Functional Genomics of *O*-glucosyltransferases from Concord grape (*Vitis labrusca*)

by

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Abstract

Grape (*Vitis* spp.) is a culturally and economically important crop plant that has been cultivated for thousands of years, primarily for the production of wine. Grape berries accumulate a myriad of phenylpropanoid secondary metabolites, many of which are glucosylated *in planta*. More than 90 *O*-glucosyltransferases have been cloned and biochemically characterized from plants, only two of which have been isolated from *Vitis* spp. The world-wide economic importance of grapes as a crop plant, the human health benefits associated with increased consumption of grape-derived metabolites, the biological relevance of glucosylation, and the lack of information about *Vitis* glucosyltransferases has inspired the identification, cloning and biochemical characterization of five novel "family 1" *O*-glucosyltransferases from Concord grape (*Vitis labrusca* cv. Concord).

Protein purification and associated protein sequencing led to the molecular cloning of UDP-glucose: resveratrol/hydroxycinnamic acid *O*-glucosyltransferase (VLRSGT) from *Vitis labrusca* berry mesocarp tissue. In addition to being the first glucosyltransferase which accepts *trans*-resveratrol as a substrate to be characterized *in vitro*, the recombinant VLRSGT preferentially produces the glucose esters of hydroxycinnamic acids at pH 6.0, and the glucosides of *trans*-resveratrol and flavonols at pH 9.0; the first demonstration of pH-dependent bifunctional glucosylation for this class of enzymes. Gene expression and metabolite profiling support a role for this enzyme in the bifuncitonal glucosylation of stilbenes and hydroxycinnamic acids *in planta*.

A homology-based approach to cloning was used to identify three enzymes from the *Vitis vinifera* TIGR grape gene index which had high levels of protein sequence

identity to previously characterized UDP-glucose: anthocyanin 5-*O*-glucosyltransferases. Molecular cloning and biochemical characterization demonstrated that these enzymes (rVLOGT1, rVLOGT2, rVLOGT3) glucosylate the 7-*O*-position of flavonols and the xenobiotic 2,4,5-trichlorophenol (TCP), but not anthocyanins. Variable gene expression throughout grape berry development and enzyme assays with native grape berry protein are consistent with a role for these enzymes in the glucosylation of flavonols; while the broad substrate specificity, the ability of these enzymes to glucosylate TCP and expression of these genes in tissues which are subject to pathogen attack (berry, flower, bud) is consistent with a role for these genes in the plant defense response.

Additionally, the *Vitis labrusca* UDP-glucose: flavonoid 3-*O*-glucosyltransferase (VL3GT) was identified, cloned and characterized. VL3GT has 96 % protein sequence identity to the previously characterized *Vitis vinifera* flavonoid 3-*O*-glucosyltransferase (VV3GT); and glucosylates the 3-*O*-position of anthocyanidins and flavonols *in vitro*. Despite high levels of protein sequence identity, VL3GT has distinct biochemical characteristics (as compared to VV3GT), including a preference for B-ring methylated flavonoids and the inability to use UDP-galactose as a donor substrate. RT-PCR analysis of *VL3GT* gene expression and enzyme assays with native grape protein is consistent with an *in planta* role for this enzyme in the glucosylation of anthocyanidins, but not flavonols.

These studies reveal the power of combining several biochemistry- and molecular biology-based tools to identify, clone, biochemically characterize and elucidate the *in planta* function of several biologically relevant *O*-glucosyltransferases from *Vitis* spp.

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Chapter 1 - General Introduction

Plants synthesize a large number of chemically diverse natural products that mediate interactions between the plant and its environment. Consumption of plant secondary metabolites by humans and the use of these compounds as neutraceuticals are associated with several health-promoting effects. Phenylpropanoids are a class of natural products derived from the amino acid phenylalanine that contribute to the pigmentation, signaling, cell wall structure, UV-protection and defense response of the plant. Phenylpropanoids can be further classified based on their structural and chemical properties and these subgroups include the flavonoids, coumarins, cinnamic acids and stilbenes. Thousands of unique phenylpropanoid compounds occur in nature, and enzymatic hydroxylation, acylation, methylation and glycosylation contribute to this amazing diversity.

Glycosyltransferases catalyze the transfer of sugar from an activated donor to a -C, -S, -N or -OH group on an acceptor substrate. In plants, glucosylation is the most prevalent reaction, and this reaction is catalyzed by glucosyltransferases that transfer glucose from uridine 5'- diphosphoglucose (UDP-glucose) to the free hydroxyl group of a low-molecular weight substrate. Glucosylation is an important biological process which is associated with changes in the subcellular location, toxicity, stability, solubility and spectral characteristics of several of these compounds. Typical acceptor substrates are plant-derived (hormones, volatiles, phenylporpanoids) or exogenous (human- or pathogen-derived xenobiotics) and are converted to glucosides or glucose esters.

Grapes contain more than 1000 secondary metabolites of which more than 200 exist as glucosides or glucose esters. These compounds are involved in the pigmentation,

aroma, and defense response of the grape berry. Some glucosides also contribute to several of the organoleptic characteristics of wine, and are well-documented as neutraceuticals.

This thesis investigates the basic biology of several *O*-glucosyltransferases that are expressed in Concord (*Vitis labrusca*) grape berry tissue. A number of tools including enzyme purification using column chromatography, molecular cloning, RT-PCR gene expression studies, metabolite profiling, and enzyme kinetics (native and recombinant protein) have been developed and exploited for functional genomic characterization of *O*-glucosyltransferases from *Vitis labrusca*.

1.1 - Outline

This thesis includes a list of references and six chapters, beginning with a general introduction (Chapter 1) and ending with a general conclusion (Chapter 6).

Chapter 2 reviews the recent advances in the literature concerning then enzymatic biosynthesis of phenylpropanoids in grapes. This pathway has been the subject of intense study in recent years in part because of the world-wide economic and agricultural importance of grapes, the high levels of human-consumption of grape-derived products (wine, juices, jams), and the relationship between the high levels of grape-derived polyphenols and the health benefits observed in humans.

Chapter 3 is a manuscript published in The Plant Journal in 2007 that describes the protein purification, molecular cloning and biochemical characterization of a mesocarp-localized bifunctional resveratrol/hydroxycinnamic acid *O*-glucosyltransferase from Concord grapes. This is the first report of glucosyltransferase which glucosylates

stilbenes *in vitro*, and the ability of this enzyme to glucosylate different substrates and functional groups at different pHs is unique for this class of enzymes.

Chapter 4 is a manuscript in preparation that describes the molecular cloning and biochemical characterization of three members of the anthocyanin 5-*O*-glucosyltransferase-like gene family from Concord grapes. *In vitro*, these recombinant enzymes glucosylate several flavonols and one xenobiotic, but not anthocyanins. The hazards of homology-based cloning approaches to clone genes with putative functions are highlighted.

Chapter 5 is a manuscript in preparation that describes the cloning and functional characterization of UDP-glucose: flavonoid 3-*O*-glucosyltransferase from Concord grape which glucosylates anthocyanins and flavonols *in vitro*. Although this enzyme has high levels of amino acid sequence identity (96 %) to the previously characterized *Vitis vinifera* flavonoid 3-*O*-glucosyltransferase, *in vitro* functional characterization reveals distinct biochemical properties for two proteins.

Chapter 2 – Literature Review

The enzymatic production of phenylpropanoids in grape (*Vitis* spp.) – A well-defined biosynthetic pathway

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The enzymatic production of phenylpropanoids in grape (*Vitis* spp.) – A well-defined biosynthetic pathway

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2.1 - Abstract

The biosynthesis of phenypropanoid compounds in plants has common enzymatic reactions among diverse plant species. These "structural" enzymes are well-regulated within the plant and have been well-studied from several plant species. Grapes produce high levels of unique phenylpropanoids which mediate the plant's interaction with its environment, contribute to the organoleptic qualities of wine and are associated with several health-promoting effects in humans. Different grape cultivars produce unique phenylpropanoid "fingerprints" which can be used to identify the origins of a specific fruit or wine.

The predominant metabolites of grapes and wine include mono- and polymeric stilbenes, flavonols, anthocyanins, hydroxybenzoic acids and the hydroxycinnamic tartaric acid esters. Modification of plant natural products by hydroxylation, methylation, acylation and glucosylation increases the diversity of these metabolites and changes their chemical properties and roles *in planta*. Recently two of the main structural enzymes of phenylpropanoid biosynthesis were crystallized from grapes and several studies have investigated the temporal, environmental and tissue-specific regulation of gene expression and the associated unique metabolite profiles of these tissues. This review

focuses on the enzymatic biosynthesis of phenylpropanoids in grapes and addresses their importance within the plant and their contribution to the organoleptic qualities of wine.

2.2 - Introduction

The health benefits associated with human consumption of natural products and their increasing use as neutraceuticals has piqued interest in their production and accumulation in plants. Grapes have been cultivated for thousands of years for wine production and can be classified based on their geographic origin. Grapes native to Europe and Asia (*Vitis vinifera*) are considered "elite", are used in viticulture and have world-wide economic and agronomic importance, whereas grapes native to North America (*Vitis labrusca, Vitis riparia, Vitis rupestris*) have limited use in wine-making or as breeding tools and are grown predominantly for the production of juices and jellies (Jackson, 2000).

Frequent consumption of grape-derived products has well-documented antioxidant and health-promoting effects that are associated with the intake of high concentrations of grape phenolic compounds (Soleas et al., 1997; Monagas et al., 2005; Kedage et al., 2007). Grape berries produce unique profiles of phenolic secondary metabolites as a result of hydroxylation, methylation, acylation and glucosylation which increases the diversity and changes the chemical, physiological and spectral properties of these compounds.

While grape is a nonclimacteric fruit, its development involves two phases of rapid berry expansion, separated by a lag phase (Coombe and McCarthy, 2000). The beginning of the second growth phase is characterized by berry softening, a decrease in

berry pH, an increase of the total soluble solutes (°Brix), development of pigmentation of the berry exocarp, and is known by the viticultural term "veraison". The grape berry can be divided into several tissue types, including the seed, the mesocarp (flesh), and the exocarp (skin) that undergo rapid metabolite profiles changes as the berry matures. In general, grape berries accumulate tartaric, malic, and hydroxycinnamic acids (Romeyer et al., 1983) and high levels of proanthocyanidins (condensed tannins) before veraison (Kennedy et al., 2000; Kennedy et al., 2001); whereas the berry produces aroma compounds and pigments (Mazza and Miniati, 1993; Wang and De Luca, 2005; Fang and Qian, 2006; Kennedy et al., 2006) during and post-veraison. While specific metabolite profiles of grapes can be influenced by environmental conditions, these are ultimately dependant on the grape species and cultivar.

Grape secondary metabolites are well studied because of their contribution to the organoleptic qualities (colour, taste, aroma, astringency) of wine, their human health benefits and the importance of viticulture as a world-wide industry. Several recent reviews have focused on the phenolic compounds which are detected in grapes and wine (Waterhouse, 2002; Brouillard et al., 2003; Flamini, 2003; 2005; Shi et al., 2003; Monagas et al., 2005), and include perspectives on the history of grape and wine phenolics (van de Wiel et al., 2001; This et al., 2006), the effect of viticultural practices on the phenolic profiles in grapes and in wine (Kennedy et al., 2006; Moreno-Arribas and Polo, 2005; Downey et al., 2006) and the effects of these metabolites on human health (Folts, 2002; Caimi et al., 2003; López-Vélez et al., 2003; Ruf, 2003). Of the three main classes of secondary metabolites (the flavonoids, the alkaloids, and the terpenes) grapes produce and accumulate terpenes and flavonoids, whereas the occurrence of alkaloids in

Vitis tissues is not well documented. Additionally, several studies have focused on the accumulation of polyphenols in grapes in response to internal and environmental cues, as well as the transcriptional regulation of the pathway enzymes (see below; Elmer and Reglinski, 2006).

The production of natural products mediate the grape's interaction with its environment and have diverse roles attracting pollinators and seed dispersers, as volatile aroma compounds, as structural components in cell wall biosynthesis, and in the plant's response to stress (Koes et al., 1994; Wink, 2003). Similarly, many plant defenses against insect and microbial pathogens rely upon the tissue-specific sequestration of potentially toxic secondary metabolites (Paquette et al., 2003), which are biosynthesized, modified and accumulated within unique cell, tissue- and organ types.

The terminal step in many biosynthetic pathways is glucosylation of a low-molecular weight metabolite that changes the molecule's toxicity, stability, spectral characteristics, and solubility (Vogt and Jones, 2000). Glucosylation is often required by the plant for the proper transport, storage, signaling and inactivation of biologically active aglycones (Jones and Vogt, 2001). Glucosyltransferases are cytosolic enzymes that transfer glucose from uridine 5'- diphosphoglucose (UDPG) to plant-derived or exogenous substrates and this reaction allows the plant to accumulate high levels of these potentially toxic compounds (Vogt and Jones, 2000; Jones and Vogt, 2001). This class of enzymes exists as a large multi-gene family (Li et al., 2001; Bowles, 2002; Lim et al., 2003) with broad but regio-selective substrate specificity that allows the plant to glucosylate several structurally unrelated compounds (Vogt and Jones, 2000). Glucosyltransferases which modify plant natural product are classified as "family 1"

glucosyltransferases and are identified by the presence of the Plant Secondary Product Glucosyltransferase (PSPG) consensus sequence in the C-terminus of the protein (Vogt and Jones, 2000). More than 90 family 1 glucosyltransferases have been functionally characterized from a number of plant species, and are the topic of several recent reviews (Vogt and Jones, 2000; Jones and Vogt, 2001; Ross et al., 2001; Bowles, 2002; Lim and Bowles, 2004; Lorenc-Kukula et al., 2004; Bowles et al., 2006).

Grapes contain more than 200 aglycones, including flavonoids, hormones, stilbenes, terpenes and hydroxycinnamic and hydroxybenzoic acids that are modified by glucosylation (Sefton, 1993; Sefton, 1994; Monagas et al., 2005). Consistent with the accumulation of a multitude of glucosylated compounds, a BLAST search of the *Vitis vinifera* TIGR grape gene index with the previously characterized *Petunia* x *hybrida* anthocyanin 5-*O*-glucosyltransferase (Accession # AB027455) identified more than 90 glucosyltransferase-like sequences. Despite the *in planta* expression of several glucosyltransferases and the widespread accumulation of glucosylated products, only a few of these enzymes have been cloned and biochemically characterized from grape (Ford et al., 1998; Meyer et al., 2003).

The recently completed *Vitis* genome sequencing and the generation of several thousands of publicly available expressed sequence tag (EST) nucleotide sequences from *Vitis vinifera* has been used to design primers and successfully clone full-length genes from *Vitis labrusca* cv. Concord (Wang and De Luca, 2005; Chapters 3-5). Additionally, the availability of large amounts of nucleotide sequence information has permitted the development of a grape Microarray, and several recent publications have used this technology to undertake the large-scale screening of the transcriptomes of several grape

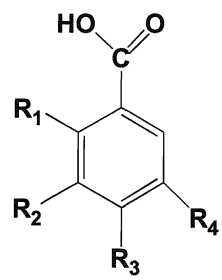
tissues under diverse physiological and environmental conditions (Waters et al., 2005; Cramer et al., 2007; Espinoza et al., 2007). By combining the grape transcriptional profiles with metabolite profiling and proteome analysis of these tissues, the basic biology of the grape can be elucidated.

This review describes the biosynthesis of grape phenylpropanoids, and focuses on the enzymatic pathway leading to their production and accumulation within the plant. The contribution of these metabolites to the organoleptic characteristics of wine and the role of phenylpropanoids within the plant is discussed, with an emphasis on the importance of glucosylation of these metabolites to their functions within the grape berry.

2.3 Grapes produce and accumulate flavonoid and non-flavonoid phenylpropanoids in several tissues

Metabolites which have a benzene ring substituted with at least one hydroxyl group are known as phenols and these compounds can be further classified as non-flavonoids or flavonoids based on their ring patterns (Monagas et al., 2005). The basic phenylpropane skeleton consists of a C_6C_3 phenylalanine-derived backbone which can be elaborated to extend the ring structure, or can be simplified to produce C_6C_1 compounds (Figure 1., Dixon et al., 2002).

The conversion of phenylalanine to cinnamic acid is the first committed step of phenylpropanoid biosynthesis and is catalyzed by the enzyme phenylalanine ammonia



	Substitution Patterns			
Acid	**R1**	**R2**	**R3**	**R4**
Benzoic	H	H	H	H
Anthranilic	NH_2	H	H	H
Salicylic	OH	H	H	H
***para*-hydroxybenzoic**	H	H	OH	H
Gentisic	OH	H	H	OH
Protocatechuic	H	OH	OH	H
Gallic	H	OH	OH	OH
Vanillic	H	OCH_3	OH	H
Syringic	H	OCH_3	OH	OCH_3

Figure 1. Hydroxybenzoic acids detected in grape berries and wine. Modified from Monagas et al., 2005.

lyase (PAL). In *Vitis vinifera*, *PAL* exists as a large, multigene family, with 15-20 copies per genome (Sparvoli et al., 1994) and although partial cDNA sequences have been identified, a full-length *PAL* gene obtained from *Vitis* spp. has not been heterologously expressed.

