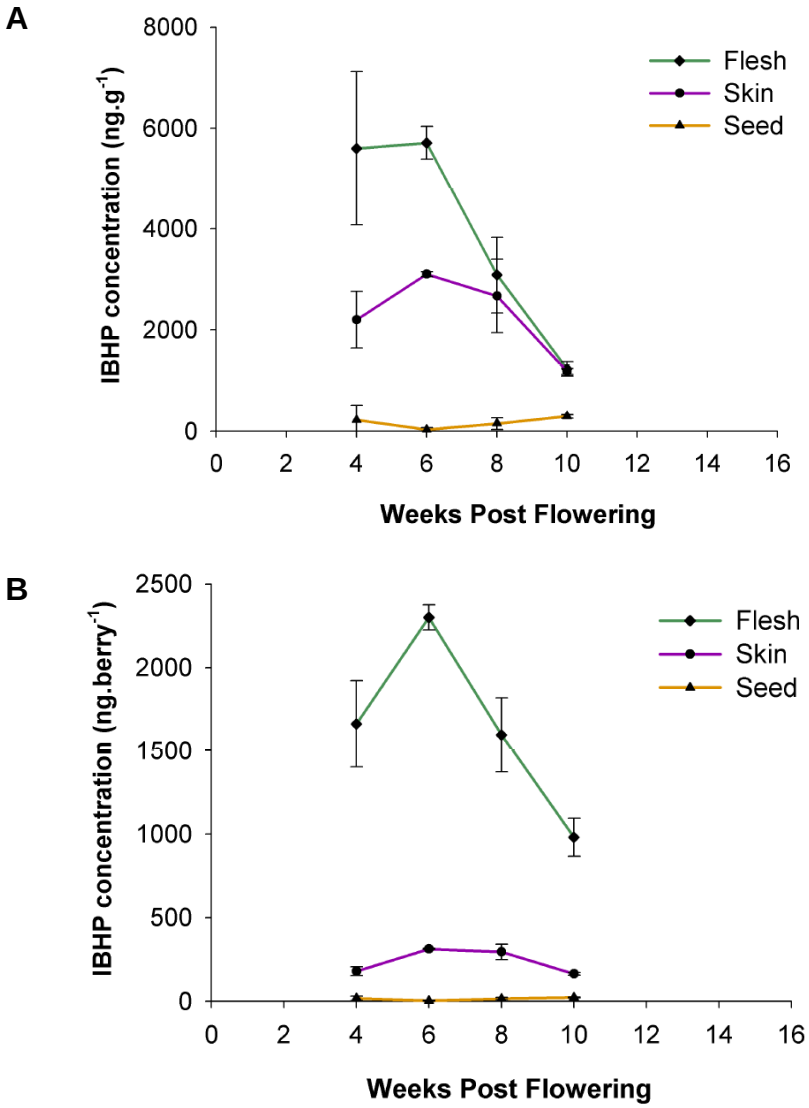


Figure 5.13 - The concentration of IBHP in the skin, flesh and seed at pre-véraison stages of CS berry development. IBHP concentration is expressed as either (**A**) ng.g^{-1} of berry fresh weight or (**B**), ng.berry^{-1}.

85-90% of the total IBHP in the berry between 4-8 wpf, when the majority of IBMP is synthesised (Fig. 5.13B).

5.4 Discussion

5.4.1 Influence of sunlight exclusion on the biosynthesis of IBMP

The exclusion of sunlight though the application of opaque boxes dramatically increased the accumulation of IBMP in grape berries (Fig. 5.3A). The effect of light exclusion on IBMP levels was far greater during pre-véraison stages of development, when synthesis of IBMP is thought to occur, than it was post-véraison, when degradation/metabolism is thought to occur. This finding is consistent with two recently published studies which both found that the manipulation of the vine canopy to alter sunlight exposure has the greatest effect on IBMP levels in berries pre-véraison (Ryona *et al.*, 2008; Scheiner *et al.*, 2010). The authors of these studies proposed that sunlight exposure reduces the biosynthesis of IBMP rather than increasing the photo-degradation of the compound, which had previously been thought to be the mechanism responsible for the effect of sunlight on IBMP levels (Ryona *et al.*, 2008). Indeed the level of *VvOMT3* expression, which the data presented in Chapters 3 and 4 suggests is the gene responsible for IBMP biosynthesis in grape berries, was significantly increased by the exclusion of sunlight in this study (Fig. 5.5A). This finding provides further evidence that the influence of sunlight exposure on IBMP concentrations acts predominantly through changes in the biosynthesis of the compound. Interestingly, the opposite response (i.e. down-regulated when light was excluded) was seen when the expression of *VvOMT4* was analysed in these samples (Fig. 5.5B). This suggests that the two OMT genes, which are separated

by less than 2,000 base pairs on chromosome 3, are under the control of separate transcriptional activators.

Although the influence of sunlight exposure on IBMP concentrations was greatest pre-véraison, the removal or addition of boxes at véraison had significant yet lesser effects on IBMP concentrations post-véraison (Fig. 5.4). As *VvOMT3* was not expressed post-véraison (Fig. 5.5A), it appears that the influence of sunlight on IBMP levels during these stages of berry development is through changes in degradation or metabolism of IBMP, rather than through biosynthesis.

Although the reduction in IBMP concentration was slowed by the boxing of grape bunches, there was still a large decline in the amount of IBMP per berry even in the absence of light (Fig. 5.3B). This finding implies that photo-degradation only accounts for a small percentage of the total decrease in IBMP concentration in post-véraison grapes. Therefore, other mechanisms of IBMP loss are likely to be responsible for the large reduction in concentration during berry ripening. Possible mechanisms include further metabolism or, alternatively, chemical degradation due to changes in the metabolic activity or chemistry of the ripening berry. It is also possible that the decline in IBMP content is due to volatilization of the compound out of the berry. This could be determined in the future by sealing in a container growing grape bunches and an absorbing solid phase chromatography fiber to detect any release of MPs.

Interestingly, IBHP concentrations in berries responded to sunlight exclusion in a similar way to IBMP, with two-fold more IBHP present in the boxed samples at all time points (Fig.5.6). From these data it cannot be determined if this change is due to an increase in biosynthesis or a decrease in the degradation or metabolism of IBHP. However, given the structural similarities between IBHP and IBMP it is possible that they both behave similarly in regards

to photo-decomposition. It is also possible that there is a feed-forward effect on the expression of *VvOMT3* by IBHP, which stimulates increased production of IBMP when more IBHP is present.

It has previously been proposed that the effect of sunlight exposure on IBMP levels may actually be due to changes in the thermal heating of the berry (Ryona *et al.*, 2008). Although the boxes used in this study have no influence on the air temperature surrounding the grape bunch, the shielding of bunches from the sunlight and solar radiation may have indeed resulted in less thermal heating of the berry. Future studies should test this hypothesis. For example, it may be possible to encase bunches in clear Perspex boxes which permit the heating of berries, but block UV rays, to help determine the nature of the stimulus that leads to increased IBMP accumulation in pre-véraison berries. Other coloured plastics or Perspex could be used to determine if the stimulus involves light quality or certain wavelengths of light. The use of temperature controlled growth chambers could also be a valuable resource for determining the individual effect of temperature on IBMP biosynthesis, accumulation and metabolism.

In Chapter 1 it was proposed that grapevines may have evolved the means to produce IBMP in immature fruits as an odorant signal to deter potential seed dispersers, and this signal then declines as the berry matures. The increase in IBMP biosynthesis in the boxed samples may be in keeping with this theory. Grapevines grown in low light conditions generally ripen slower than those grown under high light conditions (Morrison and Noble, 1990; Rojas-Lara and Morrison, 1989), and so grapevines may have evolved a mechanism to increase IBMP production in low light conditions to ensure that the concentration of IBMP persist at perceivable levels until the grape berry is fully matured. This hypothesis also appears to hold true for temperature, as low temperatures during grape ripening

also generally result in slower maturation (Parra *et al.*, 2010; Chuine *et al.*, 2004) and increased IBMP concentrations (Allen *et al.*, 1994; Belancic and Agosin, 2007; Heymann and Noble, 1987; Lacey *et al.*, 1991). Future experiments on wild species of grapevine will help establish if the phenomenon of IBMP production in immature fruit is a common aspect of natural grape populations and has been selected for or against by humans during the domestication of the grape.