PAL activity and expression is regulated by berry development and is induced in response to several endogenous and exogenous stimuli. *PAL* mRNA accumulation, protein expression and enzyme activity is biphasic, reaching maximal values in pre- and post-veraison grape berries (Boss et al., 1996a; Chen et al., 2006a). Within the vine, *PAL* transcripts accumulate in the leaves, tendrils, roots, seeds, stems, flowers and berry exocarp tissues (Boss et al., 1996a, b), and subcellular localization of *PAL* targets the transcript to the cell walls, secondarily thickened walls, and parenchyma cells (Chen et al., 2006a). *PAL* transcripts accumulate in *Vitis labrusca* cell suspension cultures in response to L-alanine (Chen et al., 2006c) and PAL enzyme activity is induced following treatment of berry exocarps with ethephon, sucrose and light (Roubelakis-Angelakis and Kliewer, 1986), or in cell suspension cultures with sugar, abscisic acid (Hiratsuka et al., 2001) and fungal elicitors (Melchior and Kindl, 1991). Similarly, the signal molecule salicylic acid activated PAL gene expression, protein accumulation and enzyme activity in intact grape berries (Chen et al., 2006b) and in berry exocarp (Wen et al., 2005), while UV-light treatment also activated the same processes in grape leaves (Fritzemeier and Kindl, 1981).

The PAL reaction product, cinnamic acid, is converted to *para (p)*-coumaric acid by the cytochrome P450 (CYP) hydroxylase, cinnamate 4-hydroxylase (C4H). Like *PAL*, *C4H* is expressed biphasically throughout grape berry development (Chen et al., 2006a)

and gene expression is induced by UV light in grape leaves (Fritzemeier and Kindl, 1981) or by treatment of *Vitis labrusca* cell suspension cultures with L-alanine (Chen et al., 2006c). Similarly, C4H protein is expressed throughout grape berry development and immunolocalization targets *C4H* transcript to the chloroplast (plastid) and nucleus of berry exocarp tissue (Chen et al., 2006a). The localization of C4H to the chloroplast and to the nucleus is surprising since most CYPs are known to be associated with the cytoplasmic face of the endoplasmic reticulum (Omura, 1999). The enzyme hydroxycoumaroyl CoA ligase (4CL) produces hydroxycoumaroyl CoA from *p*-coumaric acid (Buchanan et al., 2000). Within the grape berry, *4CL* is expressed and active biphasically throughout development, with maximal gene expression 2 weeks post-flowering (Waters et al., 2005), and like *PAL*, *4CL* is localized to the secondarily thickened cell walls and parenchyma cells of berry mesocarp tissue (Chen et al., 2006a). Although these enzymes are required to produce hydroxycinnamic acid and its CoA derivatives, which are important precursors and intermediates in the biosynthesis of flavonoids, stilbenes, hydroxybenzoic and hydroxycinnamic acids; full-length *C4H* and *4CL* genes have not been identified from grapes and few studies have described the timing and expression of these genes.

2.4 - Hydroxybenzoic acids accumulate within the grape and are involved in numerous biological processes

Pre-veraison grape berries produce and accumulate hydroxylated derivatives of the C_6C_1 compound benzoic acid including gentisic, syringic, *p*-hydroxybenzoic, salicylic, vanillic, protocatechuic, and gallic acid (Figure 1). As the grape berry matures

the concentrations of these compounds decrease and are maintained at low levels in post-veraison exocarp tissues (Robertson, 1984; Fernández de Simón et al., 1992; Chen et al., 2006a). The biosynthesis of benzoic acid is not entirely understood in plants, but it is known that at least two pathways convert L-phenylalanine to benzoic acid (Hertweck et al., 2001) and subsequent hydroxylation and methylation produce the C_6C_1 derivatives that accumulate within the grape berry.

Glucosylation of hydroxybenzoic acids produces glucose esters, which can act as high energy intermediates (Li and Steffens; 2000; Lehfeldt et al., 2000), or glucosides, which decrease the toxicity and increase the stability of these reactive metabolites (Chen et al., 1995; Lee and Raskin, 1999). Although grapes do not accumulate glucosylated derivatives of hydroxybenzoic acids, an enzyme which produces the glucose esters of several hydroxybenzoic and hydroxycinnamic acids has been cloned and partially characterized from *Vitis vinifera* (Meyer et al., 2003), however little is known about its expression and function *in planta*.

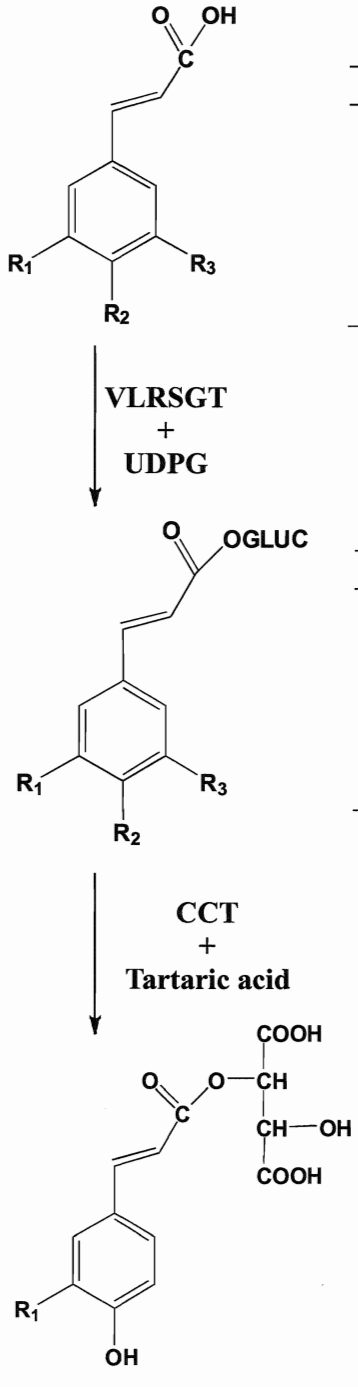
Hydroxybenzoic acids have *in vivo* roles as hormones (salicylic acid), aromatic volatiles and plant defense compounds as well as precursors for more complex plant phenols (Monagas et al., 2005). Additionally, these compounds accumulate as part of the plant defense response, or can be induced by the overexpression of alcohol dehydrogenase (*ADH*) in grape leaves (Tesniere et al., 2006). *In planta*, endogenous peroxidases enzymatically oxidize these hydroxybenzoic acids, converting them to their highly toxic and reactive quinones (Zapata et al., 1992). Grape juice and wine also contain vanillic, *p*HBA, *p*-coumaric, syringic and gallic acid (Mattila et al., 2006) in addition to their decarboxylated alcohols that contribute to the browning, aroma, acidity,

bitterness, mouth-feel and astringency of wines (Zapata et al., 1992; Soleas et al., 1997; Flamini, 2003; Flamini, 2005).

2.5 - Hydroxycinnamic acids and their tartaric acid esters are the predominant phenols in white grapes and are important to the organoleptic characteristics of wine

The hydroxycinnamic acids include *p*-coumaric acid and its hydroxylated and methylated derivatives caffeic acid, ferulic acid and sinapic acid (Figure 2). Glucosylation of hydroxycinnamic acids produces glucose esters that occur at low levels in the grape berry (Monagas et al., 2005) whereas high levels of the tartaric acid esters of caffeic, ferulic and *p*-coumaric acids (caftaric, fertaric and coutaric acid, respectively) (Figure 2., Romeyer et al., 1983; Moskowitz and Hrazdina, 1981; Jaworski and Lee, 1987; Oszmianski and Lee, 1990; Lameula-Raventós and Waterhouse, 1994; Monagas et al., 2005; Kennedy et al., 2006; Mozetič et al., 2006) accumulate in the vacuoles of *Vitis vinifera* mesocarp and exocarp tissues. Despite the abundance of these metabolites in grapes, the pathway leading to their biosynthesis is poorly characterized.

Two glucosyltransferases producing glucose esters of hydroxycinnamic acids *in vitro* have been cloned and functionally characterized from *Vitis* spp. The *p*HBA glucosyltransferase described above (Meyer et al., 2003) glucosylates hydroxycinnamic acids at low levels; while a glucosyltransferase from Concord grape (VLRSGT), preferentially produces glucose esters of hydroxycinnamic acids (Chapter 3). Gene expression profiling and metabolite analysis indicate that *VLRSGT* is expressed in the mesocarp of post-veraison *Vitis labrusca* grape berries, the site of accumulation of these



Hydroxycinnamic acids

	Substitution Patterns		
	R1	**R2**	**R3**
Cinnamic acid	H	H	H
***p*-coumaric acid**	H	OH	H
Caffeic acid	OH	OH	H
Ferulic acid	OCH_3	OH	H
Sinapic acid	OCH_3	OH	OCH_3

Hydroxycinnamic acid glucose esters

	Substitution Patterns		
	R1	**R2**	**R3**
Cinnamic acid	H	H	H
***p*-coumaric acid**	H	OH	H
Caffeic acid	OH	OH	H
Ferulic acid	OCH_3	OH	H
Sinapic acid	OCH_3	OH	OCH_3

Hydroxycinnamic acid tartaric acid esters

	Substitution Pattern
	R1
Caftaric acid (*trans*-caffeoyltartaric acid)	OH
Coutaric acid (*p*-coumaroyltartaric acid)	H
Fertaric acid (*trans*-feruloyltartaric acid)	OCH_3

Figure 2. Proposed biosynthetic pathway leading to the biosynthesis of hydroxycinnamic acid glucose esters and tartaric acid esters in grapes (*Vitis* spp.). Modified from Monagas et al., 2005.
Abbreviations: VLRSGT - *Vitis labrusca* resveratrol/hydroxycinnamic acid *O*-

metabolites, which is consistent with a role for this enzyme in the glucosylation of hydroxycinnamic acids (Figure 2; Chapter 3). In plants, glucose esters are high-energy intermediates involved in the biosynthesis of phenylpropanoid and fatty acid esters (Li and Steffens, 2000; Lehfeldt et al., 2000). Enzyme assays with crude desalted protein extracts from Concord grape berry suggest a similar biosynthetic pathway in grapes that uses the caffeic, *p*-coumaric, and ferulic acid glucose esters as high energy intermediates for acylation of tartaric acid to yield caftaric, coutaric and fertaric acid, respectively (Figure 2; Chapter 3). Additionally, hydroxycinnamic acids are precursors in the biosynthesis of flavonoids, stilbenes and coumarins or they can be converted to their corresponding alcohols which contribute to the structure of the plant cell and cell wall fortification as lignins, lignans and suberized tissue (Buchanan et al., 2000).

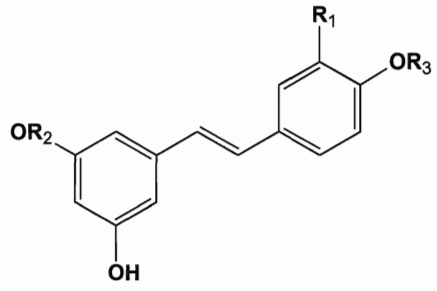
In grape juice and wine, high levels of hydroxycinnamic acids act as copigments with anthocyanins to increase anthocyanin stability and to alter the colour of wine. Wine colour is also affected during fermentation when these tartaric acid esters are oxidized to their highly reactive quinone derivatives by polyphenol oxidase and then oxidized with several other components of the must to produce an undesirable brown colour (Romeyer et al., 1983; Cheynier et al., 1990; Monagas et al., 2005).

2.6 - Grapes accumulate stilbenes which have important roles as phytoalexins and neutraceuticals

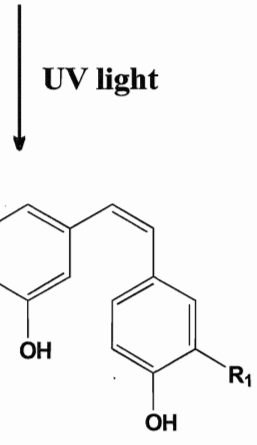
Stilbenes are phenylpropanoids which accumulate constitutively, or accumulate in response to stress in a few diverse plant species, including grapes (Fremont, 2000). The enzyme stilbene synthase (StSy) catalyzes the reaction of hydroxycoumaroyl CoA and

three molecules of malonyl CoA (from fatty acid biosynthesis) to produce *trans*-resveratrol (Figure 3). StSy is closely related to chalcone synthase (CHS, see below) in plants which accumulate stilbenes (Tropf et al., 1994; Goodwin et al., 2000), and in grapes, StSy exists as large multifamily gene (15-20 copies per genome) (Sparvoli et al., 1994). Molecular cloning of a full-length *StSy* from *Vitis vinifera* and heterologous expression of the recombinant protein provides evidence that StSy acts as a homodimer to produce resveratrol, but does not produce chalcone or naringenin (the products of the CHS reaction) (Melchior and Kindl, 1990).

The production and accumulation of stilbenes in *Vitis* is dependent upon grape cultivar, tissue and developmental stage (Jeandet et al., 1991; Ali and Strommer, 2003; Versari et al., 2001). The *trans*-isomers of stilbenes are easily converted to their *cis*-forms by exposure to ultraviolet (UV) light (Figure 3; Trela and Waterhouse, 1996), although predominantly the *trans*- isomers are found in grapes. The amount of stilbene within the grape berry is controlled by *StSy* expression whose expression is affected by several endogenous stimuli, environmental cues (wilting, elevation and heavy metals), viticultural practices, the tissue type, the developmental stage, and the exposure to fungal elicitors (Versari et al., 2001; Bavaresco et al., 1999; Bavaresco, 2003; Li et al., 2006). In grapevine, *trans*-resveratrol and its derivatives accumulate in the leaves, stems and at low levels in whole grape berries (Langcake, 1981; Langcake and Pryce, 1977a, b), with local concentrations of stilbenes within the berry exocarp reaching levels of 1 mM (Creasy and Creasy, 1998). Treatment of grape berry tissues or cell cultures with UV-light is well documented to induce *StSy* gene expression, stilbene accumulation and to



	Substitution Patterns		
	R1	**R2**	**R3**
***Trans*-resveratrol**	H	H	H
***Trans*-piceid**	H	GLUC	H
***Trans*-piceatannol**	OH	H	H
***Trans*-astringin**	OH	GLUC	H
***Trans*-resveratroloside**	H	H	GLUC



	Substitution Patterns	
	R1	**R2**
***Cis*-resveratrol**	H	H
***Cis*-piceid**	H	GLUC
***Cis*-piceatannol**	OH	H
***Cis*-astringin**	OH	GLUC

Figure 3. *trans-* and *cis-* isomers of the stilbene monomers which are detected in *Vitis* spp. (Modified from Trela and Waterhouse, 1996; Monagas et al., 2005).

produce fruit and wines with increased stilbene content (Bais et al., 2000; Cantos et al., 2000; Cantos et al., 2001; Cantos et al., 2003; Bonomelli et al., 2004; Borie et al., 2004). Several other studies have also shown that *StSy* gene expression, enzyme activity and stilbene accumulation is activated following treatment with methyl jasmonate (Krisa et al., 1999; Larronde et al., 2003; Tassoni et al., 2005; Belhadj et al., 2006) or L-alanine (Chen et al., 2006c).

Stilbenes are well-documented phytoalexins, which accumulate within the plant following fungal attack (Langcake and Pryce, 1977a; Jeandet et al., 1991) and in roots, before and after infestation by phylloxera (Kellow et al., 2004). Grape cell suspension cultures or plantlets infected with fungi (Calderón et al., 1993; Wiese et al., 1994; Vezzulli et al., 2007) or treated with general elicitors of plant defense (benzothiadiazole; crab, algal, fungal and plant cell wall and membrane metabolites and/or other glucan derivatives) activate *StSy* gene expression, enzyme activity and the extracellular accumulation of resveratrol (Melchior and Kindl, 1991; Bézier et al., 2002; Aziz et al., 2003; Aziz et al., 2004; Iriti et al., 2004; Bru et al., 2006; Laquitaine et al., 2006; Trotel-Aziz et al., 2006; Aziz et al., 2007) and demonstrate increased resistance to fungal infection (Coutos-Thévenot et al., 2001; Repka, 2001; Bézier et al., 2002).

Hydroxylation of *trans*-resveratrol produces tri-and tetra-hydroxylated stilbenes, while glucosylation at the 3-*O* or 4-*O* positions produces the *trans*-piceid and *trans*-resveratroloside respectively (Figure 3., Monagas et al., 2005). Glucosylation of *trans*-resveratrol increases its half-life by protecting it from oxidation by endogenous polyphenol oxidases (Regev-Shoshani et al., 2003), as such, *trans*-piceid is the predominant stilbene in grapes and wine. An enzyme which glucosylates *trans*-

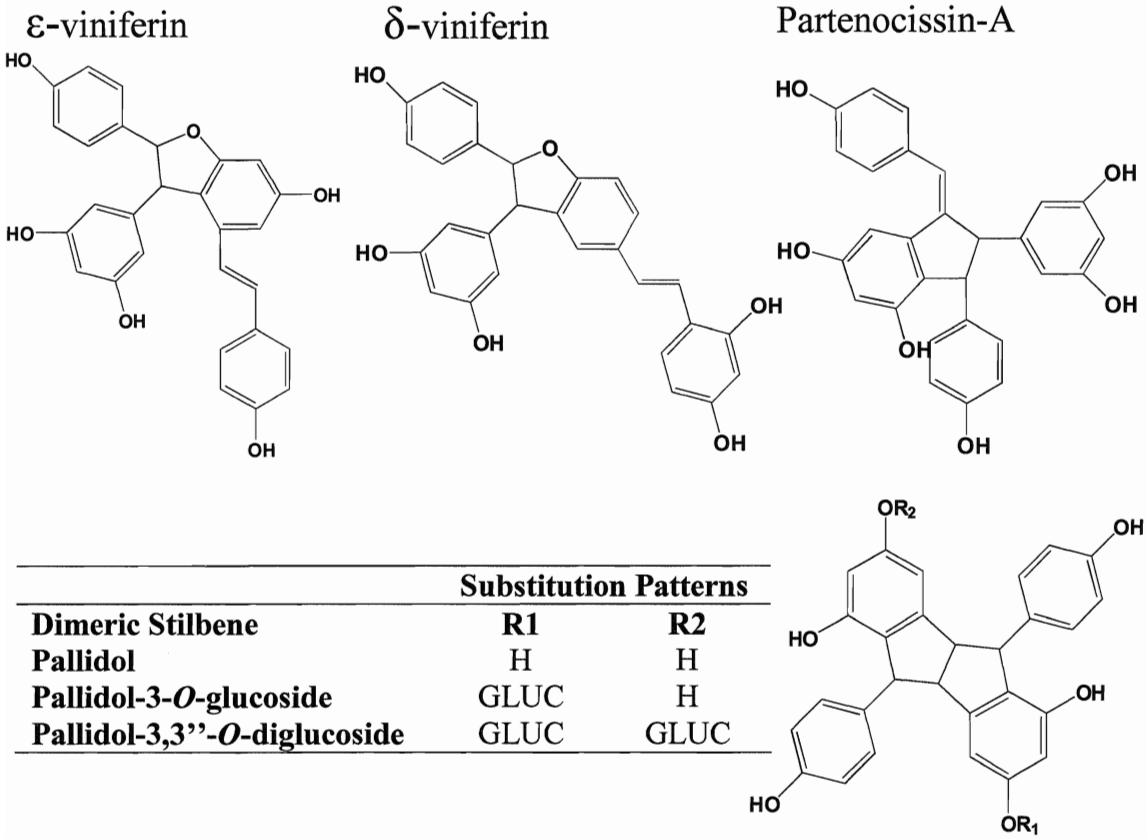
resveratrol to produce *trans*-piceid was first identified in *Vitis vinifera* cell suspension culture, and was subjected to partial purification and preliminary biochemical characterization (Krasnow and Murphy, 2004). Molecular cloning and *in vitro* characterization of rVLRSGT identified *trans*-resveratrol as an acceptor substrate, which produced *trans*-piceid and *trans*-resveratroloside (Chapter 3). Within the developing grape berry, *VLRSGT* is co-ordinately expressed with *StSy*, and kinetic analyses and metabolite profiling of these tissues are consistent with a role for this enzyme in stilbene glucosylation (Chapter 3).

Stilbene dimers, trimers and tetramers known as viniferins are present in grapes and in wine (Figure 4., Langcake and Pryce, 1977b, Jeandet et al., 2002; Guebailia et al., 2006). These polymers are more potent phytoalexins than their monomeric precursors (Langcake and Pryce, 1977b, Jeandet et al., 2002), are correlated with increased fungal resistance in grapevine (Barlass et al., 1987; Sbaghi et al., 1995) and accumulate in response to hormones and stresses including treatment with methyl jasmonate, UV light and ozone (Belhadj et al., 2006; Gonzàlez-Barrio et al., 2006). The biosynthesis of resveratrol oligomers is controlled by peroxidases, and expression of these genes is induced upon fungal attack (Calderón et al., 1993; Jeandet et al., 2002).

2.7 - The structural enzymes involved in flavonoid biosynthesis have been well characterized in *Vitis* spp.

Grapes biosynthesize several diverse flavonoids, including the flavonols, the condensed tannins (flavan-3-ols), the leucoanthocyanidins (flavan-3,4-diols) and the

A. Stilbene dimers



	Substitution Patterns	
Dimeric Stilbene	**R1**	**R2**
Pallidol	H	H
Pallidol-3-*O*-glucoside	GLUC	H
Pallidol-3,3''-*O*-diglucoside	GLUC	GLUC

B. Stilbene polymers

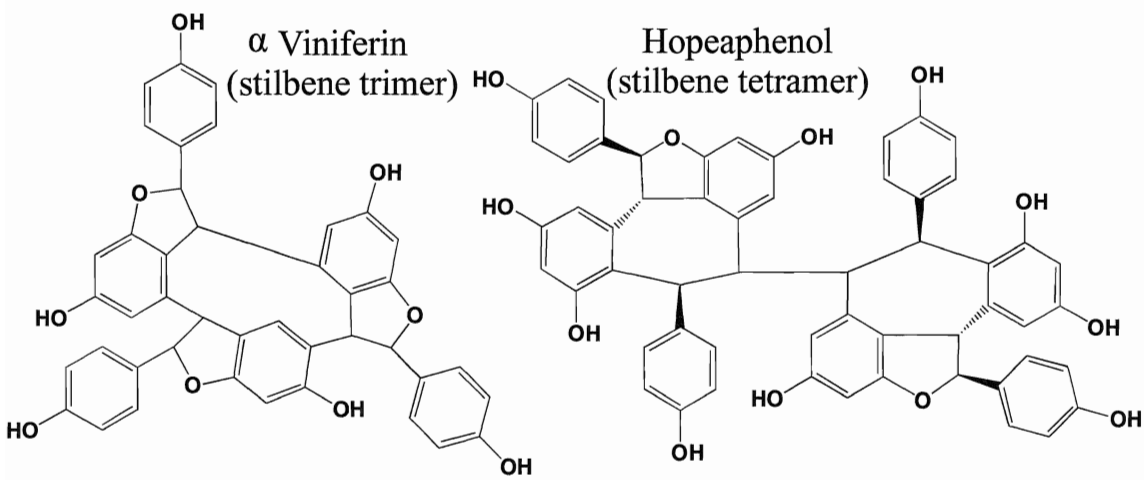


Figure 4. Dimeric and polymeric stibenes which are detected in grape (*Vitis* spp.). A. Stilbene dimers which are detected in *Vitis* spp. B. Stilbene polymers which are detected in *Vitis* spp. (Modified from Jeandet et al., 2002; Monagas et al., 2005; Guebailia et al., 2006)

anthocyanins. Hydroxylation, acylation, methylation, glucosylation, and polymerization, increases the structural diversity of these compounds and changes their biochemical properties (Soleas et al., 1997; Monagas et al., 2005). In grapes, the key enzymes of the flavonoid biosynthesis (Figure 5) have been well-characterized; and similar to other pigmented fruits, these enzymes are expressed biphasically throughout berry ripening (Boss et al., 1996a; Chen et al., 2006a; Jaakola et al; 2002; Halbwirth et al., 2006).

2.7.1 - Chalcone synthase (CHS) is the first committed step in flavonoid biosynthesis

Chalcone synthase (CHS) catalyzes the condensation of *p*-coumaroyl CoA with three molecules of malonyl-CoA to produce naringenin chalcone (4,2',4',6'-tetrahydroxychalcone; Figure 5). The CHS reaction is the first committed step in flavonoid biosynthesis and unlike StSy, CHS occurs ubiquitously throughout the plant kingdom (Goto-Yamamoto et al., 2002). In grapes, *CHS* exists as a multigene family (3-4 copies per genome) (Sparvoli et al., 1994) and transcriptional profiling of three grapevine *CHS* genes shows differential expression in the leaves and berry exocarp of red and white cultivars (Goto-Yamamoto et al., 2002).

CHS is expressed in grape cell suspension cultures, leaves, tendrils, stems, roots, seeds, flowers and within the grape berry where *CHS* transcript is detected in pre-veraison mesocarp and exocarp tissue as well as during veraison in the exocarp with maximal expression occurring 2 weeks post-flowering (Hrazdina et al., 1984; Kakegawa et al., 1995; Boss et al., 1996a, b; Waters et al., 2005). Heterologous expression of a *Vitis vinifera* CHS facilitated the production of CHS-specific antibodies to identify the grape leaves, stems and berries, as the locations of CHS protein expression (Tian et al., 2006a),

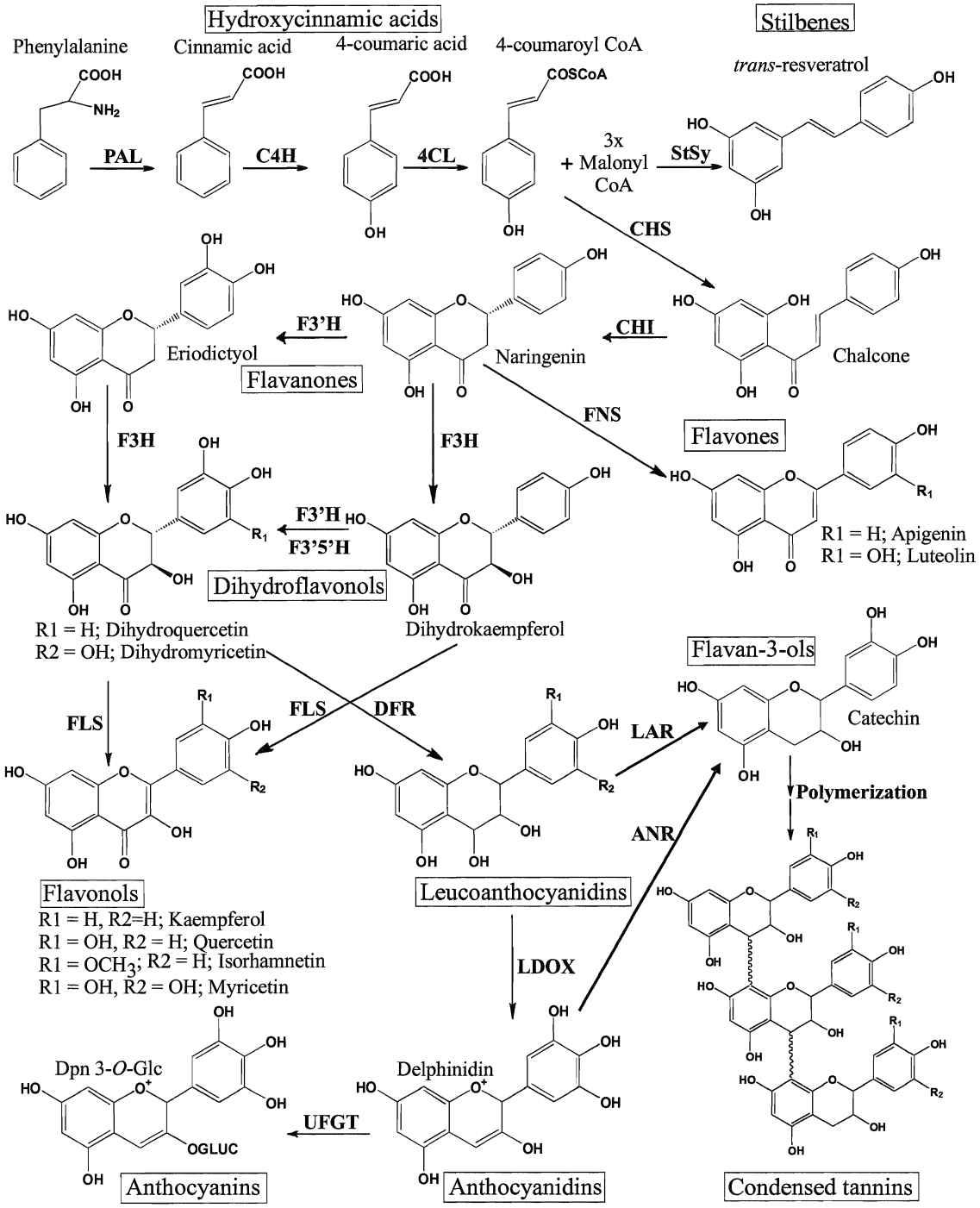


Figure 5. The phenylpropanoid biosynthetic pathway in grapes (*Vitis* spp.). Abbreviations: PAL - phenylalanine ammonia lyase; C4H - cinnamate 4-hydroxylase; 4CL - hydroxycoumaroyl coenzyme A ligase; StSy - stilbene synthase; CHS - chalcone synthase; CHI - chalcone isomerase; FNS - flavone synthase; F3H - flavanone 3-hydroxylase; F3'H - flavanone 3'-hydroxylase; F3'5'H - flavanone 3'5'-hydroxylase; FLS - flavonol synthase; DFR - dihydroflavonol 4-reductase; Ldox - leucoanthocyanidin dioxygenase; LAR - leucoanthocyanidin reductase; ANR - anthocyanidin reductase; UFGT - UDP-glucose: flavonoid 3-*O*-glucosyltransferase; Dpn 3-*O*-Glc - Delphinidin 3-*O*-glucoside. Modified from Boss et al., 1996a;

with maximal CHS protein accumulating in grape berry during veraison, consistent with *CHS* gene expression (Deytieux et al., 2007). Naringenin chalcone is highly reactive and rarely accumulates in nature, serving almost exclusively as an intermediate in the biosynthesis of more elaborate and stable compounds.

2.7.2 - Chalcone isomerase (CHI) produces the flavanone naringenin from naringenin chalcone

Chalcone isomerase (CHI) catalyzes the cyclization of naringenin chalcone to generate the flavanone naringenin (Figure 5), a common intermediate in the biosynthesis of isoflavonoids, flavones, dihydroflavonols, flavonols, flavan-3,4-diols, catechins, and anthocyanins. In grapes, *CHI* is a single copy gene (Sparvoli et al., 1994) expressed early in grape leaf development, in grape tendrils, stems, roots, seeds, flowers and biphasically in berry exocarp and mesocarp tissues (Boss et al., 1996a, b; Goes da Silva et al., 2005; Waters et al., 2005). *CHI* gene expression is induced in grape exocarp tissue treated with ABA and sugar (Hiratsuka et al., 2001), and in 2 week old seedlings after exposure to light (Sparvoli et al., 1994), while CHI protein expression is induced in transgenic grapevine overexpressing ADH (Sauvage et al., 2007).

Although grapes are not known as dietary sources of naringenin, wine contains low levels of this flavanone (Wang and Huang, 2004). Four flavonoid *O*-glucosyltransferases isolated from Concord grape were expressed heterologously and were shown glucosylate the 7-*O*-position of naringenin at low levels *in vitro* (Chapter 3, Chapter 4), however, as grapes do not accumulate naringenin 7-*O*-glucoside the biological relevance of this reaction *in planta* has not been clarified.

2.7.3 - Hydroxylation at the 3, 3', and 5' positions diversifies the in vivo flavonoid profile in Vitis spp.

In *Vitis* spp. flavanones are converted to dihydroflavonols and flavones by the CYPs, flavanone 3-hydroxylase (F3H) and flavone synthase (FNS), respectively (Figure 5). As stated earlier, CYPs are attached to the cytosolic face of the endoplasmic reticulum (ER), and hydroxylate secondary metabolites, which allows further modification by methylation, acylation and glucosylation of the hydroxyl group (Koes, 1994).

F3H converts naringenin to dihydrokaempferol, an intermediate in the biosynthesis of flavonols, flavan-3,4-diols, catechins, and anthocyanins. Flavonoid 3'hydroxylase (F3'H) converts dihydrokaempferol to dihydroquercetin, and flavonoid 3'5'hydroxylase (F3'5'H) converts dihydrokaempferol to dihydroquercetin (F3'H activity) and to dihydromyricetin (F5'H activity) (Figure 5). Within the grape genome, *F3H* and *F3'H* are present in two copies, while only one copy of *F3'5'H* is present (Sparvoli et al., 1994; Jeong et al., 2006). The *in vitro* characterization of CYP enzymes is complicated by the inability to produce large amounts of soluble protein (Yun et al., 2006). *In planta* characterization by suppression or overexpression of CYP enzymes is a common technique for elucidating the function of these enzymes. Isolation of *F3'H* and *F3'5'H* from *Vitis*, and overexpression of these transcripts in *Petunia* x *hybrida*, resulted in the accumulation of flavonoids with novel patterns of B-ring di- and tri-hydroxylation, (Bogs et al., 2006).

F3H gene expression is light-inducible (Sparvoli et al., 1994); and is expressed in leaves, tendrils, stems, roots, seeds, flowers and biphasically in grape berry exocarp and

mesocarp tissues (Boss et al., 1996a,b), with maximal transcript in berries detected two weeks after flowering (Waters et al., 2005). While F3H protein accumulates to high levels in post-veraison grape berries (Deytieux et al., 2007), its expression is down-regulated in response to salinity and drought stress (Vincent et al, 2007).

F3'H and *F3'5'H* transcripts are detected in grape leaf, flower, root and seed tissue and accumulate biphasically in the exocarp of red (but not white) berries throughout development (Castellarin et al., 2006; Jeong et al., 2006, Bogs et al., 2006; Mori et al., 2007). Additionally, *F3'5'H* gene expression that is induced by high vineyard temperatures (Mori et al., 2007), has been suggested to participate in the regulation of anthocyanin biosynthesis in white grape cultivars, whereas the ubiquitous expression of F3H implies that pathway control is exerted elsewhere.