5.4.2 Influence of yield on the biosynthesis of IBMP

CS vines of low crop yield were found to produce grapes with higher IBMP concentrations than high-cropping vines (Fig. 5.8), consistent with previous findings (Chapman *et al.*, 2004). However, the effect of yield manipulation on IBMP content was not as large as the effect observed by the elimination of light exposure on the bunches. Unlike sunlight exposure, neither *VvOMT3* expression (Fig. 5.9) nor IBHP concentrations (Fig. 5.10) were significantly different in the berries from the yield treatments and, therefore, do not appear to be responsible for the differences in IBMP concentrations. It is unlikely that changes in sunlight exposure were responsible for the differences in IBMP as there were no visual differences in the canopy structure of the two treatments and, furthermore, this would be expected to change the abundance of the *VvOMT3* transcript, which was not seen (Fig. 5.9A).

The manipulation of yield did have a significant influence on both berry weight and the initial rate of sugar accumulation during ripening (Fig. 5.7), suggesting significant differences in the primary metabolism of the grapes under the two yield regimes. Changes in fruit yield ultimately alters the ratio between the berries and the leaves, which are the source of the majority of the berries

primary metabolites (Conde *et al.*, 2007). A key indicator of primary metabolism in grape berries is malic acid, which accumulates at high concentrations in the vacuoles of berries pre-véraison. Just prior to the onset of véraison, malic acid is released into the cytosol to act as the major carbon source (Sweetman *et al.*, 2009; Terrier *et al.*, 2001), which result in major physical and biochemical changes in berry cells (Conde *et al.*, 2007; Terrier *et al.*, 2001; Zhang *et al.*, 2006). A number of authors have noted that the decline in IBMP levels just prior to véraison coincides with the decline in malic acid levels (Allen *et al.*, 1991; Roujou de Boubee *et al.*, 2000; Ryona *et al.*, 2008). Therefore, it seems likely that the major changes that occur in the cellular metabolism of the berries at this stage are somehow linked with the decline in IBMP concentrations. It would be interesting to investigate this decline or metabolism of IBMP and so future studies should be conducted in which ripening grape berries are fed deuterium labeled IBMP and the potential breakdown products identified using a combination of gas and liquid chromatography. This may help to identify the mechanism that leads to the reduction in IBMP levels and help determine the timing of this reduction to see if it can be linked to the major shift in malic acid metabolism observed in grape berries. Another possible reason behind the differences in IBMP concentrations caused by yield effects is that changes in primary metabolism alter the stability of the VvOMT3 enzyme or the regulation of its translation. Future studies could test these scenarios by developing antibodies to the VvOMT3 enzyme and determining its abundance due to different viticultural treatments.

As mentioned in Chapter 4, it is likely that the VvOMT3 enzyme possesses activity against numerous substrates, which is typical of Class II OMTs (Lam *et al.*, 2007). Manipulation of fruit yield may result in changes in the concentrations of any potentially competitive substrates and subsequently alters

the flux of IBHP to IBMP. However, given the extremely low K_m observed for VvOMT3 against HPs, it would appear that a high concentration of any competitive substrate would be required to affect the binding of HPs to the active site of VvOMT3.

The compound SAM not only acts as the methyl donor for IBMP biosynthesis but is also a substrate in the synthesis of ethylene, polyamines and other methyl acceptor molecules (Adams and Yang, 1977; Grillo and Colombatto, 2008). In plants the biosynthesis of SAM is dependent on ATP (Moffatt and Weretilnyk, 2001) and so it is possible that by increasing yield this decreases the availability of SAM in the berries leading to the reduction seen in IBMP concentrations. This hypothesis could be tested in the future as a method for quantifying SAM in fruit has recently been developed (Van de Poel *et al.*, 2010).

5.4.3 IBMP biosynthesis in different berry tissues

In Chapter 2 it was shown that the majority of IBMP accumulation occurs in the skins, consistent with previous findings (Roujou de Boubee *et al.*, 2002). The expression of *VvOMT3* was analysed in these samples to determine if its expression pattern coincided with the high levels of IBMP present in the skins. However, *VvOMT3* was found to be predominantly expressed in the flesh, with less transcript detected in the skins and only at 4 wpf (Fig. 5.12A). The flesh was also found to contain approximately twice the amount of IBHP than in the skins in pre-véraison berries when biosynthesis of IBMP occurs (Fig. 5.13A). Therefore, differences in either *VvOMT3* expression levels or IBHP concentrations fail to explain why the skins of CS grapes contain the greatest concentrations of IBMP. To eliminate the effects of dilution and changes in skin to flesh ratio as the berry expands, calculations were made to determine how much each tissue contributes

to the total amount of IBMP and IBHP in the whole berry. These data show that pre-véraison the majority of IBMP actually accumulates in the flesh (Fig. 5.11B), consistent with the high levels of *VvOMT3* expression in this tissue. However, following véraison the amount of IBMP in the flesh decreases while the amount in the skins remains relatively high, until 12 wpf when IBMP concentrations decline rapidly (Fig. 5.11B). These results imply that IBMP is synthesised in the flesh and is subsequently transported or diffuses into the skin. The level of IBMP that is synthesised could also be affected by differences in the translation or activity of VvOMT3, differences in the concentrations of SAM or postulated competitive substrates. As mentioned above, each of these factors should be examined in the future to gain a greater understanding of variables that impact IBMP biosynthesis in grape berries.

It appears that the decrease in IBMP content is greater in the flesh than in the skin of the berry (Fig. 5.11B). As mentioned above, the decline in IBMP coincides with major biochemical chemical changes associated with the primary metabolism of the berry. The majority of primary metabolites (malic acid and the sugars, glucose and fructose) that are involved in cellular respiration accumulate primarily in the flesh (Conde *et al.*, 2007; Coombe, 1987). This could explain why IBMP decreases in the flesh at this stage but does not decrease in the skin, but, at present, the mechanisms causing the reduction in IBMP concentration in these tissues remain unknown. The measurement of primary metabolites such as malic acid in the samples from the different treatments may provide some insight into IBMP metabolism/degradation processes.

Despite the uncertainties surrounding the post-véraison decline in IBMP concentrations, the key factors that influence IBMP biosynthesis are now clear. Furthermore the results of this chapter confirm that sunlight exposure influences

IBMP accumulation by reducing its biosynthesis, which is a direct result of reduced *VvOMT3* expression.

Chapter 6 - Conclusion

MPs are a family of potent volatile grape-derived flavour compounds that can impart green/vegetative characters to wines of certain varieties including CS. Depending on the intensity of the flavours imparted by MPs they are considered to either add varietal complexity to wine or are overpowering and detrimental to its overall quality. The impact MPs have on the sensory attributes of wine depends on their concentration, although other compounds can have a masking effect and may allow greater amounts to be tolerated in some instances (Hein *et al.*, 2009). MP concentrations in wine are, in turn, dependent on the abundance of these compounds in the grapes (Kotseridis *et al.*, 1999; Roujou de Boubee *et al.*, 2002; Ryona *et al.*, 2009). The concentrations of MPs in grape are known to be influenced by a number of environmental stimuli (Allen *et al.*, 1994; Belancic and Agosin, 2007; Chapman *et al.*, 2004; Ryona *et al.*, 2008), however little information exists on the biochemical pathway of MP biosynthesis or the metabolism and degradation of these compounds in grapes. A previous study has shown that the final step in MP biosynthesis is catalysed by an OMT enzyme (Hashizume *et al.*, 2001a). OMTs are a class of enzymes that catalyse the methylation of a wide array of secondary metabolites in plants, and the grapevine genome contains at least 56 unique OMT sequences (da Silva *et al.*, 2005). The primary aim of this study was to identify the OMT gene(s) responsible for the final step in MP biosynthesis in grape berries as the first step in developing an in-depth understanding of the major factors that influence MP accumulation in grape berries.

In a study published in 2001, a protein was purified from CS shoots with the ability to methylate HP substrates forming MPs, and a 22 amino acid sequence of the protein's N-terminus was obtained (Hashizume *et al.*, 2001b). Chapter 2 describes the identification of the gene *VvOMT1* which has 100 % identity to this 22 amino acid N-terminal sequence, while a second gene, *VvOMT2* was also identified showing 87% sequence identity to *VvOMT1*. The initial hypothesis of this study was that one or both of these genes were responsible for the OMT activity responsible for MP biosynthesis in grape berries. Indeed, *in vitro* functional analyses of recombinant VvOMT1 and VvOMT2 revealed they both have activity against HP substrates. Furthermore, transcript analysis showed that *VvOMT1* is highly expressed during the pre-véraison stages of grape berry development when MP biosynthesis occurs, suggesting that *VvOMT1* plays a role in MP accumulation in grape berries.