Dihydroflavonols accumulate within the grape berries and stem and are precursors for the production of flavan-3-ols, flavonols and anthocyanins with identical B-ring hydroxylation patterns (Trousdale and Singleton, 1983; Souquet et al., 2000; Pozo-Bayón et al., 2003; Monagas et al., 2005; Castellarin et al., 2006; Mattivi et al., 2006). The ratio of F3'H and F3'5'H activity and the production of differentially hydroxylated dihydroflavonols corresponds to the final ratios of hydroxylated anthocyanins and flavonols within the grape berry and ultimately dictate the colour characteristics of the berry and of wine. In wine, the predominant dihydroflavonols are dihydroquercetin, dihydroquercetin 3-*O*-rhamnoside (astilbin; Souquet et al., 2000; Landrault et al., 2002; Monagas et al., 2005), dihydrokaempferol 3-*O*-rhamnoside (Souquet et al., 2000) and dihydrokaempferol 3-*O*-glucosides (Pozo-Bayón et al., 2003). In addition to serving as intermediates in polyphenol biosynthesis, dihydroflavonols have antifungal properties

(Landrault et al., 2002) and their oxidation contributes to the browning of wines (Trousdale and Singleton, 1983).

Whereas *F3'H* and *F3'5'H* have been cloned and characterized from *Vitis* spp., the F3H enzyme has not been cloned or characterized. Several enzymes that glucosylate dihydroflavonols *in vitro* have been identified, including the UDP-glucose: 3-*O*-glucosyltransferase (UFGT; see below) and four glucosyltransferases isolated from Concord grape that glucosylate the 7-*O*-position of dihydroquercetin at low levels (Chapter 3, Chapter 4), however the *in planta* relevance of these reactions is unknown.

2.7.4 - Flavonol synthase (FLS) converts dihydroflavonols to their corresponding flavonols

Flavonol synthase (FLS), a 2-oxoglutarate dependent dioxygenase, that converts dihydroflavonols (dihydrokaempferol, dihydroquercetin, dihydromyricetin) to their corresponding flavonols (kaempferol, quercetin, myriciten; Figure 5), has been cloned and biochemically characterized from several plant species (Holton et al., 1993; Wellmann et al., 2002; Lukačin et al., 2003; Takahashi et al., 2007). A full-length *FLS* gene has not been cloned from *Vitis* spp., however five putative *FLS* transcripts are differentially expressed in grape tissues (leaves, buds, inflorescences, berries) (Downey et al., 2003; Fujita et al., 2006) and in response to shading or hormone treatments (Fujita et al., 2006; Pereira et al., 2006)

Flavonol accumulation in grapes is dependant on the variety, environmental conditions, developmental stage and tissue (Souquet et al., 2000; Cantos et al., 2002; Castillo-Muñoz et al., 2007). The predominant flavonols in all grape berries are the 3-*O*-

glucosides of quercetin, isorhamnetin, and kaempferol, with smaller amounts of 3-*O*-galactosides, 3-*O*-glucuronides, 7-*O*-glucosides and the methoxylated derivatives of these metabolites (Park and Cha., 2003; Monagas et al., 2005; Wu and Prior, 2005; Castillo-Muñoz et al., 2007); whereas grape leaves, also produce quercetin 3-*O*-glucoside-7-*O*-glucuronide, and kaempferol 3,7-*O*-diglucoside (Park and Cha, 2003). Consistent with the lack of F3'5'H gene expression and enzyme activity in white grapes, only red grapes accumulate the 5' hydroxylated flavonols (myricetin, laricitrin, syringetin) (Mattivi et al., 2006).

UFGT is capable of glucosylating flavonols at the 3-*O* position *in vitro*, however kinetic analysis of the recombinant enzyme suggests that anthocyanidins, and not flavonols are the preferred substrates for this enzyme (Boss et al., 1996a, b; Ford et al., 1998; Offen et al., 2006; Chapter 5). Four enzymes from Concord grapes have been characterized that glucosylate flavonols at the 7-*O* position at low levels *in vitro*; however, kinetic analysis indicates that these are not likely to be the preferred *in vivo* substrates for these enzymes (Chapter 3, Chapter 4).

Within the grape, flavonols appear yellow in colour and are bitter in taste, characteristics which are important for their functions as UV-protectants and insects repellants, respectively (Flint et al., 1985; Koes et al., 1994; Simmonds et al., 2003). In wine making, glycosylated flavonols are hydrolyzed and the corresponding aglycones are found in wine, where they form complexes with volatiles, tannins and anthocyanins influencing the final colour, flavour and aroma of the wine (Monagas et. at., 2005).

2.7.5 - Flavone synthase (FNS) catalyzes the production of luteolin and apigenin in grape tissues

Flavone synthase (FNS) is a nicotinamide adenine dinucleotide phosphate (NADPH)- and O_2-dependent CYP monooxygenase which has two proposed reaction mechanisms in plants (Martens and Mithöfer, 2005). In grapes, little is known about the FNS reaction and the corresponding gene has not been cloned or characterized from *Vitis* spp. Grape leaves, but not berries contain the 7-*O*-glucosides of apigenin and luteolin (Moore and Giannasi, 1994; Hmamouchi et al., 1996), while these glucosides as well as baicalein have been detected in wine (Wang and Huang, 2004; Bevilacqua et al., 2004). Although little is known about flavone biosynthesis in grapes, three glucosyltransferases that can glucosylate the 7-*O* position of luteolin are expressed in Concord grape leaves and berries (Chapter 4), and these enzymes may produce flavones *in planta*.

Flavones are involved in copigmentation, UV-protection and signaling between the plant and its environment (reviewed in Martens and Mithöfer, 2005), and may be involved in nodulation (Peters et al., 1986; Zhang et al., *in press*). The contribution of these metabolites to the organoleptic attributes of wine is poorly studied; however, flavones impart a bitter taste (Shin et al., 1995) and can copigment with anthocyanins to influence the flavour, aroma and colour of wine (reviewed in Ellestad, 2006).

2.7.6 - Dihydroflavonol 4-reductase (DFR) is essential for the biosynthesis of anthocyanins and condensed tannins

Dihydroflavonol 4-reductase (DFR) catalyzes the NADPH-dependent reduction of dihydroflavonols to their corresponding leucoanthocyanidins (flavan-3,4-diols; Figure

5), key intermediates in the biosynthesis of anthocyanins and condensed tannins. Two copies of *DFR* are present in the *Vitis* genome (Sparvoli et al., 1994) and the full-length gene was recently cloned and biochemically characterized from post-veraison *Vitis vinifera* cv. Cabernet sauvignon grape berry tissue (Petit et al., 2007). Additionally, the recent crystal structure of DFR has been resolved, yielding information about the structure and function of this enzyme including the identification of amino acid residues important for substrate specificity (Petit et al., 2007).

DFR is expressed almost ubiquitously in grape leaves, tendrils, stems, roots, seeds, flowers and throughout ripening in mesocarp and exocarp tissues (Boss et al., 1996a, b). Gene expression analysis of *DFR* and its promoter (expressed as a GUS fusion protein) indicates that expression of this gene is induced in response to a number of exogenous stimuli including light (Sparvoli et al., 1994), night temperature (Mori et al., 2005), ethanol (El-Kereamy et al., 2002), methyl jasmonate (Tassoni et al., 2005) calcium and sucrose (Gollop et al., 2002), but is not affected by application of ethylene (El-Kereamy et al., 2003).

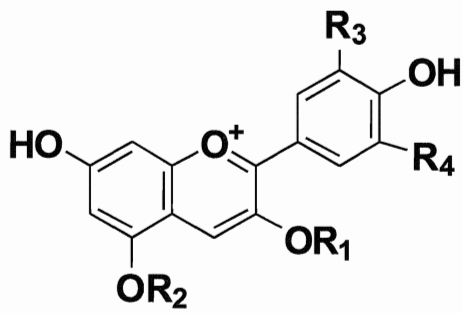
Leucoanthocyanidins are colourless, unstable metabolites that do not accumulate within grape berries. They appear to function exclusively as intermediates in the biosynthesis of anthocyanins, catechins and proanthocyanidins (condensed tannins) that do accumulate to high levels in grapes (Adams, 2006).

2.7.7 - Leucoanthocyanidin dioxygenase (Ldox) converts the colourless leucoanthocyanidins to the brightly coloured anthocyanidins

Leucoanthocyanidin dioxygenase (Ldox) is a 2-oxoglutarate-dependent enzyme that converts leucoanthocyanidins to anthocyanidins (Figure 5). Functional characterization of this enzyme is complicated by the instability of the both the substrate and the product of this reaction and has not been well studied. In grapes, *Ldox* is a single copy gene (Sparvoli et al., 1994; Gollop et al., 2001) expressed in red cell suspension cultures, intact plantlets, leaves, tendrils, stem, roots, seeds, flower and berries (Boss et al., 1996a, b; Gollop et al., 2001).

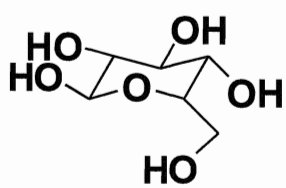
Ldox gene expression is biphasic in grape berry exocarp tissues, whereas the berry mesocarp only accumulates *Ldox* transcript before veraison (Boss et al., 1996a, b). *Ldox* gene and protein expression in grape berries is directly related to the biosynthesis and accumulation of condensed tannins before veraison and anthocyanins during veraison (Bogs et al., 2005; Deytieux et al., 2007). Additionally, *Ldox* gene expression can also be induced by external stimuli, including light (Sparvoli et al., 1994), night temperature (Mori et al., 2005), sucrose and calcium (Gollop et al., 2001).

Anthocyanidins are unstable, water-soluble, positively charged aglycones that are immediately glucosylated at the 3-*O*-position to produce stable and colourful anthocyanins (Kong et al., 2003). Five anthocyanidin backbones occur within grapes, cyanidin, delphinidin, malvidin, peonidin, petunidin (Figure 6) that differ in their B-ring hydroxylation and methylation pattern. The role of anthocyanidins in plants and in wine will be discussed below in the context of their stable glucosylated derivatives (see below).

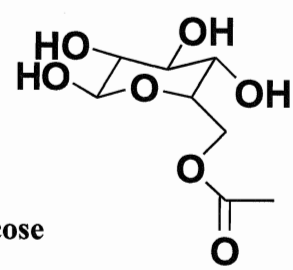


	Substitution Patterns			
Aglycone	**R1**	**R2**	**R3**	**R4**
Delphinidin	OH	OH	OH	OH
Cyanidin	OH	OH	OH	H
Malvidin	OH	OH	OCH_3	OCH_3
Petunidin	OH	OH	OCH_3	OH
Peonidin	OH	OH	OCH_3	H

R1 and/or R2 = Glucose



R1 = *p*-acetoyl Glucose



R1 = *p*-coumaroylated Glucose

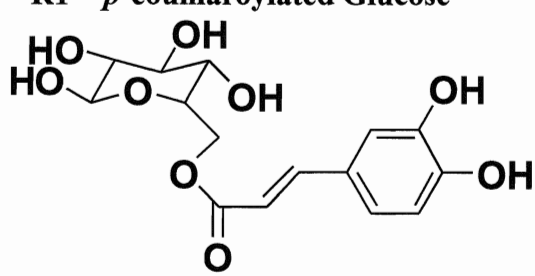


Figure 6. Anthocyanin substitution patterns detected in *Vitis* spp. Modified from Cantos et al., 2002; Monagas et al., 2005; and Wu and Prior, 2005.

2.7.8 - Leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) produce flavan-3-ol substrates for polymerization into condensed tannins

Grapes biosynthesize and accumulate the flavan-3-ols (proanthocyanidins) catechin, and epicatechin (Souquet et al., 1996; Monagas et al., 2005; Adams, 2006) that polymerize to form condensed tannins. Further modification of catechins and condensed tannins by galloylation and unique polymerization patterns increases the diversity of these metabolites within the grape (Kennedy et al., 2001; Adams, 2006). Proanthocyanidins are biosynthesized from either leucoanthocyanidin or cyanidin in reactions catalyzed by the enzymes leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR), respectively (Figure 5). Two *LAR* genes and one *ANR* gene from grape have been cloned and functionally characterized (Bogs et al., 2005; Fujita et al., 2005; Pfeiffer et al., 2006) to confirm their role in proanthocyanidin biosynthesis. In grape leaves, berry exocarp and seeds, LAR and ANR are involved in the biosynthesis of catechins and condensed tannins throughout development, and specific temporal and spatial gene expression profiles correlate to the polymer profile in these tissues (Bogs et al., 2005; Fujita et al., 2005; Waters et al., 2005).

Flavan-3-ols accumulate and are stored within vacuoles, whereas condensed tannins with comparatively higher degrees of polymerization are generally cell-wall associated (Gagné et al., 2006). While both compounds accumulate to high levels in pre-veraison grape berry seeds and in berry exocarp tissue, their concentrations and degrees of polymerization change throughout berry ripening (Jaworski and Lee, 1987; Souquet et al., 1996; Harbertson et al., 2002; Harbertson et al., 2003; Adams, 2006). Condensed tannins and catechins participate in the plant's resistance to fungal pathogens, insects and

herbivores (Wink, 1988; Koes et al., 1994; Peters and Constabel, 2002) while catechin also has antimicrobial and allelopathic properties that inhibits invasion of other plant species (reviewed in Field et al., 2006). In grape cell suspension cultures, the level of catechin and condensed tannins increases in response to osmotic stress (Decendit and Mérillon, 1996; Larronde et al., 1998), and overexpression of ADH in grape leaves increases the degree of proanthocyanidin polymerization (Tesniere et al., 2006).

In humans, catechins and condensed tannins precipitate salivary proteins that produce the bitterness and astringency characteristics of wine (Rossi and Singleton, 1966; Arnold et al., 1980; Gawel., 1998). Throughout wine fermentation and aging, catechins and condensed tannins prevent oxidation (Berg and Akiyoshi, 1956), and form complexes with anthocyanins or proteins (Singleton and Trousdale, 1992), producing unique spectral and organoleptic characteristics, especially in wine fermented with tissues that accumulate these metabolites (berry seeds and berry exocarps) (Adams, 2006). Additionally, catechin is a substrate for polyphenol oxidase and can copigment with anthocyanins, acetaldehyde and tartaric acid to contribute to the browning of wine (Monagas et al., 2005)

2.7.9 - Flavonoid 3-O glucosyltransferase (UFGT) glucosylates anthocyanins and flavonols

Glucosylation of anthocyanidins at the 3-*O*-position is essential for stabilization and accumulation of these colourful compounds within the vacuoles of red grape berry exocarp tissue (Moskowitz and Hrazdina, 1981). These metabolites are essential for the pigmentation of the grape berry and are responsible for the red and blue colours of grape

juice and wine (Offen et al., 2006; Prior and Wu, 2006). The enzyme which glucosylates anthocyanidins at the 3-*O*-position is the UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UFGT) (Figure 5). This enzyme was first partially purified and biochemically characterized from *Vitis vinifera* cell suspension cultures (Do and Cormier, 1995), and since then, the gene for this enzyme, and the corresponding recombinant protein has been the focus of extensive molecular and biochemical characterization. The *UFGT* has been cloned and biochemically characterized from both *Vitis vinifera* (Ford et al., 1998) and *Vitis labrusca* (Chapter 5) and the crystal structure of the *Vitis vinifera* UFGT protein has recently been elucidated (Offen et al., 2006).

In grapes, *UFGT* is a single copy gene (Sparvoli et al., 1994) expressed in anthocyanin-accumulating tissues including post-veraison berry exocarp tissue, which is also the site of UFGT protein expression and enzyme activity (Boss et al., 1996a, b; Kobayashi et al., 2001; Deytieux et al., 2007). *In vitro* kinetic analysis of the recombinant UFGT suggests that this enzyme preferentially glucosylates anthocyanins, as compared to flavonols; consistent with a role for UFGT in anthocyanin (but not flavonol) biosynthesis in grapes (Ford et al., 1998; Offen et al., 2006; Chapter 5). Additionally, tissues such as pre-veraison grape berries, berry mesocarp tissue or grapevine vegetative tissues do not accumulate anthocyanins and do not express UFGT (Boss et al., 1996a, b; Kobayashi et al., 2001). Unlike plant species that regulate anthocyanin biosynthesis at early reactions in the biosynthetic pathway (Mol et al., 1998; Koes et al., 2005), grapes exert transcriptional control of anthocyanin biosynthesis by regulating *UFGT* expression. For example, *UFGT* is only expressed in the post-veraison exocarp of red, but not white *Vitis* grape berries (Boss et al., 1996a, b; Kobayashi et al., 2001) and regulation of UFGT

gene expression is controlled by two *MYBA* transcription factors (see below; Kobayashi et al., 2002); which are mutated and incapable of activating UFGT transcription in white grapes (Kobayashi et al., 2004; Walker et al., 2007).

UFGT gene expression is also transcriptionally regulated by a number of plant hormones and external stimuli. Treatment of grape cell suspension cultures with synthetic auxin or indole-3-acetic acid and treatment of grape berries with ethylene increased the transcription of *UFGT* (El-Kereamy et al., 2003); interestingly, treatment of grape berries with synthetic auxin delayed *UFGT* gene expression and anthocyanin biosynthesis (Davies et al., 1997; Kokubo et al., 2001). *UFGT* transcription and anthocyanin biosynthesis decrease in response to increased night temperatures (Mori et al., 2005) and treatment of cell cultures with a fungal metabolite (Afifi et al., 2003), but increased in response to sucrose (Decendit and Mérillon, 1996; Larronde et al., 1998), ethanol (El-Kereamy et al., 2002), light exposure and jasmonate elicitation in seedlings and cell suspension cultures (Sparvoli et al., 1994; Curtin et al., 2003).

The predominant anthocyanins in red grape berries, juice and wine are the anthocyanidin 3-*O*-glucosides, but smaller amounts of anthocyanin 3,5-*O*-diglucosides and 6'' acylated anthocyanin monoglucosides also accumulate (Figure 6., Mazza and Miniati, 1993; Winkel-Shirley, 2001; Monagas et al., 2005). The wide variety of grape colours in nature is a reflection of the myriad of anthocyanin compounds and polyphenol copigments and these unique, cultivar-specific anthocyanin profiles can be used to identify berry and wine origins (Nùñez et al., 2004).

In general, the anthocyanin profiles of the "elite" *Vitis vinifera* cultivars are simple, and accumulate predominantly anthocyanin 3-*O*-monoglucosides; whereas the

anthocyanin profiles of North American and hybrid cultivars are intricate and often consist of more than 25 differentially modified anthocyanins (Wang et al., 2003; Wu and Prior, 2005). The complex anthocyanin profiles of North American and hybrid varieties, and specifically the accumulation of 3,5-*O*-diglucosides and complex acylation patterns at the 6'' position results in an undesirable blue berry colour, and reduces the utility of these grapes in viticulture and breeding. The enzymes responsible for glucosylation of anthocyanins at the 5-*O*-position has not been identified in grapes, however several attempts at homology-based cloning and protein purification of this enzyme from grape exocarp tissue have been unsuccessful (Chapter 4).

Within the grape, the red, blue and purple colours of anthocyanins play important roles to attract seed dispersers and pollinators (Winkel-Shirley, 2001), and in UV-protection and DNA photorepair (Hada et al., 2003). Accumulation of anthocyanins often indicates cell stress (Winkel-Shirley, 2002) and roles for anthocyanins as antioxidants and phytoalexins suggests the involvement of these metabolites in the defense response of plants against pathogens and insects (Koes, 1994; Boss et al., 1996b; Kong et al., 2003; Brouillard et al., 2003).

In red wine, anthocyanins are the major colourants that impart the desirable sensory spectral characteristics to viticulturally important grape cultivars. As described above, undesirable blue and purple colouration arises from modification of the anthocyanin by glucosylation and acylation, which decreases the agronomic importance of these varieties. Copigmentation and intermolecular stacking of anthocyanins with different phenolics including the hydroxycinnamic acids, catechins, condensed tannins and flavonols changes the stability and spectral characteristics of the wine as it ages,

which is essential for the red colour of "elite" wines (Monagas et al., 2005; Schwarz et al., 2005; Gómez-Míguez et al., 2006).

2.8 - Regulation of polyphenol biosynthesis in grape by MYB transcription factors

The regulation of anthocyanin biosynthesis in grapes has been extensively studied in an effort to correlate the observable phenotypic differences in exocarp colouring (red and white), with the genotype and gene expression profiles in these tissues. Anthocyanin biosynthesis in grape is controlled by the developmental and tissue-specific expression of a series of *MYBA* transcription factors in grape berry exocarp (Kobayashi et al., 2002). A retrotransposon inserted into the upstream sequence of the *Vitis vinifera* (*Vv*) *MYBA1* transcription factor blocks *VvMYBA1* expression and is the molecular basis for a mutation that produced the white grape phenotype from a red grape (Kobayashi et al., 2004; Lijavetzky et al., 2006). A second adjacent transcription factor (*VvMYBA2*) also regulates grape berry colour, and in white (but not red) grape berries, *VvMYBA2* is mutated twice, resulting in the expression of a truncated inactive protein unable to activate *UFGT* transcription (Walker et al., 2007). *VvMYB5A* also contributes to the transcriptional control of polyphenol metabolism and is expressed in berry exocarp, mesocarp and seeds early stages in berry development. *VvMYB5A* function was defined by overexpression of the *VvMYB5A* gene in tobacco, which correlated to an increase in anthocyanin, flavonol, tannin and lignin biosynthesis and accumulation (Deluc et al., 2006).

Proanthocyanidin biosynthesis is controlled by the *VvMYBPA1* transcription factor that regulates the expression of *LAR*, *ANR,* and other flavonoid pathway genes, but

does not regulate anthocyanin biosynthesis (Bogs et al., 2007). WRKY transcription factors are involved in the defense response of grapes to pathogen attack, and expression of these transcription factors is affected by the developmental stage of the grape berry and leaves, and the signal molecules to which the tissue is exposed (Marchive et al., 2007). Generally, polyphenol biosynthesis in grapes is a strictly regulated pathway by temporal and spatial expression of multiple transcription factors, only a few of which have been characterized. Further studies on additional transcription factors and their control on phenylpropanoid metabolism in grapes would contribute to the overall understanding of these complex pathways.

2.9 - Grape tissue culture provides a year-round supply of plant material, enzymes and secondary metabolites which facilitates the study of the biosynthesis of plant natural products

Plant tissue culture (micropropagation) produces a year-round supply of sterile biological tissue, enzymes and secondary metabolites. Grapes have been vegetatively propagated for several years and *in vitro* micropropagation of grapevine has been used for more than 10 years to aseptically multiply many of the "elite" cultivars of grapevine to preserve their viticulturally desirable genotypic and phenotypic attributes (Deloire et al., 1995).

Callus tissue is generated by manipulation of sterile grape tissues on solid media or in liquid cell suspension cultures with different concentrations of hormones. Solid and liquid cultures have been established and maintained for *Vitis vinifera* cv. Gamay cells for more than 30 years and recently, an equivalent cell culture system has been produced

from *Vitis labrusca* (Chen et al., 2006c). The establishment of tissue culture systems for both European and North American varieties allows the comparison of their biochemical and metabolic properties in response to a number of endogenous and exogenous stimuli.

The accumulation of secondary metabolites in grape cell cultures is well-documented and the selection of cells with increased anthocyanin content allows for a nearly homogenous and synchronized source of pigmented material. Similarly, *in vitro* cultivation allows the adjustment of conditions such as light, temperature, hormones and media composition (osmotic potential i.e. sucrose and ammonium concentration) to induce and increase anthocyanin production in these cells (Do and Cormier, 1990; Do and Cormier, 1991a, b, c; Zhang and Furusaki, 1999; Hiratsuka et al., 2001) and to facilitate the molecular and biochemical characterization of these responses. Throughout this review, grape tissue culture has been used extensively to evaluate and characterize the molecular and biochemical responses of cultured grape cells to osmotic stress, several hormones and the addition of pathogen-derived metabolites to the culture media.

The establishment of grape tissue culture facilitates the production of transgenic tissues either transiently, by biolistic transformation (Kikkert et al., 2005), or stably, by *Agrobacterium tumefaciens*-mediated transformation (Iocco et al., 2001; Bornhoff et al., 2005). Grapevine is an ideal crop plant for genetic modification due to the recent genome sequencing efforts, and since classical breeding techniques often introduce undesirable characteristics in addition to the desired trait. Whole-plant stable transformation systems in grapevine are undesirable due to the long regeneration time (several years) of intact plants whereas cells cultured *in vitro* can be transformed and regenerate within days or weeks. Similarly, somatic embryos of grape have been

established for *Vitis vinifera* (Kikkert et al., 2005; Ben Amar et al., 2007) which can be transformed and regenerated to produce transgenic plants with unique metabolic and biochemical characteristics (Hébert et al., 1993; Scorza et al., 1995; Kikkert et al., 1996, 2005; Vidal et al., 2003; 2006)

Although plant tissue culture has many advantages, the results obtained from experiments which depend on cultured plant tissues must be interpreted with caution since the biology, metabolism and biochemistry of a whole organism with multiple cell types is very different than the single cell-type present in cell suspension cultures.

2.10 – Future studies concerning the phenylpropanoid biosynthetic pathway of *Vitis* spp.

2.10.1 - Anthocyanin Vacuolar Transport

Detoxification of reactive metabolites is often achieved by conjugation to glutathione followed by vacuolar sequestration. In *Zea mays* and *Petunia* x *hybrida*, anthocyanin vacuolar transport requires glutathione *S*-conjugation of the anthocyanin, a reaction catalyzed by glutathione *S*-transferase proteins (GST) (Alfenito et al., 1998; Mueller et al., 2000). A recent study demonstrated that long distance intercellular movement of flavonoids in *Arabidopsis thaliana* is mediated by energy-dependent ABC transporters (Buer et al., 2007). Vacuolar sequestration of glutathione *S*-conjugated anthocyanins also requires energy-dependent transporters located in the vacuolar membrane. In *Arabidopsis thaliana*, a magnesium-requiring ATP binding cassette transporter (*AtMRP1*) has been identified and shown to transport glutathione *S*-conjugated anthocyanins and herbicides (Lu et al., 1997). Additionally, in *Zea mays* an

identical energy-dependent transporter is required for anthocyanin transport and is expressed coincident with the structural genes involved in anthocyanin biosynthesis (Goodman et al., 2004).

It is well established that anthocyanins accumulate in the vacuoles of *Vitis* spp (Moskowitz and Hrazdina, 1981). Equivalent mechanisms of glutathione *S*-conjugation and energy dependent vacuolar transport are likely involved in the sequestration of anthocyanins in *Vitis* spp. The genes and proteins responsible for these reactions have not been identified from grape, however the TIGR grape index contains 24 ESTs with sequence identity to the previously characterized *Petunia* x *hybrida GST* and 54 ESTs with sequence identity to *AtMRP1*, suggesting that in fact, similar mechanisms of anthocyanin conjugation and transport are present but uncharacterized in *Vitis* spp.

2.10.2 - Anthocyanin decoration reactions

Several grape cultivars accumulate anthocyanins with elaborate methylation and acylation patterns (Mazza and Miniati, 1993; Wang et al., 2003). Methylation of the hydroxylated anthocyanins (cyanidin and delphinidin) elaborates the pigment backbone producing peonidin, petunidin and malvidin (Figure 6). An *S*-adenosyl-L-methionine dependent cyanidin 3-*O*-glucoside 3'-*O*-methyltransferase activity has been identified and partially biochemically characterized from *Vitis vinifera* cell suspension cultures (Bailly et al., 1997). The gene corresponding to this activity has not been identified from grape and further studies should concentrate on the cloning and *in vitro* biochemical characterization of these enzymes, including substrate specificity studies and in depth kinetic.

Although several *p*-coumaroylated and malonylated anthocyanins are found in several grape cultivars, and are implicated as increasing the stability of these reactive metabolites (Monagas et al., 2005; Prior and Wu, 2006), the enzymes responsible for these acylation reactions have not been identified. Anthocyanin acyltransferases have been identified from several plant species (Fujiwara et al., 1998; Yonekura-Sakakibara et al., 2000; Suzuki et al., 2001, 2002, 2003, 2004a, 2004b; Luo et al., 2007), and typically belong to the BAHD family of acyltransferases which require the acyl CoA derivative for catalysis. Alternatively, a recent study reports an acyl CoA independent acylation, which instead requires the activated acyl glucose ester for catalysis by a serine-carboxypeptidase like protein (Fraser et al., 2007). Whether anthocyanin acylation in grapes is catalyzed by BAHD-type or serine carboxypeptidase-type acyltransferases (or both) has yet to be established, however, the importance of these enzymes to the colour and stability of grape anthocyanins demands additional study of these reactions in *Vitis* spp.

2.10.3 – Outstanding questions concerning the structural enzymes involved in phenylpropanoid biosynthesis

Although the structural enzymes involved in grapevine phenylpropanoid biosynthesis have been well characterized, several outstanding questions remain to be addressed. Several studies have investigated the transcriptional control of phenylpropanoid biosynthesis in grapes, however studies concerning the additional mechanisms of control, including post-translational modifications of these proteins has not been studied. Several of the enzymes described in this review accept multiple

substrates, have differentially expressed isoforms and catalyze non-specific reactions. The reactions catalyzed by these enzymes *in vivo* are likely dependent on the cellular microclimate, substrate availability, subcellular location and associations with other enzymes within the cell, parameters which require thorough characterization.

2.11 - Conclusion

The biosynthesis of phenylpropanoids in grapes has been well-characterized from a molecular, enzymatic and biochemical perspective. These metabolites have several roles within grapes and they contribute to the organoleptic characteristics of wine. Although several of the enzymes involved in the biosynthesis of these compounds have been well studied, many of the enzymes responsible for the "decoration" (hydroxylation, acylation, methylation and glucosylation) of polyphenols have not been described in detail. Grapes accumulate more than 200 glucosylated metabolites, and analysis of grape ESTs is consistent with the expression of more than 90 GTs in the plant. The agronomic value of grape, the diversity of glucosylated metabolites within the berry, their importance to the biology of the plant, their contribution to the organoleptic attributes of wine and their use as neutraceuticals suggests that the biosynthesis of phenylpropanoids is a relevant target for continued biochemical and molecular characterization.

Chapter 3.

Mesocarp localization of a bifunctional resveratrol/hydroxycinnamic acid glucosyltransferase of Concord grape (*Vitis labrusca*)

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Mesocarp localization of a bifunctional resveratrol/hydroxycinnamic acid glucosyltransferase of Concord grape (*Vitis labrusca*)

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3.1 - Abstract

Resveratrol is a stilbene with well-known health-promoting effects in humans that is produced constitutively or accumulates as a phytoalexin in several plant species including grape (*Vitis* spp.). Grape berries accumulate stilbenes in the exocarp as *cis*- and *trans*-isomers of resveratrol, together with their respective 3-*O*-monoglucosides. An enzyme glucosylating *cis*- and *trans*-resveratrol was purified to apparent homogeneity from Concord (*Vitis labrusca*) grape berries, and peptide sequencing associated it to an uncharacterized *Vitis vinifera* full-length clone (TC38971, TIGR database). A corresponding gene from *Vitis labrusca* (VLRSGT) had 98% sequence identity to clone TC38971 and 92% sequence identity to a *Vitis vinifera* *p*-hydroxybenzoic acid glucosyltransferase that produces glucose esters. The recombinant enzyme was active over a broad pH range (5.5–10), producing glucosides of stilbenes, flavonoids and coumarins at higher pH and glucose esters of several hydroxybenzoic and hydroxycinnamic acids at low pH. *Vitis labrusca* grape berries accumulated both stilbene glucosides and hydroxycinnamic acid glucose esters, consistent with the bi-functional role of VLRSGT in stilbene and hydroxycinnamic acid modification. While phylogenetic analysis of VLRSGT and other functionally characterized glucosyltransferases places it

with other glucose ester-producing enzymes, the present results indicate broader biochemical activities for this class of enzymes.

3.2 - Introduction

Plants produce an amazing diversity of phenolic compounds that are derived from the aromatic amino acid L-phenylalanine, a few of which play essential structural roles in the formation of plant vasculature and in cell-wall composition. Remarkably, the many thousands of different phenols produced by plants appear to play many additional, dynamic and evolving roles in mediating interactions with the environment, including attracting pollinators while defending against most herbivores, as constitutive and inducible products that prevent plant diseases while at the same time playing crucial roles in mediating chemical communications and symbiosis between species, and as protectants against some abiotic stresses. The phenolic compounds of grape berry include hydroxybenzoic and hydroxycinnamic acids, stilbenes, flavan-3-ols, proanthocyanidins and flavonoids (Monagas et al., 2005).

The enzyme stilbene synthase (StSy) catalyzes the formation of resveratrol from one molecule of hydroxycinnamoyl CoA and three molecules of malonyl CoA. Within the plant, stilbenes may accumulate constitutively or may be induced to accumulate as phytoalexins within infected tissues. While stilbenes accumulate at high levels in grape leaves and stems, they occur at significantly lower levels in grape berries (Langcake, 1981; Langcake and Pryce, 1977a,b). Epidemiological studies suggest that stilbene consumption by humans has health-promoting effects (Fremont, 2000; Kris-Etherton et al., 2002), and most studies have focused on the concentration of resveratrol and its

glucosides in wine (Gambuti et al., 2004; Moreno-Labanda et al., 2004; Vitrac et al., 2005) as opposed to the grape berries. Within the berry, stilbenes accumulate to high concentrations within the berry exocarp (up to 1 mM; Creasy and Creasy, 1998), whereas little resveratrol is detected in the berry mesocarp (Creasy and Coffee, 1988). Stilbenes may exist within the berry as aglycones and monoglucosides whose concentration and composition varies with each grape cultivar (Ali and Strommer, 2003; Versari et al., 2001). The glucosylation of stilbenes may protect them from enzymatic oxidation by polyphenol oxidases, thereby increasing their half-life (Regev-Shoshani et al., 2003).

Additionally, grape berry mesocarp and exocarp tissues contain gallic acid, chlorogenic acid, the tartaric acid esters of coumaric, caffeic and ferulic acid, as well as the glucose esters of *para* (*p*)-coumaric and ferulic acid (Monagas et al., 2005). Within plants, such glucose esters may serve as activated intermediates in the biosynthesis of other phenolic compounds (Lehfeldt et al., 2000; Li and Steffens, 2000).