However, partial silencing of both *VvOMT1* and *VvOMT2* in grapevine hairy-roots did not result in a reduction of MPs suggesting that they are not solely responsible for MP biosynthesis in grapevine roots. While the silenced hairy-root lines did not display full down-regulation of the *VvOMT1* and *VvOMT2* genes, these results raised doubts about their role in the production of MPs in berries as well. This work demonstrates the need for conclusive *in planta* evidence when attempting to determine gene function. While the determination of *in vitro* activity and expression profiling can provide clues to the function of a gene, this information is not sufficient to prove gene function and may be misleading. The need for *in planta* evidence is especially true for OMT genes which are abundant in plant genomes and which encode proteins that often possess *in vitro* activity against a wide range of substrates creating numerous possibilities for *in planta* function.

The mapping of a gene to a trait provides extremely strong evidence of gene function. In grapevines, certain physiological characteristics (for example, length of life cycle, juvenility and plant size) have previously hindered transformation and mapping experiments commonly employed in other plant species to obtain *in planta* evidence of gene function. However, the discovery of the grapevine dwarf derived from Pinot Meunier (Boss and Thomas, 2002) has provided a model plant for grapevine genetic studies. Phenotypically, the PM dwarf was also of interest in this study because it does not accumulate MPs in the berries, which allowed a population segregating for this trait to be generated via crosses with CS. The mapping work presented in Chapter 3 demonstrates the effectiveness of the PM dwarf as a valuable new tool for identifying genes responsible for phenotypic traits. The combination of linkage mapping and directed association mapping could potentially allow the identification of major genes and QTLs responsible for many other grapevine traits. As well as providing a tool for genetic research, the short generation time of the PM dwarf could be valuable for increasing the speed of traditional breeding programs to create new wine grape varieties.

The linkage and association mapping results clearly showed that neither *VvOMT1* nor *VvOMT2* were responsible for MP biosynthesis in berries. However, strong evidence was generated that suggests the gene *VvOMT3* is responsible for IBMP accumulation in CS berries. *VvOMT3* was identified within a 2.3 Mb locus which segregated with the dominant trait of IBMP-accumulation in an F2 population resulting from a cross between CS and the PM dwarf. Furthermore, a marker located within the *VvOMT3* gene was closely association with the trait of IBMP-accumulation in the berries of 91 grapevine cultivars.

In Chapter 4, recombinant VvOMT3 was found to have methylating activity against HP substrates *in vitro*, which was 500 - 5000 fold greater than that of VvOMT1 and VvOMT2. Additionally, the timing of *VvOMT3* expression coincided with the biosynthesis of MPs during CS berry development and *VvOMT3* was not expressed in the berries of Pinot varieties, which is likely to be the reason why IBMP does not accumulate in Pinot grapes.

The method of MP quantification used in this study did not permit the quantification of IPMP or SBMP in all samples as they were below the limit of detection. Consequently it is prudent not to conclude that *VvOMT3* is also responsible for IPMP or SBMP accumulation in certain grape varieties. Recently, more sensitive methods of MP quantification have been published (Lopez *et al.*, 2011; Schmarr *et al.*, 2010) which may enable the quantification of IPMP and SBMP in the grape samples used in these mapping studies to determine if *VvOMT3* expression is also associated with the biosynthesis of these MPs in berries.

In Chapter 5, the expression of *VvOMT3* was found to be enhanced by the elimination of sunlight exposure to CS grape berries, resulting in significantly increased concentrations of IBMP in such treated berries. The results of this study also implied that factors other than light are responsible for the majority of the reduction in IBMP concentrations in post-véraison berries. During the pre-véraison stage of berry development, the majority of IBMP was located in the flesh, coinciding with the location of greatest *VvOMT3* expression and IBHP concentrations. However, after véraison the levels of IBMP in the flesh rapidly declined, while the levels in the skin did not peak until 10 wpf and contributed the majority of the IBMP in berries at harvest, consistent with previous findings (Roujou de Boubee *et al.*, 2002).

MPs have previously been proposed to act as deterring odorants in warningly coloured plants and insects (Guilford *et al.*, 1987; Rothschild *et al.*, 1984; Siddall and Marples, 2011). It is possible that MPs have a similar function in unripe grapes, by deterring organisms that may eat the fruit before the grapes are fully matured. The finding that elimination of sunlight results in increased *VvOMT3* expression and IBMP biosynthesis appears to support this hypothesis, as low sunlight is associated with longer ripening times (Morrison and Noble, 1990; Rojas-Lara and Morrison, 1989). Therefore, under these conditions, it would be of benefit to the grapevine to have increased levels of IBMP for them to remain perceivable throughout the lengthened ripening stage and only decrease when the seed is ready to be dispersed. Interestingly, grapevine roots were found to contain high levels of MPs, which could imply that in this tissue they act to deter herbivorous insects or other predators that feed on grapevine roots. A recent study found that IPMP and SBMP are present at high concentrations (120 and 230 $ng.g^{-1}$ respectively) in the roots of *Gynura bicolor*, an edible plant native to tropical Asia (Shimizu *et al.*, 2010). As MPs, and IPMP in particular, are often described as having sensory characteristics that are "earthy" or "root-like" it is plausible that the presence of MPs in roots may be widespread in the plant kingdom, and this warrants further investigation.

To date there has been very little commercial success in the breeding of new grape varieties for wine production, in part due to the dominance of a handful of traditional wine grape cultivars, but also due to the difficulties associated with grapevine breeding. However, the recent sequencing of the grapevine genome (Jaillon *et* al., 2007; Velasco *et al.*, 2007), together with the identification of large numbers of SNPs between different grape cultivars (Lijavetzky *et al.*, 2007; Troggio *et al.*, 2007) and the development of the PM dwarf, means the assigning

of grapevine genes or QTLs to phenotypic traits of economic importance should become more common in the future. The use of high-throughput molecular marker screening targeted to important genes and QTLs will enable the breeding of new varieties specifically tailored to suit certain needs (Troggio *et al.*, 2008; Myles *et al.*, 2011). As mentioned in Chapter 3, some major genes or QTLs have already been identified in grapevines for important traits such as disease resistance, flower sex, berry colour, fleshlessness, seedlessness and monoterpene content (Battilana *et al.*, 2009; Dalbo *et al.*, 2000; Donald *et al.*, 2002; Fernandez *et al.*, 2006; Lijavetzky *et al.*, 2006; Mejia *et al.*, 2007). The identification of *VvOMT3* as the major gene responsible for IBMP accumulation is an important step towards the development of another genetic marker that will be of major benefit to current and future breeding programs, by allowing the screening of grapevine seedlings for the trait of IBMP-accumulation in berries. However, before a definitive marker can be attributed to IBMP accumulation, the genetic variation responsible for the suppression of *VvOMT3* transcription needs to be determined. Preliminary evidence presented in Chapter 4 suggests that, in Pinot varieties, the lack of IBMP production may be due to the presence of a large MULE transposon immediately downstream of the *VvOMT3* coding region. Future work investigating the presence of this transposon in different grapevine varieties will not only provide the evidence for its involvement in suppressing *VvOMT3* expression and reducing IBMP biosynthesis, but will also provide a valuable case study for understanding the involvement of transposable elements in genetic and phenotypic variability in grapevines.

The identification of *VvOMT3* as the major gene responsible for IBMP accumulation in grapes is not only beneficial to breeding programs, but will also benefit future studies investigating the effect of environmental and viticultural

practices on IBMP accumulation. In this study it was found that sunlight exposure affects IBMP accumulation predominantly through changes in *VvOMT3* expression. Other factors such as temperature and water status are also thought to influence IBMP concentration in berries, however, the mechanism for this action is unknown. In future studies, the quantification of *VvOMT3* expression and IBHP concentration will provide a means to gauge the potential for IBMP biosynthesis in a sample and allow the distinction between degradation/metabolism and synthesis when investigating the influence of variables on IBMP accumulation in berries. This information will ultimately provide grape growers with a greater ability to manipulate the IBMP content of grapes at harvest.

Appendix A - Primer sequences

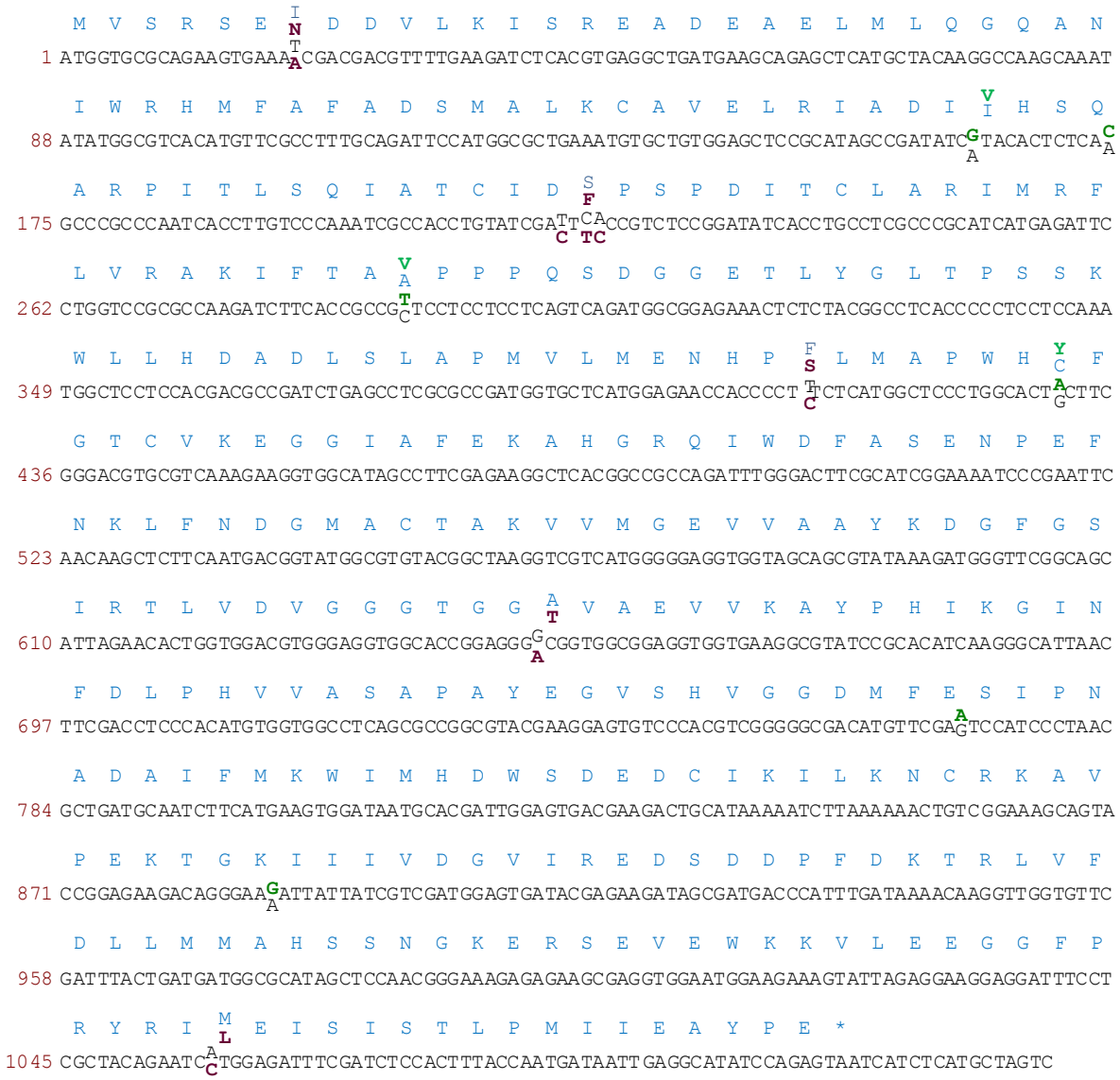
Names and sequence of oligonucleotide primers used throughout this study.

Primer name	Sequence 5'-3'
Gene amplification	
*MT1*1F1	ATGGTGAGCAGAAGTGAAAT
*MT1*1R1	GACTAGCATGAGATGATTAC
*MT2*F1	ATGGTGGGCACAAGTGAAAA
*MT2*R1	ACACGATGTAGATTACATCT
*MT3*F1	GGTTGAGATATTTCCTTGAAC
*MT3*R1	CTAAGGATAAGCTTCCATGACA
*MT4*F1	TCTTAGTGAAGAGTGAACAT
*MT4*R1	AATTTAGAGGGATTTACCTTGCGAT
qPCR quantification	
*MT1*RTF1	GGCCTCAGCGCCGGCGTACG
*MT1*RTR1	TCTCTTTCCCGTTGGAGCT
*MT2*RTF1	TCCGAGAAGATGGCTATGAG
*MT2*RTR2	CTGCAAAGTTGGAATCTTTAA
*MT3*RTF1	CAATCTTAAAGACGTGGGGT
*MT3*RTR1	CTAAGGATAAGCTTCCATGACA
*MT4*RTF1	TTTCATCATGAAAGTCCTACA
*MT4*RTR1	TTTGCCTGTAGTAGTTGC
*MT3*RTPNF1	GGCAACAATCTTAAAGACGT
*MT3*RTPNR1	AAACGAAAAAGAAGGTGGCA
UbiqF1	GTGGTATTATTGAGCCATCCTT
UbiqR1	AACCTCCAATCCAGTCATCTAC
ActinF1	GCATCCCTCAGCACCTTCCA
ActinR1	AACCCCACCTCAACACATCTCC
ELF1F1	CGGGCAAGAGATACCTCAAT
ELF1R1	AGAGCCTCTCCCTCAAAAGG
Vector cloning	
*MT1*F1BamHI	GCGGATCCATGGTGAGCAGAAGTG
*MT1*R1XhoI	GCTCGAGGCATGAGATGATTAC
*MT2*F1BamHI	GCGGATCCATGGTGGGCACAAGT
*MT2*R1XhoI	GCTCGAGCGATGTAGATTACATC
*MT3*F1BamHI	GCGGATCCATGGAGAAAGTGGTA
*MT3*R1XhoI	GCTCGAGCTAAGGATAAGCTTCG
*MT4*F1BamHI	GCGGATCCATGACAGAGGCAACGAGG
*MT4*R1XhoI	GCTCGAGAGGGATTTACCTTGCG
T7 promoter	TAATACGACTCACTATAGG
T7 terminator	GCTAGTTATTGCTCAGCGG

Primer name	Sequence 5'-3'
M13 forward	GTAAAACGACGGCCAGT
M13 reverse	CAGGAAACAGCTATGACC
attbMTF1	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGAGCTCAT GCTACAAGGCC
attbMTR1	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTGGAGGA GCCATTTGGAG
CAPS and dCAPs markers	
VvChrom1TF1	CAACATCTCACTCTCCCACAC
VvChrom1TR1	AACTCAACAACATCCCCTTTC
VvChrom1BF2	TTGCAAGAATCCTACCATA
VvChrom1BR2	AACACTGCAGAAAGAAGTATGGAGCAGC
VvChrom2TbF1	GATTGCTCAGCGGCTTACTT
VvChrom2TbR1	GGTCAACATGGACTTATTTTGGT
27461F1	GATTCAGTGTAGTTTTTGTA
27461R1	TTTCTTGATTTCTTGAGAGG
VvChrom3@1.6F1	GCGAAGGAAAGGAAGAAGAAG
VvChrom3@1.6R2	CGATCTTAAACCACAATCACTGAAAATGCGTAAGC
VvChrom3@1.9F3Ear	CCTTCTCTTTGAGCACTTCCCAACTAATGC
VvChrom3@1.9R1	TGAAACTTCAAATTGTGTAA
Vv23989exon1F1	TCTTAGTGAAGAGTGAAGAT
Vv23989exon1R1	CATGATGAAAACAGCATCAG
VvChrom3@2.21F1NcoI	AAGTGTGCCATTGAGCTCGGCATAGCTGATGCCAT
VvChrom3@2.21R1	GCGTTCCCTTTCCCTAGAAG
8789F1	CTTTCACTCGGATTCGGACG
8789R1	TAGCCGTAATTTCCACCACC
12860F2	TGTAGGGCAGTCAATTCTAG
12860R2	AGGCAAAAGGGGAATGTTGG
VvChrom3@2.8F3	TACCACGCGTTCACCAGCGGGTTCGATGAC
VvChrom3@2.8R3	ACGGAATCTACCTGATTCTG
VvChrom3TF1	AAATATAACCAACCGAAGCA
VvChrom3TR1	AATGAAAAAGGCAGCATAGCA
VvChrom3BF1	CCTCCGCTACCACCATAAAC
VvChrom3BR1	ACCAACTCAAAATTATAGATGCCGTCGA
VvChrom6TF1	GGCTTGAGGGACAGCTAATC
VvChrom6TR1	GTGCCATATCTTGGGAACATC
VvChrom16TF1	GGGAGGCAAAATGTGTTGTT
VvChrom16TR1	TGAAGTCCCACCGATTAAGG

Appendix B - VvOMT gene and protein sequences

VvOMT1. Nucleotide and predicted amino acid sequence of the *VvOMT1* coding region amplified from CS and PN cDNA. CS and PN each share one common allele and each posses a unique allele. Differences present in the unique CS allele are highlighted in green and differences present in the unique PN allele are highlighted in purple.