Glucosyltransferases are a class of cytosolic enzymes that are responsible for the production of both glucose esters and glucosides, and generally exhibit a strict regio-specificity towards the acceptor substrate (Vogt and Jones, 2000). Many glucosyltransferases that produce glucose esters of hydroxybenzoic and hydroxycinnamic acids have been cloned, and have been shown to accept a wide range of structurally similar substrates (Lim et al., 2001; Lim et al., 2002; Lunkenbein et al., 2006; Meyer et al., 2003; Milkowski et al., 2000a, b). In contrast, only preliminary studies with resveratrol glucosyltransferase have been performed using crude extracts obtained from Gamay Freaux grape cell suspension cultures (Krasnow and Murphy, 2004), and the gene responsible for this reaction remains to be isolated. This report describes the biochemical

purification, molecular cloning and functional characterization of a novel bi-functional glucosyltransferase from *Vitis labrusca* cv. Concord that produces glucosides of stilbenes and glucose esters of hydroxycinnamic acids. RT-PCR analysis and metabolite profiling suggest this possible bifunctional role for VLRSGT within the grape.

3.3 - Experimental procedures

3.3.1 - Plant Material

Grape berries (*Vitis labrusca* cv. Concord) were harvested weekly from the G.H. Wiley vineyard (St Catharines, Ontario, Canada) from early June to late October in 2003–2005. *Vitis vinifera* cv. Gamay was harvested from the Château des Charmes Vineyard (Niagara on the Lake, Ontario, Canada) in September 2004. Fresh berries were peeled by hand, leaving small amounts of berry mesocarp, and the exocarps were quick-frozen and stored at −80°C. Alternatively, whole berries were frozen and stored at −80°C for future use. Berry maturity is indicated as weeks after flowering.

To determine the ratio of exocarp to mesocarp in the exocarp-enriched *Vitis labrusca* week 15 berry tissue, the tissue from ten berries was partially thawed and the exocarp was gently scraped with tweezers. This time-consuming procedure allowed the isolation of exocarp and mesocarp, which were weighed in three replicates to obtain the mean weight ± standard deviation for each tissue.

3.3.2 - Physiological parameters of grape berry development

Five grape berries from each developmental stage (weeks after flowering (AF)) were thawed to room temperature and macerated by hand in a plastic bag to produce

grape juice (Wang and De Luca, 2005). Juice pH was determined with a pH meter (Fisher Scientific, http://www.fishersci.ca), and the total soluble solids content (degrees Brix) was determined with an Abbe refractometer (Sola International Inc., http://www.sola.com). Additionally, a representative grape from each stage of development was thawed and photographed.

3.3.3 - Chemicals

Most of the substrates used, including aromatic carboxylic acids, phenylpropanoids, quercetin, *trans*-resveratrol and UDP-glucose (UDPG), were purchased from Sigma (http://www.sigmaaldrich.com/). Anthocyanin substrates, dihydroquercetin and kaempferol were purchased from Indofine Chemical (http://www.indofinechemical.com). *Cis*-resveratrol was produced by converting *trans*-resveratrol to the *cis*-form by exposure to light for 3 h (Trela and Waterhouse, 1996). Generally, 2 mM stocks of all acceptor substrates were prepared in 100% MeOH. Quercetin and kaempferol were first diluted in DMSO then diluted to 2 mM final concentration with MeOH (1% final DMSO concentration). UDPG stock solutions were prepared in water to 50 mM final concentration.

3.3.4 - Crude protein extraction and glucosyltransferase enzyme assay

Concord grape tissue (exocarp or mesocarp) was ground in liquid nitrogen to a fine powder in a chilled mortar and pestle. Three times the volume of grape extraction buffer (500 mM Tris-HCl, pH 8.0, 0.1 % β-mercaptoethanol, 5 mM sodium metabisulfite ($Na_2S_2O_5$), 1% PVP-40 (polyvinyl polypyrrolidone), 10% glycerol, 1 mM phenylmethyl

sulfonyl fluoride) and 10% insoluble polyvinylpyrrolidine (PVPP) was added to the powder and homogenized to a concentrated slurry. The slurry was filtered through one layer of 20 μM nylon mesh and centrifuged at 21000 *g* for 10 min at 4°C. The supernatant was desalted on a PD-10 desalting column (GE Healthcare, http://www.gehealthcare.com/caen) pre-equilibrated in 100 mM Tris, pH 9.0, with 0.1% *β*-mercaptoethanol. For each assay, 100 μl of crude extract was used, together with 100 μM acceptor substrate, 2.5 mM UDPG, and assay buffer (100 mM MES, pH 6.0, or 100 mM Tris, pH 9.0) to a final reaction volume of 150 μl. The reactions were incubated at 30°C for 30 min and terminated by adding 150 μl of 100 % MeOH. Reaction products were processed as described below prior to analysis by HPLC. The amount of protein in each extract was determined using a protein assay kit (Bio-Rad Laboratories Inc.; http://www.bio-rad.com/). Specific activities are calculated as picomoles of glucoside or glucose ester produced in one second by 1 mg of total protein.

3.3.5 - HPLC analysis of enzyme assays

Prior to HPLC, reaction products were centrifuged at 21000 *g* for 10 min at 4°C, and supernatants were filtered through a 0.45 μM white nylon filter (Millipore, http://www.millipore.com) for analysis by reverse-phase HPLC on an Inertsil ODS-3 (GL Sciences, http://www.gls.co.jp/index-e.html) C18 column (4 × 250 mm) equipped with a 3 × 4 mm guard column (Phenomenex, http://www.phenomenex.com) using a method modified from that described by Ali and Strommer (2003). Between 1 and 100 μL of each reaction product was injected onto the column, and products were eluted (1 ml min^{-1}) with solvent A (5% formic acid in H_2O) and solvent B (33:60:70 MeOH:MeCN:H_2O)

using the following gradient: 0–9 min, 10–47% B; 9–12 min, 47–85% B; 12–13 min, 85–100% B; 13–21 min, 100% B; 21–24 min, 100–50% B; 24–26 min, 50% B; 26–27 min, 50–10% B; 27–37 min, 10% B. Reaction products were monitored at 325 nm (sinapic acid, caffeic acid and ferulic acid), 275 nm (cinnamic acid), 310 nm (coumaric acid), 306 nm (*trans*-resveratrol), 365 nm (kaempferol), 285 nm (*cis*-resveratrol), 290 nm (naringenin), 520 nm (anthocyanins), 370 nm (quercetin) and 345 nm (esculetin) using a 2996 photodiode array detector (Waters, Milford, MA, USA). Alternatively, *trans*- and *cis*-resveratrol, *p*HBA and benzoic acid were monitored using a Waters 2475 fluorescence detector with set excitation and emission wavelengths of 330 and 374 nm, respectively, for *trans*- and *cis*-resveratrol, and 265 and 350 nm, respectively, for benzoic acid and *p*HBA. The amount of each product was determined using integrated peak areas and pre-determined calibration curves for each substrate.

3.3.6 - Protein purification and sequencing

Week 15 AF Concord exocarp-enriched berry tissue (100 g) was pulverized with liquid nitrogen in a chilled mortar and pestle. The powder was transferred to a chilled Waring blender and homogenized with 200 ml pre-chilled grape extraction buffer containing 30 g of insoluble PVPP. The slurry was filtered through 20 μM nylon mesh and centrifuged at 18500 *g* for 30 min at 4°C. The dark-blue anthocyanin-containing supernatant was filtered through 20 μM nylon mesh and mixed with 70 g of Dowex AG 1-X8 resin (Bio-Rad), pre-equilibrated in the same buffer for 20 min at 4°C. The mixture was centrifuged at 18500 *g* for 30 min at 4°C. The supernatant was then submitted to the same procedure except that 50 g of Dowex was used the second time.

After filtration, the blue supernatant (150 ml) was applied to a 450 ml Sephadex G25 (Sigma) column pre-swollen and equilibrated in buffer A (100 mM Bis-Tris, pH 7.0, 0.1 % β-mercaptoethanol, 0.1 % Tween-20). The light-blue desalted fractions (150 ml) were applied to a 20 ml Q Sepharose anion exchange column (GE Healthcare) pre-equilibrated in buffer A. The column was washed with 50 ml of buffer A, and bound proteins were eluted with a 20 min 0–1 M NaCl (in buffer A) gradient and 12 min final wash with 2 M NaCl (in buffer A). Fractions (2 ml) were collected and assayed for glucosyltransferase activity. Fractions containing glucosyltransferase activity eluted with 2 M NaCl were pooled (8 ml total), concentrated, and desalted using an Amicon ultra-centrifuge filter with a molecular weight cut-off at 10 000 Da (Millipore; Mississauga, ON, Canada) according to the manufacturer's instructions.

The concentrated sample (2.5 ml) was incubated with 250 mg of Cibacron blue affinity chromatography resin (Sigma) pre-equilibrated in buffer B (100 mM Bis-Tris, pH 7.0, 0.1 % β-mercaptoethanol) for 24 h at 4°C with gentle mixing. The mixture was centrifuged at 9300 *g* for 10 min at 4°C in a benchtop microcentrifuge. The supernatant (unbound fraction) was saved, and the resin was washed three times (W1–W3) with 2.5 ml of buffer B. Bound proteins were eluted three times (E1–E3) with 2.5 ml of 20 mM UDPG in buffer B, and once (E4) with 2.5 ml of 1.5 M NaCl, 20 mM UDPG in buffer B. The unbound, wash and eluted fractions were assayed for glucosyltransferase activity. Eluted fractions (E1–E3) with enzyme activity were pooled, concentrated and desalted with an Amicon ultra-centrifuge filter as described above.

The concentrated sample (560 µl) was applied to a Superdex 75 gel filtration column (Pharmacia, New York, NY, USA) pre-equilbrated in buffer C (0.15 M NaCl,

100 mM Bis-Tris, pH 7.0, 0.1 % β-mercaptoethanol). The column was washed and eluted with 30 ml of buffer C (0.5 ml min^{-1}), and 0.5 ml fractions were collected and assayed for glucosyltransferase activity. Active fractions were concentrated, subjected to SDS–PAGE and stained with colloidal Coomassie blue (Invitrogen; http://www.invitrogen.com/). A 55 kDa protein band which positively correlated to glucosyltransferase activity was excised from the gel and forwarded for sequence analysis performed at the Harvard Microchemistry Facility (Harvard University) by microcapillary reverse-phase HPLC nano-electrospray tandem mass spectrometry (μLC/MS/MS; Thermo Scientific, Waltham, MA, USA) on a Finnigan LCQ DECA XP Plus quadrupole ion trap mass spectrometer. Eight peptide sequences were obtained from this protein (GLLVTFTTTPESIGT, KASNITDQPTPVGDGMIR, RAILGQYK, ICPIKPVGPLYK, CLIEATTGEK, KAAEEAVAEGGSSDR, NLQEFVDEVR, MSMELVCK) corresponding to a full-length EST TC38971 from the *Vitis vinifera* TIGR grape index.

3.3.7 - Separation of trans-resveratrol glucosylating enzymes in exocarp (free of mesocarp) and in mesocarp by anion exchange chromatography

The exocarp (with no mesocarp attached) of week 15 AF *Vitis labrusca* berries was prepared as described above. Glucosyltransferase activity was separated by anion exchange chromatography as described above and assayed for activity with *trans*-resveratrol with the following changes. Exocarp (5 g) and mesocarp (20 g) were independently ground to a powder in liquid nitrogen and extracted in 20 ml of pre-chilled grape extraction buffer containing 6 g of insoluble PVPP. The slurries were filtered through 20 μM nylon mesh and centrifuged at 21000 *g* for 15 min at 4°C. The

supernatants were then mixed twice with Dowex AG 1-X8 resin (5.25 and 3.75 g), filtered and centrifuged at 21000 *g*. The resulting supernatants were then applied to 50 ml Sephadex G25 columns, and the active fractions were subjected to anion exchange chromatography (Q Sepharose). Fractions (2 ml) were collected and assayed for glucosyltransferase activity.

3.3.8 - Molecular cloning of VLRSGT and recombinant glucosyltransferase expression and purification

The full-length EST TC38971 from the *Vitis vinifera* TIGR grape gene was used to design forward primers in the 5' UTR (F1) and at the 5' end of the ORF (F2) corresponding to the sequences 5'-GAATATTCATTTCTCCCTGTG-3' and 5'-GCAAGAATTCATGGGGTCTGAATCAAAGCTAG-3', respectively. Reverse primers were designed in the 3' UTR (R1) and at the 3' end of the ORF (R2), corresponding to the sequences 5'-CAAGTGCCATGAGACGACACCGT-3' and 5'-GCTTCTCGAGTCAAA TTTTCTTTGACTTGC-3', respectively.

PCR with F1, R1 and 1 µl of Concord week 7 exocarp-specific cDNA was run for 25 cycles. The amplified reaction was diluted 1000-fold and used as a template for a second PCR amplification using F2 and R2 to amplify the full-length gene (1440 bp). The PCR product was sequenced (Robarts Research Institute, http://www.robarts.ca) and cloned into the pGEX 4T-1 GST fusion expression vector (GE Healthcare). The vector containing the full-length amplified product–GST was transformed into *Escherichia coli* (*E. coli*) BL21 (DE3) cells. A 3 ml culture in 2 × yeast tryptone (YT) medium containing 50 µg ml^{-1} ampicillin was inoculated, grown overnight at 37°C, and induced using a

method modified from Lim et al. (2001). Briefly, 1 ml of the saturated 3 ml culture was inoculated to 50 ml of ampicillin-containing 2 × YT medium, grown at 37°C to OD 1.0, induced with a 1 mM final isopropyl-1-thio-β-D-galactoside (IPTG) concentration, and grown at room temperature (23°C) for 24 h. After 24 h, the culture was centrifuged at 2800 *g* for 10 min to harvest the cells, and stored at −20°C until they were processed for extraction.

For protein purification, cell pellets were washed once and resuspended in 2.5 ml of 1× PBS buffer with 0.1 % *β*-mercaptoethanol. The cells were incubated with lysozyme (1 mg ml^{-1} final) for 20 min at room temperature, sonicated and centrifuged at 2800 *g* at 4°C for 10 min. The supernatant was then incubated with glutathione Sepharose 4B (GE Healthcare) for 45 min at room temperature with gentle mixing. The protein was purified batchwise according to the manufacturer's instructions, except that bound protein was eluted with 20 mM glutathione (50 mM Tris, pH 8.0, 0.1% *β*-mercaptoethanol).

3.3.9 - Recombinant glucosyltransferase activity assays

Typically, 0.25 µg of recombinant protein, 100 µM acceptor substrate and 2.5 mM UDPG in either 100 mM MES, pH 6.0, with 0.1 % *β*-mercaptoethanol or 100 mM Tris, pH 9.0, with 0.1 % *β*-mercaptoethanol to a final reaction volume of 125 µl were incubated at 30°C for 25 min, and the reaction was stopped by the addition of either 125 µl of 100% MeOH (or 125 µl of 0.12 N HCl in MeOH for anthocyanidin aglycone substrates).

Determination of the pH optimum involved a buffer system consisting of MES for assays between pH 5.0 and 6.5, Bis-Tris for those between pH 6.5 and 7.0, Tris for those

between pH 7.0 and 9.0, carbonate for those between pH 9.0 and 9.5, and CAPS for those between pH 9.5 and 10.0, together with 0.1 % β-mercaptoethanol, 0.25 µg of pure recombinant protein, 2.5 mM UDPG and 100 µM acceptor substrate in a final assay volume of 125 µl.

For the determination of kinetic parameters, kaempferol and *trans*-resveratrol were assayed in 100 mM Tris, pH 9.0, with 0.1 % β-mercaptoethanol, containing 0.25 and 0.5 µg of recombinant protein, respectively. Sinapic acid was assayed in 100 mM MES, pH 6.0, with 0.1 % β-mercaptoethanol, containing 0.25 µg of recombinant protein. For all assays, the UDPG concentration was held constant at 9 mM as the acceptor substrate concentration was varied.

UDPG saturation curve and kinetics were determined with *trans*-resveratrol as the acceptor substrate (100 µM final concentration) in 100 mM Tris, pH 9.0, with 0.1 % β-mercaptoethanol and 0.5 µg of recombinant protein. All assays were repeated at least in triplicate. The identification of reaction products as glucose esters was performed using a modified alkaline hydrolysis (adapted from Lim et al., 2001 and Mock and Strack, 1993). Following the enzyme assay, 1 N NaOH was added (to a final concentration of 0.1 N NaOH), and the mixture incubated for 1 h at room temperature, and hydrolysis was terminated with an equal volume of 0.12 N HCl in MeOH. Specific activities are calculated as picomoles of glucoside or glucose ester produced in one second by one mg of purified protein extract.

3.3.10 - RT-PCR analysis of VLRSGT and stilbene synthase (StSy) gene expression

RNA was extracted from exocarp and mesocarp tissue of Concord grape berry (0.1–5 g FW) and *Vitis vinifera* cv. Pinot Noir and cv. Pinot Noir Droit (up to 1 g FW)

using the Concert plant RNA reagent (Invitrogen) according to the manufacturer's protocols. One microgram of RNA was used for reverse transcription to produce cDNAs using the oligo(dT) primer from the Takara RNA PCR kit version 2.1 (Fisher Scientific). Gene expression was monitored using 1 µl of cDNA with the following gene-specific primers: stilbene synthase (StSy), forward 5'-ATAACGCAGGAGCACGAGTT-3', reverse 5'-TCAACTGCATCGAGAATTGC-3'; VLRSGT, forward 5'-TGTTGCAGCTGATCTTGGAC-3', reverse 5'-TACAAGAACCCCAAAGTGCC-3'; Actin, forward 5'-GATTCTGGTGATGGTGTGAGT-3', reverse 5'-GACAATTTCCCGTTCAGCAGT-3'. The PCR was carried out using ExTaq DNA polymerase (Fisher Scientific) at 96°C for 2 min, 32 cycles of 96°C for 30 sec, 51°C for 30 sec, 72°C for 30 sec, and then a final extension at 72°C for 7 min.