VvOMT2. Nucleotide and predicted amino acid sequence of the *VvOMT2* coding region amplified from CS cDNA.

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    M  V  G  T  S  E  N  G  D  V  L  K  V  S  S  E  A  D  E  T  E  L  M  L  Q  G  Q  A  N
1   ATGGTGGGCACAAGTGAAAACGGCGACGTTTTGAAGGTCTCGTCTGAGGCCGATGAAACAGAGCTCATGCTACAAGGCCAAGCAAAT
    I  W  R  H  M  F  A  F  A  D  S  M  A  L  K  C  A  V  E  L  R  I  A  D  I  I  H  S  H
88  ATATGGCGTCACATGTTCGCCTTTGCAGATTCCATGGCGCTGAAATGTGCTGTGGAGCTCCGCATAGCCGATATCATACACTCTCAC
    A  R  P  I  T  L  S  Q  I  A  T  C  I  D  S  P  S  P  D  I  T  C  L  A  R  I  M  R  F
175 GCCCGCCCAATCACCTTGTCCCAAATCGCCACCTGTATCGATTCACCGTCTCCGGATATCACCTGCCTCGCCCGCATCATGAGATTC
    L  V  R  A  K  I  F  T  A  A  P  P  P  Q  S  D  G  G  E  T  L  Y  G  L  T  P  S  S  K
262 CTGGTCCGCGCCAAGATCTTCACCGCCGCTCCTCCTCCTCAGTCAGATGGCGGAGAAACTCTCTACGGCCTCACCCCCTCCTCCAAA
    W  L  L  H  D  A  E  L  S  L  A  P  M  V  L  M  E  N  H  P  S  L  M  A  P  W  H  C  F
349 TGGCTCCTCCACGACGCCGAGCTGAGCCTCGCGCCGATGGTGCTCATGGAGAACCACCCCTCTCTCATGGCTCCCTGGCACTGCTTC
    G  T  C  V  K  E  G  G  I  A  F  E  K  A  H  G  H  Q  I  W  D  L  A  S  E  K  P  E  F
436 GGGACGTGCGTCAAAGAAGGTGGCATAGCCTTCGAGAAGGCTCACGGCCACCAGATTTGGGACTTAGCATCGGAAAAACCCGAATTC
    N  K  L  F  N  D  G  M  A  C  T  A  K  I  S  I  K  A  V  I  A  A  Y  K  D  G  F  G  S
523 AACAAGCTCTTCAATGACGGTATGGCGTGTACGGCTAAGATCTCCATCAAGGCGGTGATAGCAGCATATAAAGATGGGTTCGGCTCC
    I  G  T  L  V  D  A  G  G  G  T  G  G  A  V  A  E  V  V  K  A  Y  P  H  I  K  G  I  N
610 ATTGGAACTCTGGTGGACGCGGGAGGTGGCACCGGAGGGGCGGTGGCGGAGGTGGTGAAGGCGTATCCGCATATCAAGGGCATTAAC
    F  D  L  P  H  V  V  A  T  A  P  A  Y  K  G  V  S  H  V  G  G  D  M  F  E  S  I  P  D
697 TTCGACCTCCCGCATGTGGTCGCCACAGCGCCAGCGTACAAAGGAGTGTCCCACGTCGGGGGTGACATGTTCGAGTCCATCCCTGAC
    A  D  A  I  F  M  K  R  I  L  H  D  W  N  D  E  D  C  V  K  I  L  K  N  C  R  K  A  I
784 GCTGATGCAATCTTCATGAAGCGGATATTGCACGATTGGAATGACGAAGACTGCGTCAAGATCTTAAAAAACTGTCGGAAAGCAATA
    P  E  K  T  G  K  V  I  I  V  D  G  V  I  R  E  D  G  Y  E  P  F  D  E  T  R  L  V  L
871 CCGGAGAAAACTGGGAAAGTTATAATCGTTGATGGAGTGATCCGAGAAGATGGCTATGAGCCATTTGATGAAACAAGGCTGGTGTTG
    D  L  V  M  M  A  H  S  S  H  G  Q  E  R  T  E  V  E  W  K  K  L  L  E  E  G  G  F  P
958 GATTTAGTGATGATGGCGCATTCCTCTCACGGGCAAGAGAGAACCGAGGTTGAATGGAAGAAATTATTGGAGGAAGGAGGGTTTCCT
    R  Y  R  I  L  K  I  P  T  L  Q  M  I  I  E  A  Y  P  V  *
1045 CGTTACAGAATCTTAAAGATTCCAACTTTGCAGATGATAATCGAGGCTTATCCAGTGTAGATGTAATCTACATCGTGT
```

VvOMT3. Nucleotide and predicted amino acid sequence of the *VvOMT3* coding region amplified from CS and PN cDNA. Sequence given represents CS, with the 13 SNPs present in PN highlighted in purple and one three base deletion highlighted in green.

M E K E E A E A E V E M W K Y I

1 GATTCACTAATCAATTTTCATGGAGAAAGTGGTAAAAATCATGGAGAAAGAAGAGGCGGAAGCTGAAGTAGAGATGTGGAAGTACAT

F G F V E M A V V K C [A/G] I E L G I A D V [M/I] E S H [A/S] G P I T

88 CTTTGGATTTGTGGAAATGGCTGTAGTGAAGTGTG[C/G]CATTGAGCTCGGCATAGCTGATGTCAT[G/A]GAAAGCCAT[T/G]CAGGCCCCATCAC

L S S L S S S L G C S P S G L Y R I M R F L V N R R [L/I] F K

175 TCTTTCGTCACTGTCGTCTTCCCTCGGGTGCTCGCCTTCTGGTTTGTACCGGATCATGAGGTTCCTGGTGAACCGGAGA[C/A]TATTCAA

E V A T S Q G D T G Y Q Q T P L S R R L M T R S E N G M A

262 GGAGGTGGCGACAAGCCAAGGCGACACAGGCTATCAGCAGACACCTCTCTCGCGGCGCCTGATGACCCGCAGCGAGAATGGCATGGC

A L L L L E S S P V M L A P W H G L S A R L L G K G N A T

349 TGCCTTACTGTTGCTGGAGAGCAGCCCAGTGATGCTGGCTCCATGGCATGGCCTAAGCGCCCGGCTTCTAGGGAAAGGGAACGCGAC

F D A A H G Q D V W G Y A A S H P A H S K L I N D A M A C

436 GTTCGACGCTGCCCACGGGCAGGATGTCTGGGGCTATGCAGCTTCGCATCCTGCTCACAGTAAGCTCATAAA[T/C]GATGCGATGGCTTG

D A R M A V S A I V N G C P E V F D G V S T L V D V G G G

523 TGATGCTAGAATGGCGGTTTCCGCCATTGTGAATGGCTGTCCGGAGGTGTTTGATGGGGTGAGCACCCTGGTGGATGTCGGTGGGGG

D G T A L R T L I K A R P L I R G I N F D L P H V V S S A

610 CGATGGCACTGCCCTG[C/A]GGACGTTGATCAAGGCCCGTCCATTGATCAGAGGCATCAACTTTGATCTTCCTCACGTTGTTTCCTCCGC

P K C N G V E Y A S G D M F D T V P K A D A A F L M W V L

697 CCCTAAATGTAACGGTGTTGAGTATGC[T/C]AGTGGGGATATGTTTGACACTGTCCCTAAAGCGGATGCTGCTTTCCTCATGTGGGTTCT

H D W G D E E C I Q I L E K C R Q A I P G D K G K V I I V

784 ACACGACTGGGGAGACGAGGAGTGCATCCAAATTCTAGAAAAATGCAGACAAGC[C/T]ATTCCTGGGGACAAAGGAAGGTGATAAT[T/A]GT

E A V I Q E N E K E G D H N N L K D V G L M L D M V M M A

871 GGAAGC[T/C]GTGATACAAGAAAACGAAAAAGAAGGTGATCACAACAATCTTAAAGACGTGGGGTTGATGCTAGACATGGTTATGATGGC

(H and TCA in green)

H T T T G K E R T L K E W D Y V L K K A G F N R Y T [M/V] K P

958 TCATACTACTACCGGCAAAGAAAGGACCTTAAAGGA[G/A]TGGGACTATGTCCT[G/T]AAGAAGGCCGGTTTTAATCGATACACC[A/G]TGAAACC

I R A V K S V I E A Y P *

1045 TATTCGTGCCGTCAAATCTGTCATCGAAGCTTATCCTTAG

VvOMT4. Nucleotide and predicted amino acid sequence of the *VvOMT4* coding region amplified from CS cDNA.

```
                                M  K  T  L  E  E  E  E  E  G  G  A  G  I  E  M  W  K  Y
   1TTAATCTTCTATGACAGAGGCAACGAGGGAGATGAAAACCTTGGAGGAGGAAGAAGAAGGAGGAGCTGGAATTGAGATGTGGAAGTA

      I  Y  G  F  A  A  M  A  A  V  K  C  G  I  E  L  G  I  A  D  V  M  E  S  H  G  G  P  I
  88 CATATATGGATTTGCTGCAATGGCCGCAGTGAAGTGTGGCATTGAGCTAGGCATAGCTGATGTCATGGAAAGCCATGGAGGCCCCAT

      T  L  S  A  L  S  S  S  L  G  C  P  P  S  G  L  N  R  I  M  R  F  L  V  S  R  R  I  F
 175 CACTCTTTCTGCTTTGTCTTCTTCCCTGGGGTGCCCGCCTTCTGGTTTGAACCGGATCATGAGGTTCCTGGTGAGCCGGAGAATATT

      R  E  V  A  T  S  Q  G  D  T  G  Y  Q  Q  T  P  M  S  R  C  L  M  T  S  R  G  D  G  M
 262 CAGGGAGGTGGCGACAAGCCAAGGCGATACAGGCTATCAACAAACACCCATGTCACGGTGCCTGATGACGAGCAGAGGAGACGGCAT

      A  A  F  V  L  L  E  S  S  P  V  M  L  A  P  W  H  G  L  S  A  R  V  L  G  K  G  N  S
 349 GGCTGCCTTTGTGTTGCTGGAGAGCAGCCCAGTGATGCTGGCTCCATGGCATGGCCTTAGCGCCCGGGTTCTAGGGAAAGGGAACTC

      T  F  E  A  A  H  G  D  D  I  W  G  Y  A  A  K  N  P  D  H  S  K  L  I  N  D  A  M  A
 436 AACGTTCGAGGCTGCCCACGGGGATGATATCTGGGGCTATGCAGCTAAAAACCCTGATCACAGCAAGCTTATAAATGACGCAATGGC

      S  N  A  R  R  V  V  P  A  M  V  N  D  C  P  E  V  F  D  G  V  G  T  L  V  D  V  G  G
 523 TTCTAATGCCAGGAGGGTAGTGCCAGCAATGGTGAATGACTGTCCAGAGGTGTTTGATGGGGTGGGCACCCTGGTGGATGTAGGTGG

      G  N  G  T  A  L  R  T  L  V  K  A  C  P  W  I  R  G  I  N  F  D  L  P  H  V  V  A  T
 610 GGGCAATGGCACCGCCCTGCGGACGTTGGTCAAGGCCTGCCCATGGATTAGAGGCATCAACTTTGATCTTCCTCATGTCGTGGCCAC

      A  P  K  C  N  D  V  E  H  V  G  G  N  M  F  D  S  V  P  K  A  D  A  V  F  I  M  K  V
 697 TGCTCCTAAATGTAACGATGTTGAGCATGTTGGAGGAAACATGTTCGACAGCGTCCCCAAAGCTGATGCTGTTTTCATCATGAAGGT

      L  H  D  W  G  D  E  K  C  I  K  I  L  E  K  C  R  E  A  I  P  E  D  K  G  K  V  I  I
 784 CCTACATGATTGGGGGGATGAAAAGTGCATCAAGATCCTAGAAAAATGCAGAGAAGCCATTCCGGAGGACAAAGGGAAGGTGATAAT

      V  E  A  V  I  E  G  D  D  P  Q  G  D  D  Q  K  L  K  D  V  K  L  M  V  D  M  V  M  M
 871 TGTAGAAGCCGTGATTGAAGGTGATGATCCTCAAGGTGATGATCAGAAACTCAAGGATGTGAAGTTGATGGTAGACATGGTTATGAT

      A  H  T  T  T  G  K  E  R  T  F  K  E  W  D  Y  V  L  L  N  A  G  F  S  R  Y  T  I  K
 958 GGCACATACTACTACAGGCAAAGAAAGGACCTTCAAGGAATGGGACTACGTCCTTCTCAATGCTGGTTTTAGTCGATATACCATAAA