For each primer set, PCR conditions were optimized for T_m using gradient PCR, and optimized for the number of cycles by running the above PCR for 35 cycles and removing the reaction product every third cycle from cycles 15–35 to obtain the following cycling parameters: StSy, 30 cycles, T_m 50°C; Actin, 26 cycles, T_m 52°C; VLRSGT, 26 cycles, T_m 53°C). The PCR products were analyzed following electrophoresis on a 1% agarose gel containing ethidium bromide. RT-PCR without reverse transcriptase was used as a negative control for genomic DNA contamination. *Actin*, *VLRSGT* and *StSy* gene expression was repeated in at least duplicate, was quantified using Multigauge ver 3.0 (Fujifilm, Tokyo, Japan; www.fujifilm.ca), and the mean values were divided by the mean actin gene expression value to obtain a relative value for gene expression (% expression) in these tissues.

3.3.11 – Metabolite extraction and profiling

Metabolites were extracted from 0.3 g FW of *Vitis labrusca* week 12 and 14 berry exocarp tissue and 1.0 g of berry mesocarp tissue using a method modified from that described by Ali and Strommer (2003). Briefly, grape tissue was pulverized in liquid nitrogen to a fine powder and extracted by grinding with 3 ml of pre-chilled 80% MeOH. Extracts were transferred to 15 ml conical tubes (Sarstedt, Montreal, QC, USA), and the volumes were adjusted to 8 ml with 80% MeOH. Samples were gently mixed in the dark at 4°C for 20 h, at which time 1 ml of the extract was removed and centrifuged at 21000 *g* for 10 min at 4°C. Two hundred microlitres of the extract were filtered through a 0.45 µM Millipore membrane and subjected to HPLC analysis as described above by injecting 5 µl of berry exocarp extract and 20 µl of mesocarp extract. Exocarp and mesocarp fresh weights per berry were determined in quintuplicate and averaged for week 12 and 14 AF berries.

3.4 - Results

3.4.1 - Purification and molecular cloning of a resveratrol/hydroxycinnamic acid glucosyltransferase from Concord grape (Vitis labrusca)

Crude protein was extracted from week 12 (Figure 1) grape berry exocarp and mesocarp, desalted twice in order to remove most of the contaminating small molecular weight blue pigment (anthocyanins), and assayed for glucosyltransferase activity using *trans*-resveratrol and sinapic acid as acceptor substrates. Glucosylation of sinapic acid was detected in both mesocarp (2.50 pkat mg^{-1}) and exocarp (2.73 pkat mg^{-1}) tissue, to yield the glucose ester. Similarly, *trans*-resveratrol was glucosylated by both mesocarp

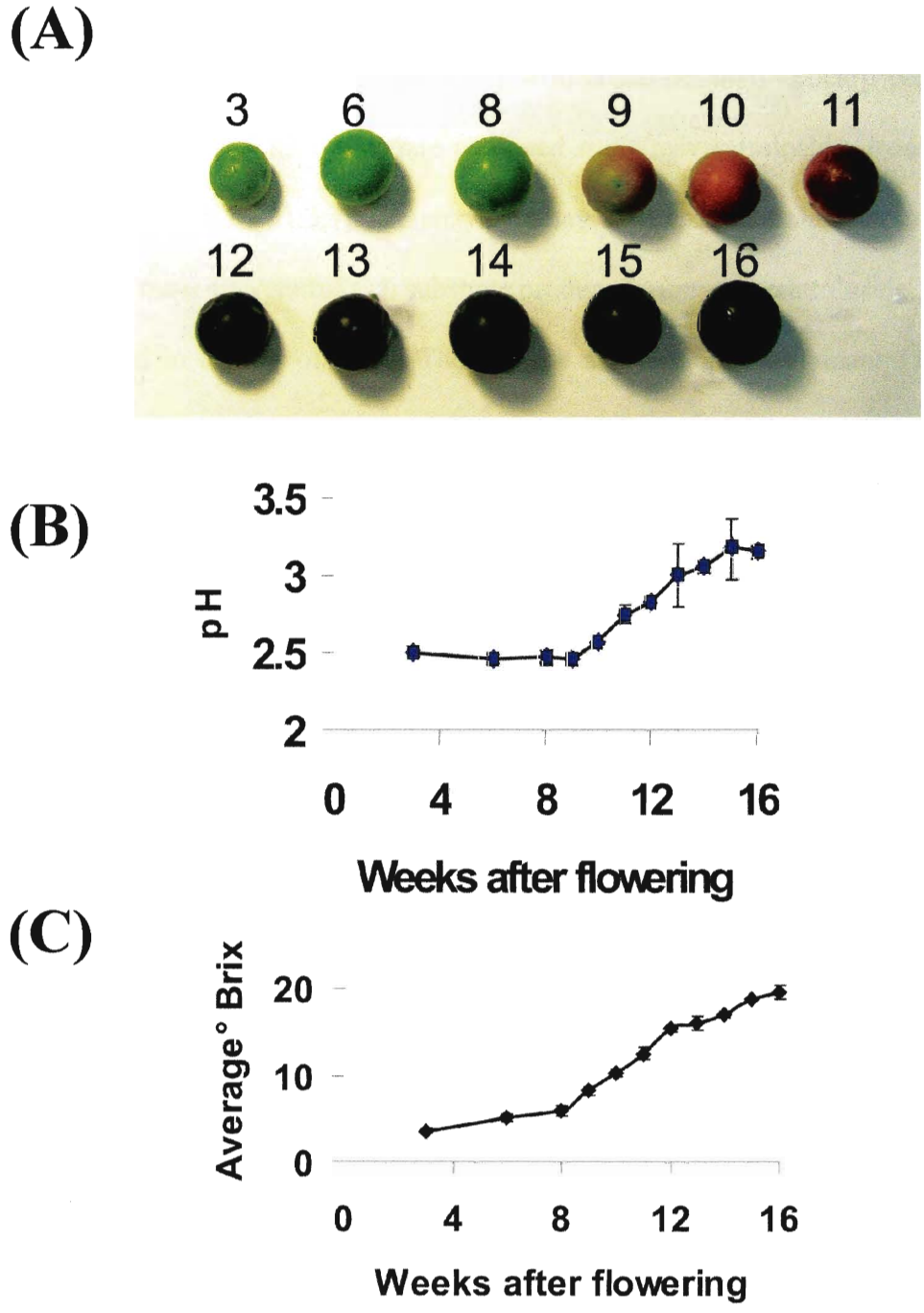

Figure 1. Characteristic developmental profile of grape ripening. A) Appearance of grapes at different stages after flowering (Weeks after flowering); B) Changes in grape pH in whole grape extracts at different stages after flowering (Weeks after flowering); C) Changes in Brix content whole grape extracts at different stages after flowering (Weeks after flowering)

(0.64 pkat mg^{-1}) and exocarp (3.40 pkat mg^{-1}) extracts to produce a combination of three separate mono-*O*-glucosylated products (*trans*-resveratroloside, *trans*-piceid and *cis*-piceid) in both tissues. The enzyme assay with crude desalted exocarp protein extract using *trans*-resveratrol as a substrate produced *trans*-resveratroloside, *trans*-piceid and *cis*-piceid in a ratio of 21:3:1. The enzyme assay with crude desalted mesocarp protein extract using *trans*-resveratrol as a substrate produced *trans*-resveratroloside, *trans*-piceid and *cis*-piceid in a ratio of 7:2:1. The enzyme assay with recombinant *Vitis labrusca* resveratrol/sinapic acid glucosyltransferase (VLRSGT) using *trans*-resveratrol as a substrate produced *trans*-resveratroloside, *trans*-piceid and *cis*-piceid in a ratio of 101:27:1. If *cis*-piceid production is removed from the ratio calculations, the exocarp, mesocarp and rVLRSGT extracts produce ratios of *trans*-resveratroloside:*trans*-piceid of 6.8:1, 3.4:1 and 3.7:1, respectively. These ratios suggest that the exocarp contains more than one resveratrol glucosyltransferase activity, compared with the mesocarp extract whose ratio is more similar to that of the VLRSGT.

Although berry exocarp contains the highest VLRSGT specific activities for both *trans*-resveratrol and sinapic acid, the berry mesocarp weighs an average of 1.06 g fresh weight per berry and the berry exocarp weighs an average of 0.12 g fresh weight per berry. Similarly, one berry mesocarp contains an average of 0.83 μg protein per berry, compared with exocarp that contains only 0.04 μg protein per berry. As a strategy to increase the amount of *trans*-resveratrol glucosylating protein, week 15 AF *Vitis labrusca* cv. Concord berry exocarp was harvested as a protein source, with small amounts of mesocarp which remained attached. In order to establish the relative contributions of the exocarp and mesocarp to this exocarp-enriched tissue, 10 berry exocarp-enriched samples

were separated more carefully into the two tissues to yield berry exocarp and berry mesocarp fresh weights of 2.62 ± 0.1 and 4.18 ± 0.3 g, respectively (average fresh weight ± standard deviation when analyzed in triplicate). These measurements suggested that berry exocarp-enriched tissues still contain considerable amounts of grape mesocarp. Protein was extracted from 100 g of exocarp-enriched grape tissue, incubated with Dowex resin to remove interfering pigments, desalted (Sephadex G25), subjected to anion exchange chromatography (Q Sepharose), and to affinity chromatography (Cibacron Blue 3GA), concentrated, desalted and applied to a gel filtration column (Superdex 75) as described in Experimental procedures and Table 1.

Glucosyltransferase activity with cyanidin, quercetin, sinapic acid, kuromanin and *trans*-resveratrol as acceptor substrates was monitored following each purification step. Anion exchange and dye ligand affinity chromatography eliminated glucosyltransferase activity towards quercetin and cyanidin in fractions that retained their activities towards resveratrol, sinapic acid and kuromanin (data not shown). Following Superdex 75 gel filtration, active fractions that were capable of *O*-glucosylating *trans*-resveratrol, sinapic acid and kuromanin were purified 38.6-, 15.4- and 79.8-fold, respectively (Table 1). Active fractions were submitted to SDS–PAGE and Coomassie staining to reveal a 55 kDa protein band corresponding to the glucosyltransferase. This was sequenced at the Harvard Microchemistry Facility to yield amino acid sequence for eight peptides (see Experimental procedures).

Peptides obtained from protein sequencing were subjected to a BLAST search of the *Vitis vinifera* TIGR grape gene database which identified a full-length EST (TC 38971) with 100% sequence identity to all eight peptides. Primers based on the *Vitis*

Table 1. Purification tables of a glucosyltransferase from week 15 *Vitis labrusca* exocarp-enriched tissues using sinapic acid (A); *trans*-resveratrol (B); and kuromanin (C) as substrates

(A) Purification of a glucosyltransferase with activity towards sinapic acid from week 15 Concord grape exocarp-enriched tissue

Purification step	Total protein (mg)	Total activity (pkatal)	Specific activity (pkat/mg prot)	Purification (-fold)	Yield (%)
Crude	167.9	2289.2	13.6	1	100
Dowex	89.2	1296.5	14.5	1.07	56.6
Sephadex G25	82.6	1223.3	14.8	1.09	53.4
Anion Exchange	3.65	1588.4	434.7	31.9	69.4
Affinity chromatography	0.11	41.1	367.3	26.9	1.8
Gel filtration	0.045	9.5	209.3	15.3	0.4

(B) Purification of a glucosyltransferase with activity towards *trans*-resveratrol from week 15 Concord grape exocarp-enriched tissue

Purification step	Total protein (mg)	Total activity (pkatal)	Specific activity (pkat/mg prot)	Purification (-fold)	Yield (%)
Crude	167.9	55.4	0.33	1	100
Dowex	89.2	33.0	0.37	1.1	59.7
Sephadex G25	82.6	18.9	0.23	0.70	34.2
Anion Exchange	3.65	0.91	0.25	0.76	1.6
Affinity chromatography	0.11	0.64	5.7	17.3	1.2
Gel filtration	0.045	0.57	12.7	38.6	1.0

(C) Purification of a glucosyltransferase with activity towards kuromanin from week 15 Concord grape exocarp-enriched tissue

Purification step	Total protein (mg)	Total activity (pkatal)	Specific activity (pkat/mg prot)	Purification (-fold)	Yield (%)
Crude	167.9	175.0	1.05	1	100
Dowex	89.2	311.2	3.49	3.35	177.8
Sephadex G25	82.6	782.4	9.48	9.09	447.0
Anion Exchange	3.65	104.3	28.5	27.4	59.6
Affinity chromatography	0.11	19.3	172.6	165.5	11.0
Gel filtration	0.045	3.8	83.2	79.8	2.2

vinifera sequence were used to clone the corresponding gene product from *Vitis labrusca*, which had 98% sequence identity to clone TC 38971 and 92% sequence identity to a *Vitis vinifera* *p*-hydroxybenzoic acid glucosyltransferase that produces glucose esters of *p*-hydroxybenzoic acid (*p*HBA) and, to a lesser extent, of hydroxycinnamic acids (Figure 2) (Meyer et al., 2003). The open reading frame (ORF) of the full-length *Vitis labrusca* clone (*VLRSGT*) is 1440 bp in length and encodes a putative protein of 479 amino acids with a theoretical molecular weight of 53.8 kDa. The presence of the plant secondary product glucosyltransferase (PSPG) consensus sequence at the C-terminus of the protein places it in the type I glucosyltransferase family (Vogt and Jones, 2000) that currently has over 80 functionally characterized members.

3.4.2 - Recombinant expression of VLRSGT

The ORF of *VLRSGT* was cloned into a GST fusion N-terminal expression vector, transformed into DE3 *Escherichia coli* cells and expressed at room temperature for 24 h. Protein was extracted and the soluble fraction was subjected to affinity purification using glutathione Sepharose 4B. Purified fractions obtained from a 50 ml *E. coli* culture contained 300–500 μg of the fusion protein (80 kDa) that was functionally active for at least 10 days at 4°C, and for at least 1 month as a 50% glycerol stock stored at −20°C. Further purification of recombinant VLRSGT (rVLRSGT) by removal of the GST tag did not change its activity, but there was a rapid 100% loss in enzyme activity after storage of the protein for 2 days at 4°C. For this reason, all subsequent characterization of the enzyme was performed using the fusion protein. Preliminary screening of potential

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Peptides                                                GLLVTFTTPESIGK   KASNITDQP
VLRSgt          MGSESKLVHVFLVSFPGQGHVNPLLRLGKRLASKGLLVTFTTPESIGKQMRKASNITDQP
VV_38971        MGSESKLVHVFLVSFPGQGHVNPLLRLGKRLASKGLLVTFTTPESIGKQMRKASNITDQP
VV_pHBAgt       MGSESKLVHVFLVSFPGQGHVNPLLRLGKRLASKGLLVTFSTPESIGKQMRKASNITDQP
                ****************************************:*******************

Peptides        TPVGDGMIR
VLRSgt          TPVGDGMIRFEFFEDGWDENEPKRQDLDLYLPQLELVGKKIIPEMIKKNAEQDRPVSCLI
VV_38971        TPVGDGMIRFEFFEDGWDENEPKRQDLDLYLPQLELVGKKIIPEMIKKNAEQDRPVSCLI
VV_pHBAgt       TPVGEGLIRFEFFEDEWDENEPKRQDLDLYLPQLELVGKKVLPQMIKKHAEQDRPVSCLI
                ****:*:******** ************************::*:****:************

Peptides
VLRSgt          NNPFIPWVSDVAADLGLPSAMLWVQSCACLSTYYHYYHGLVPFPSEAEPEIDVQLPCMPL
VV_38971        NNPFIPWVSDVAADLGLPSAMLWVQSCACLSTYYHYYHGLVPFPSEAEPEIDVQLPCMPL
VV_pHBAgt       NNPFIPWVSDVAADLGIPSAMLWVQSCACFSTYYHYYHGLVPFPSEAEPEIDVQLPCMPL
                ****************:************:********************************

Peptides                              RAILGQYK                              ICPIKP
VLRSgt          LKYDEIASFLYPTTPYPFLRRAILGQYKNLDKPFCILMDTFQELEPEVIEYMSKICPIKP
VV_38971        LKYDEIASFLYPTTPYPFLRRAILGQYKNLDKPFCILMDTFQELEPEVIEYMSKICPIKP
VV_pHBAgt       LKYDEVASFLYPTTPYPFLRRAILGQYRNLDKPFCILMDTFQELEPEVIEYMSKICPIKP
                *****:**********************:************************************

Peptides        VGPLYK
VLRSgt          VGPLYKSPKVPNAAVRGDFMKADDCIEWLDSKPPSSVVYISFGSVVYLKQEQVDEIAYGL
VV_38971        VGPLYKNPKVPNAAVRGDFMKADDCIEWLDSKPPSSVVYISFGSVVYLKQEQVDEIAYGL
VV_pHBAgt       VGPLYKNPKVPNAAVRGDFMKADDCIEWLDSKPPSSIVYVSFGSVVYLKQDQVDEIAYGL
                ******.*****************************:**:**********:*********

Peptides
VLRSgt          LNSGVQFLWVMKPPHKDAGLELLVLPEGFLEKAGDKGKVVQWSPQEQVLAHPSVACFVTR
VV_38971        LNSGVQFLWVMKPPHKDAGLELLVLPEGFLEKAGDKGKVVQWSPQEQVLAHPSVACFVTH
VV_pHBAgt       LNSGLQFLWVMKPPHKDAGLELLVLPEGFLEKAGDKGKVVQWSPQEQVLAHPSVACFVTH
                ****:*************************************************:

Peptides                                                                C
VLRSgt          CGWNSSMEALSSGMPVVAFPRWGDQVTDAKYLVDVFKVGVRMCRGEAENKLITRDEVEKC
VV_38971        CGWNSSMEALSSGMPVVAFPQWGDQVTDAKYLVDVFKVGVRMCRGEAENKLITRDEVEKC
VV_pHBAgt       CGWNSSMEALSSGMPVVAFPQWGDQVTDAKYLVDEFKIGVRMCRGEAENKLITRDEVEKC
                ********************:************ **:***********************

Peptides        LIEATTGEK             KAAEEAVAEGGSSDRNLQEFVDEVR MSMELVCK
VLRSgt          LIEATTGEKAAELKENTMKWKKAAEEAVAEGGSSDRNLQEFVDEVRRMSMELVCKSKKI
VV_38971        LIEATTGEKAAELKENTMKWKKAAEEAVAEGGSSDRNLQEFVDEVRRMSMELVCKSKKI
VV_pHBAgt       LIEATTGPKAAELKQNAMKWKKAAEQAVAEGGSSERNLQGFVDEVRRRSIEIIYKTKI-
                ******* ******:*:********:********:**** ******* *:*:: *:*
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Figure 2. Protein sequence alignment of the *Vitis vinifera p*-hydroxybenzoic acid glucosyltransferase (VV_pHBAgt) (Meyer et al., 2003),*Vitis vinifera* EST 38971 (VV_38971), the corresponding *Vitis labrusca* gene (VLRSgt) and the 8 peptides obtained from protein sequencing. The plant secondary product glucosyltransferase (PSPG) consensus sequence, characteristic of type I glucosyltransferases is underlined.

substrates indicated that the purified rVLRSGT could glucosylate *trans*-resveratrol, sinapic acid and kaempferol, whereas the boiled enzyme and bacteria expressing the corresponding empty vector (without the VLRSGT sequence) did not catalyze these reactions.