      P  I  R  S  I  V  Q  S  I  I  E  A  Y  P  *
1045 ACCTATTCGCAGTATTGTGCAGTCTATCATTGAAGCATACCCTTAATATATCGCAAGGTAAATCCCTCTAAATTATC
```

Appendix C - Genetic marker details

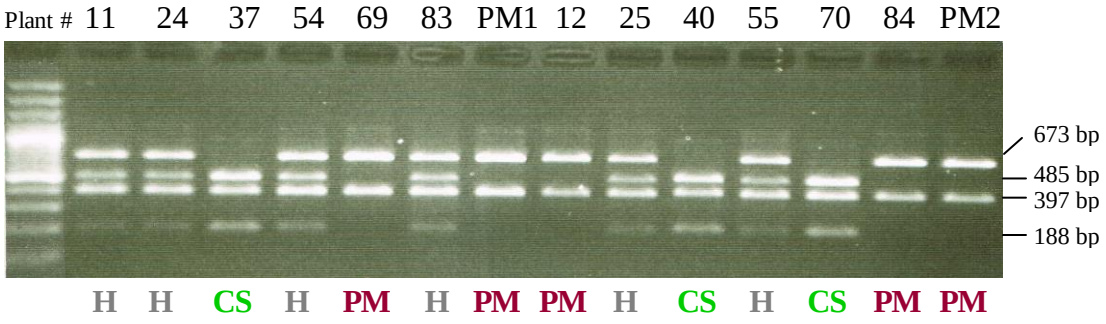
Cleavage amplified polymorphic sequence (CAPS) and derived CAPS (dCAPS) markers designed to distinguish between the genomes of CS and PM at different loci during the mapping of the IBMP-accumulation trait (Section 3.2.6). Marker names represent the respective chromosome number, and numbers which are preceded by a dash represent the marker location in Mb according to the 12× annotated genome sequence.

Name	Primer names (Appendix A)	Restriction enzyme	CS products (bp)	PM products (bp)
1	VvChrom1TF1 / VvChrom1TR1	MspA1I	210 + 280	490
2	VvChrom2TbF1 / VvChrom2TbR1	BspHI	700 + 210	910
3 - 0.3	27461F1 / 27461R1	ScaI	485+397+188	673 + 397
3 - 1.6*	VvChrom3@1.6F1 / VvChrom3@1.6R2	HindIII	235 + 32	267
3 - 1.9*	VvChrom3@1.9F / VvChrom3@1.9R1	EarI	723	684 + 39
3 - 2.19	Vv23989exon1F1 / Vv23989exon1R1	PstI	890	140 + 750
3 - 2.20*	VvChrom3@2.21F1/ VvChrom3@2.21R1	NcoI	284 + 32	316
3 - 2.4	8789F1 / 8789R1	AflII	450 + 750	1200
3 - 2.6	12860F2 / 12860R2	SnaBI	130 + 90	220
3 - 2.8*	VvChrom3@2.8F3 / VvChrom3@2.8R3	BglII	278 + 31	309
3 - 3.3	VvChrom3TF1 / VvChrom3TR1	BtsI	400 + 150	550
3 - 8*	VvChrom3BF1 / VvChrom3BR1	SalI	125	88 + 37
6	VvChrom6TF1 / VvChrom6TR1	Sau96I	600	310 + 290
16	VvChrom16TF1 / VvChrom16TR1	AccI	210 + 280	490

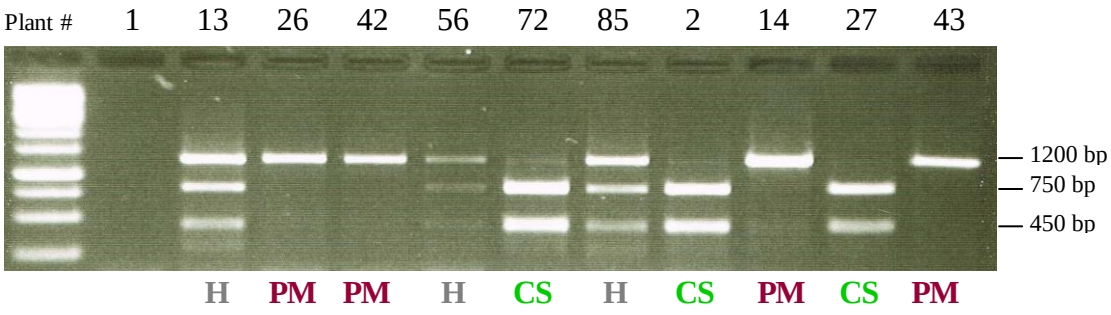
* Denotes dCAPS markers

The two figures below represent examples of restriction digests from the CAPS marker analysis of the PM × CS F2 progeny. Each gel lane shows the results of a single marker analysis of a single F2 progeny plant. The results of the restriction digests are shown below each gel with **CS** representing the plant homozygous for the Cabernet Sauvignon allele, **PM** representing the plant homozygous for the Pinot Meunier allele and **H** representing the plant is heterozygous at the respective allele.

Marker 3 - 0.31 Mb

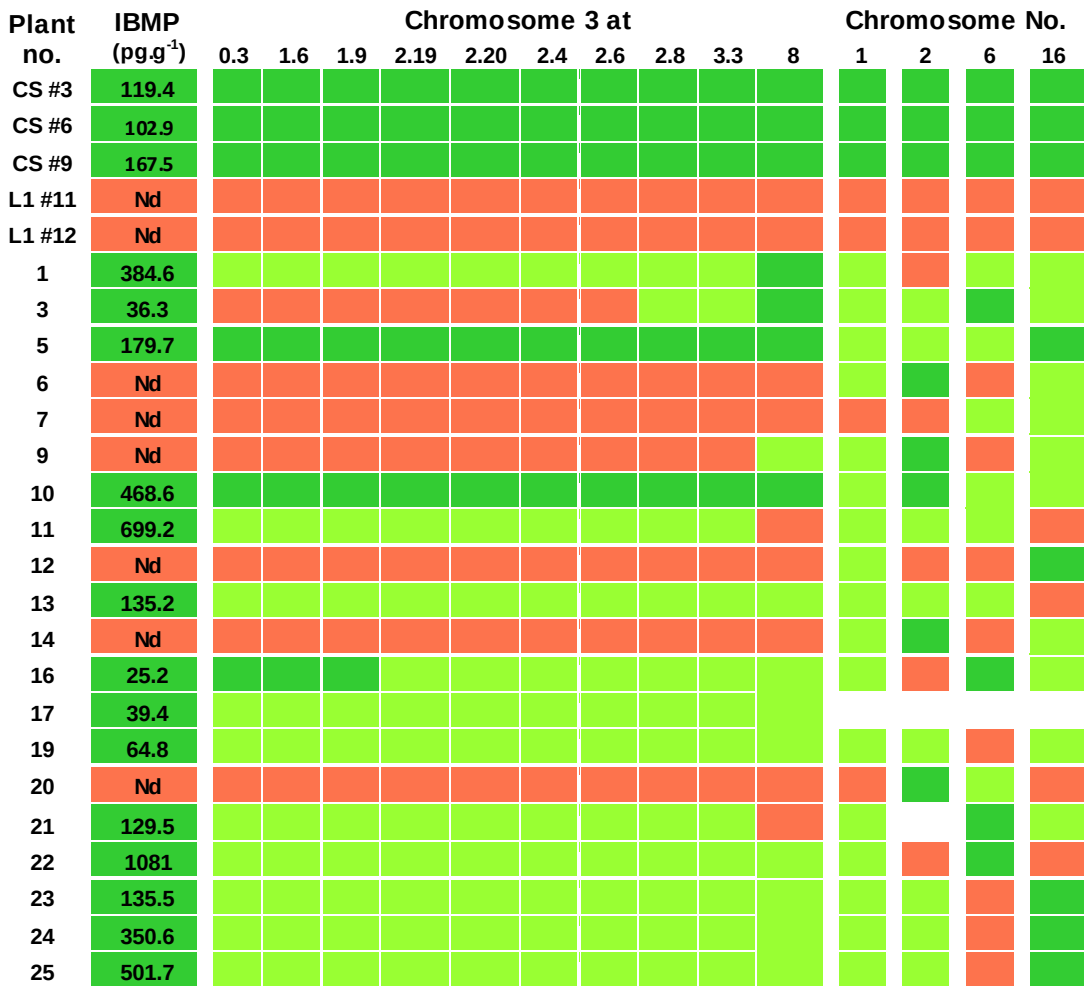


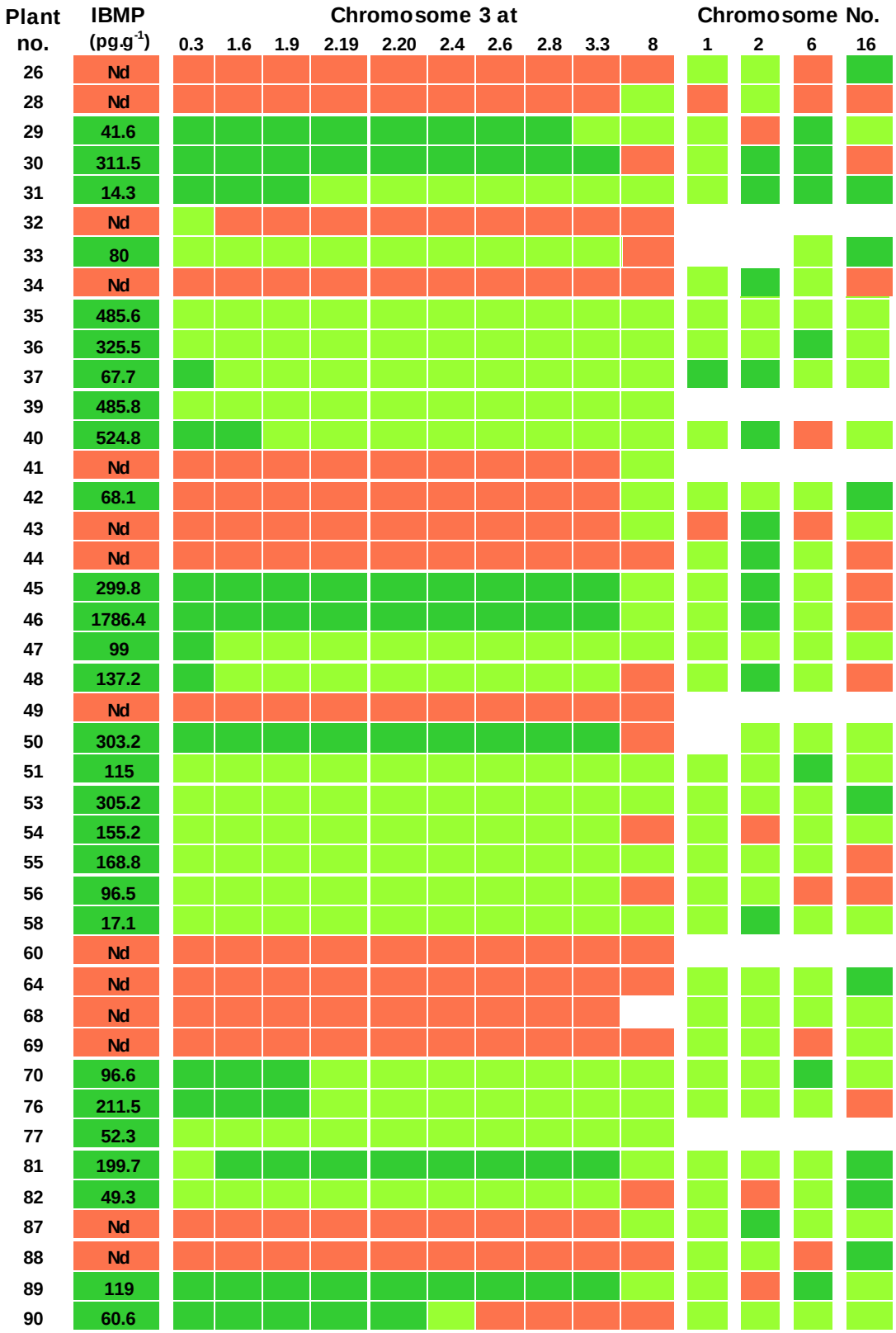
Marker 3 - 2.4 Mb



Appendix D - Marker-trait scoring in grapevine populations

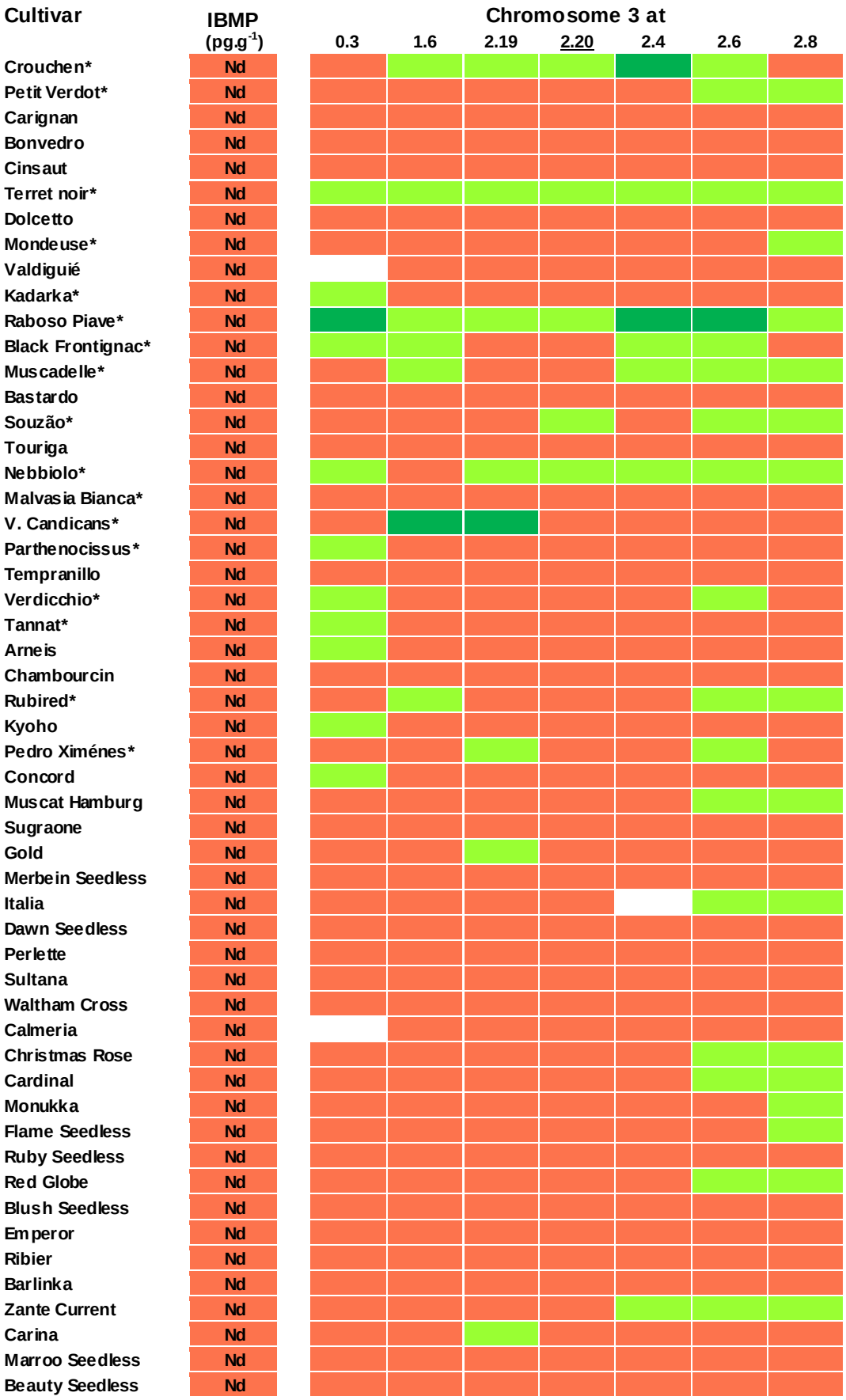
Marker-trait determinations in the segregating F2 progeny resulting from a cross between CS × PM dwarf cross. Within the IBMP trait column, green or red squares signify individuals are positive or negative (Nd) for IBMP accumulation at 7 wpf respectively. Within the marker columns red, dark green and light green squares represent individuals are either homozygous for the PM genome, homozygous for the CS genome, or heterozygous at that marker location respectively.




Plant no.
IBMP (pg.g-1)
Chromosome 3 at
0.3
1.6
1.9
2.19
2.20
2.4
2.6
2.8
3.3
8
Chromosome No.
1
2
6
16
26 Nd
28 Nd
29 41.6
30 311.5
31 14.3
32 Nd
33 80
34 Nd
35 485.6
36 325.5
37 67.7
39 485.8
40 524.8
41 Nd
42 68.1
43 Nd
44 Nd
45 299.8
46 1786.4
47 99
48 137.2
49 Nd
50 303.2
51 115
53 305.2
54 155.2
55 168.8
56 96.5
58 17.1
60 Nd
64 Nd
68 Nd
69 Nd
70 96.6
76 211.5
77 52.3
81 199.7
82 49.3
87 Nd
88 Nd
89 119
90 60.6

Marker-trait determinations in the 91 cultivars of the Coombe vineyard collection. Within the IBMP trait column, green or red squares signify individuals are positive or negative (Nd) for IBMP accumulation at 7 wpf respectively. Within the marker columns red, dark green and light green squares represent individuals are either homozygous for the PM genome, homozygous for the CS genome, or heterozygous at that marker location respectively. *, signifies cultivars with unique haplotypes used for statistical analysis.

Cultivar	IBMP ($pg.g^{-1}$)	Chromosome 3 at 0.3	1.6	2.19	2.20	2.4	2.6	2.8
Cabernet Franc	464							
Semillon	205							
Sauvignon Blanc*	175							
Merlot	136							
Ruby Cabernet	120							
Rousanne	57							
Aleatico*	42							
Tinta Cão*	31							
Tinta Amarella*	27							
Biancone	23							
Durif*	15							
Riesling*	Nd							
Chardonnay*	Nd							
Viognier*	Nd							
Verdelho*	Nd							
Marsanne*	Nd							
Colombard	Nd							
Chenin Blanc*	Nd							
Müller Thurgau*	Nd							
Traminer	Nd							
Muscat Gordo	Nd							
Shiraz	Nd							
Grenache	Nd							
Pinot noir	Nd							
Malbec	Nd							
Gamay	Nd							
Zindfandel	Nd							
Mataro	Nd							
Sangiovese	Nd							
Barbera*	Nd							
Canocazo	Nd							
Rkaziteli	Nd							
Rkaziteli	Nd							
Ondenc	Nd							
Clairette	Nd							
Farana	Nd							
Trebbiano	Nd							
Chasselas*	Nd							
Doradillo	Nd							


Cultivar
IBMP (pg.g-1)
Chromosome 3 at
0.3
1.6
2.19
2.20
2.4
2.6
2.8
Crouchen*
Petit Verdot*
Carignan
Bonvedro
Cinsaut
Terret noir*
Dolcetto
Mondeuse*
Valdiguié
Kadarka*
Raboso Piave*
Black Frontignac*
Muscadelle*
Bastardo
Souzão*
Touriga
Nebbiolo*
Malvasia Bianca*
V. Candicans*
Parthenocissus*
Tempranillo
Verdicchio*
Tannat*
Arneis
Chambourcin
Rubired*
Kyoho
Pedro Ximénes*
Concord
Muscat Hamburg
Sugraone
Gold
Merbein Seedless
Italia
Dawn Seedless
Perlette
Sultana
Waltham Cross
Calmeria
Christmas Rose
Cardinal
Monukka
Flame Seedless
Ruby Seedless
Red Globe
Blush Seedless
Emperor
Ribier
Barlinka
Zante Current
Carina
Marroo Seedless
Beauty Seedless
Nd

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