3.4.3 - A dual function VLRSGT catalyzes the pH-dependent substrate-specific biosynthesis of glucose esters or glucosides

Recombinant VLRSGT was assayed for glucosyltransferase activity towards *trans*-resveratrol, sinapic acid and kaempferol between pH 5.0 and 10.0 using MES (pH 5.0–6.5), Tris (pH 7.0–9.0) and CAPS (pH 9.5–10.0) buffer systems. The effect of the assay buffer on rVLRSGT activity was also investigated using Bis-Tris (pH 6.5–7.0) and carbonate (pH 9.0–9.5) to show no significant buffer-based variation in enzyme activity (data not shown). Maximal glucosyltransferase activity with sinapic acid as substrate was observed between pH 5.5 and 6.5, and this activity decreased several-fold at higher pH (Figure 3). Treatment of reaction products produced between pH 6.0 and 9.0 with 1 N NaOH (to a final concentration of 0.1 N NaOH) converted them back into the aglycone, and this suggested that the glucose ester of sinapic acid was being produced (Lim et al., 2001; Mock and Strack, 1993). The hydrolysis results are consistent with the ability of VLRSGT to glucosylate cinnamic and benzoic acids that have free carboxyl hydroxyl groups but do not possess phenolic hydroxyl groups in their structures (see below).

The rVLRSGT also glucosylated a number of other substrates, in addition to *trans*-resveratrol, *cis*-resveratrol and kaempferol, at low levels from pH 6.0–7.5 and at

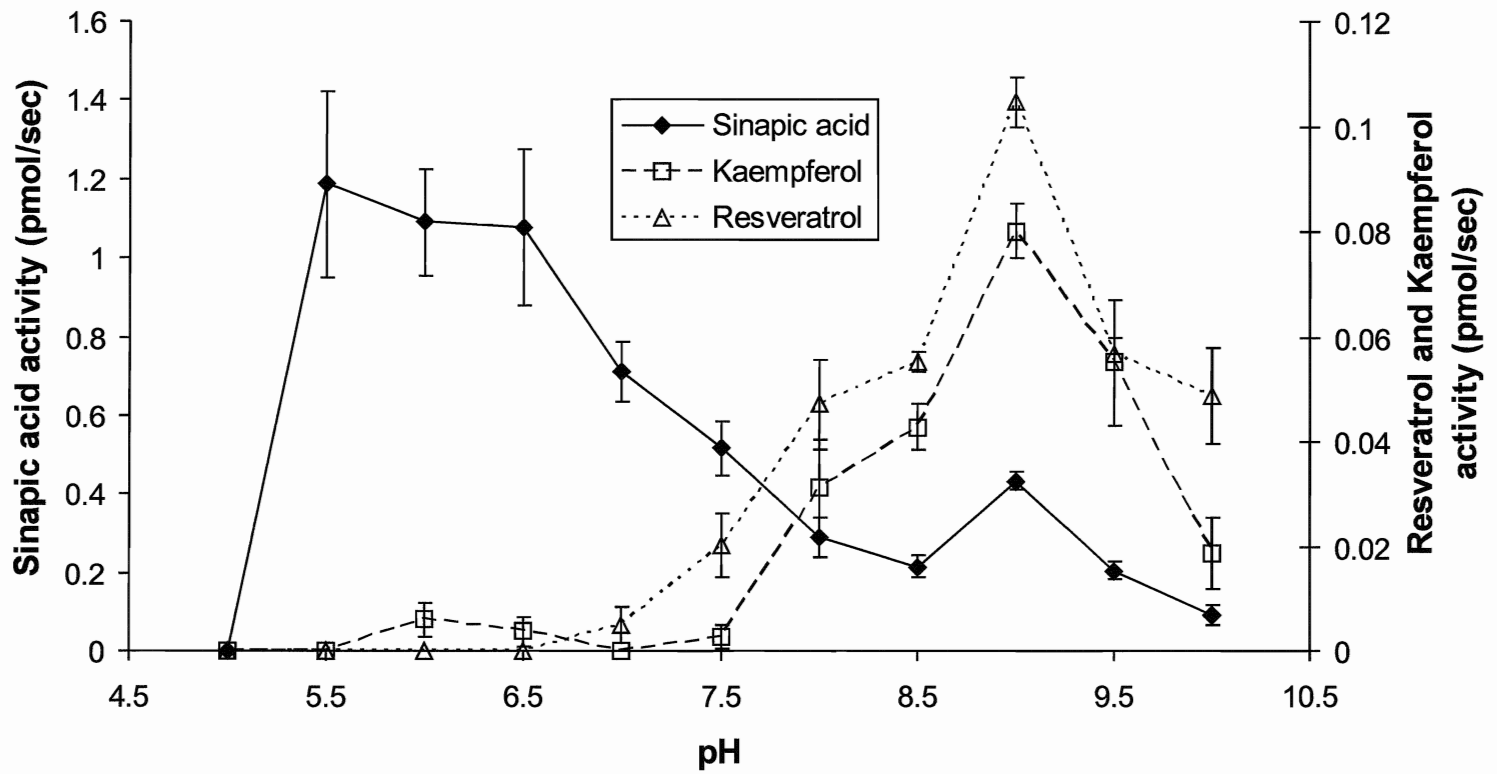


Figure 3. Effect of assay pH on *in vitro* rVLRSgt activity with sinapic acid, kaempferol and *trans*-resveratrol as substrates. Each point represents the mean of three trials ± standard deviation.

maximal levels at pH 9.0 (Figure 3). In addition, glucosylation of *trans*-resveratrol by rVLRSGT yielded two glucosylated products observed throughout the pH range of 7.5–10, with maximal production occurring at pH 9.0. Based on the UV spectra obtained, *trans*-resveratrol 3-*O* glucoside (piceid, UV_{max} = 320 nm; Hillis and Hasegawa, 1962; Waterhouse and Lamuela-Raventos, 1994) and the *trans*-resveratrol 4'-*O* glucoside (resveratroloside, UV_{max} = 304 nm; Teguo et al., 1998) were produced. For kaempferol, the reaction products co-chromatographed on HPLC with and shared an identical UV absorbance spectrum (UV_{max} = 365.5 nm) to kaempferol 7-*O*-glucoside, a minor contaminant of the kaempferol substrate (Offen et al., 2006) used in the assay.

3.4.4 - Substrate specificity of VLRSGT

To further characterize the substrate specificity of this enzyme, rVLRSGT was assayed at pH 6.0 and 9.0 using 23 different substrates. The glucose esters of sinapic, ferulic, caffeic, coumaric, *p*HBA, benzoic and cinnamic acid were preferentially formed at pH 6.0 compared with pH 9.0, and sinapic acid displayed the highest specific activity (Figure 4A). In contrast, *trans*-resveratrol, *cis*-resveratrol, kaempferol, quercetin, naringenin, dihydroquercetin, kuromanin and esculetin were good substrates for rVLRSGT at pH 9.0, whereas only kaempferol, naringenin and dihydroquercetin were glucosylated at low rates at pH 6.0 (Figure 4B). Sinapic acid glucose ester production was 10-fold higher at pH 6.0 than the maximum amount of *trans*-resveratrol-glucoside produced at pH 9.0, as reflected by the specific activities obtained for these two substrates (Figure 4). Abscisic acid, cyanidin, cyanin, chlorogenic acid, catechin,

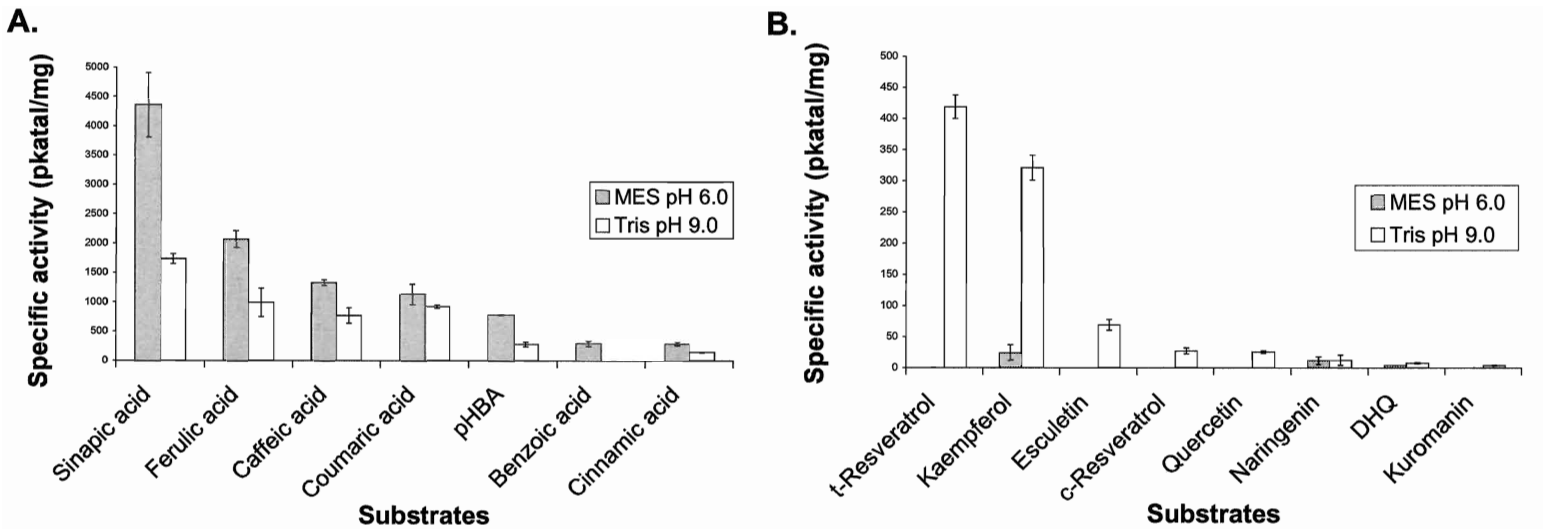


Figure 4. Substrate specificity of rVLRSgt *in vitro* at pH 6.0 and 9.0. **A**. Specific activity of substrates glucosylated to form glucose esters. B. Specific activity of substrates glucosylated to form phenolic glucosides. Each bar represents the mean of three trials ± standard deviation. Abscisic acid, cyanidin, cyanin, chlorogenic acid, catechin, epicatechin, indole-3-acetic acid, salicylic acid and 3,6,2',3'-tetrahydroxyflavone were not accepted as substrates.

epicatechin, indole-3-acetic acid, salicylic acid and 3,6,2',3'-tetrahydroxyflavone were not glucosylated by rVLRSGT.

3.4.5 - Kinetic analysis of the recombinant VLRSGT

The kinetic parameters of the rVLRSGT were investigated using the best *in vitro* substrates as suggested by substrate specificity studies: kaempferol, sinapic acid and *trans*-resveratrol as acceptor substrates and UDP-glucose (UDPG) as the donor substrate. The linearity of the reaction was maintained in assays (30°C for 25 min) carried out with 2.5 mM UDPG and varying concentrations of sinapic acid, *trans*-resveratrol or kaempferol assayed at pH 6.0 (ester formation) or pH 9.0 (glucoside formation). The data were fitted to Michaelis–Menton kinetics, and the resulting plots for sinapic acid, kaempferol and *trans*-resveratrol yielded K_m values of 27, 2.8 and 8.6 μM (Table 2), indicating that rVLRSGT has 3.1- and 9.5-fold higher affinities for *trans*-resveratrol and kaempferol, respectively, than for sinapic acid.

The calculated catalytic efficiency ($K_{cat}K_m^{-1}$) values for sinapic acid (172.7 mM^{-1} sec^{-1}), kaempferol (99.6 mM^{-1} sec^{-1}) and *trans*-resveratrol (20.9 mM^{-1} sec^{-1}) suggest that sinapic acid remains the best substrate for rVLRSGT as the turnover numbers with sinapic acid as substrate ($K_{cat} = 4.68$ sec^{-1}) are 16.7- and 26.7-fold higher than for kaempferol ($K_{cat} = 0.28$ sec^{-1}) or *trans*-resveratrol ($K_{cat} = 0.18$ sec^{-1}), respectively. Kinetic analysis for VLRSGT with varying concentrations of UDPG as the donor substrate using *trans*-resveratrol (100 μM) as the acceptor substrate gave a K_m value of 1260 μM and a $K_{cat}K_m^{-1}$ value of 0.17 mM^{-1} sec^{-1} (Table 2)

Table 2. Kinetic data for kaempferol, *trans*-resveratrol, sinapic acid and UDPG as substratesfor rVLRSGT. Mean values from three independent experiments ± Standard deviation are shown.

Substrate	K_m (mM)	K_{cat} (s^{-1})	K_{cat}/K_m ($mM^{-1}s^{-1}$)
Sinapic acid	0.027 ± 0.0040	4.68 ± 0.61	172.7
Kaempferol	0.00283 ± 0.000642	0.282 ± 0.0440	99.6
***trans*-Resveratrol**	0.00862 ± 0.00571	0.180 ± 0.053	20.88
UDPG	1.26 ± 0.285	0.216 ± 0.029	0.17

3.4.6 - Gene expression profile of VLRSGT throughout grape berry development is consistent with stilbene synthase gene expression

Total RNA was extracted from 8, 10, 12, 13, 14, 15 and 16 weeks after flowering Concord grape berry mesocarp tissue, 3, 6, 8, 10, 12, 14 and 16 weeks after flowering from Concord grape exocarp tissue, and from post-veraison *Vitis vinifera* cv. Pinot Noir and cv. Pinot Noir Droit exocarp and mesocarp tissues. Template generated from reverse transcription without reverse transcriptase was checked for genomic DNA contamination (data not shown). Primers designed to amplify *Vitis vinifera* actin were used with the cDNA templates to determine baseline expression between samples. Gene expression was quantified and divided by the mean *Actin* gene expression values to obtain the relative % expression of *VLRSGT* and stilbene synthase (*StSy*)compared to those of *Actin* throughout grape berry development in exocarp and mesocarp tissues (Figure 5). Low levels of *VLRSGT* and *StSy* transcript were observed at 10 weeks after flowering in Concord berry mesocarp, and these levels increased and were maintained for both transcripts throughout berry ripening (Figure 5A, Appendix I). *StSy* transcripts were also detected in the mesocarp of ripening (14 weeks after flowering) *Vitis vinifera* varieties (Pinot Noir and Pinot Noir Droit), whereas *VLRSGT* transcript is observed at lower levels in these tissues (Figure 5B, Appendix I).

Vitis labrusca grape berry exocarp did not appear to express detectable levels of *VLRSGT* and *StSy* at any stage of berry development (Figure 5A), in contrast to the two *Vitis vinifera* varieties (Pinot Noir and Pinot Noir Droit) that do express *StSy* but not *VLRSGT* transcripts in ripening exocarps (Figure 5B). However, *VLRSGT* transcript

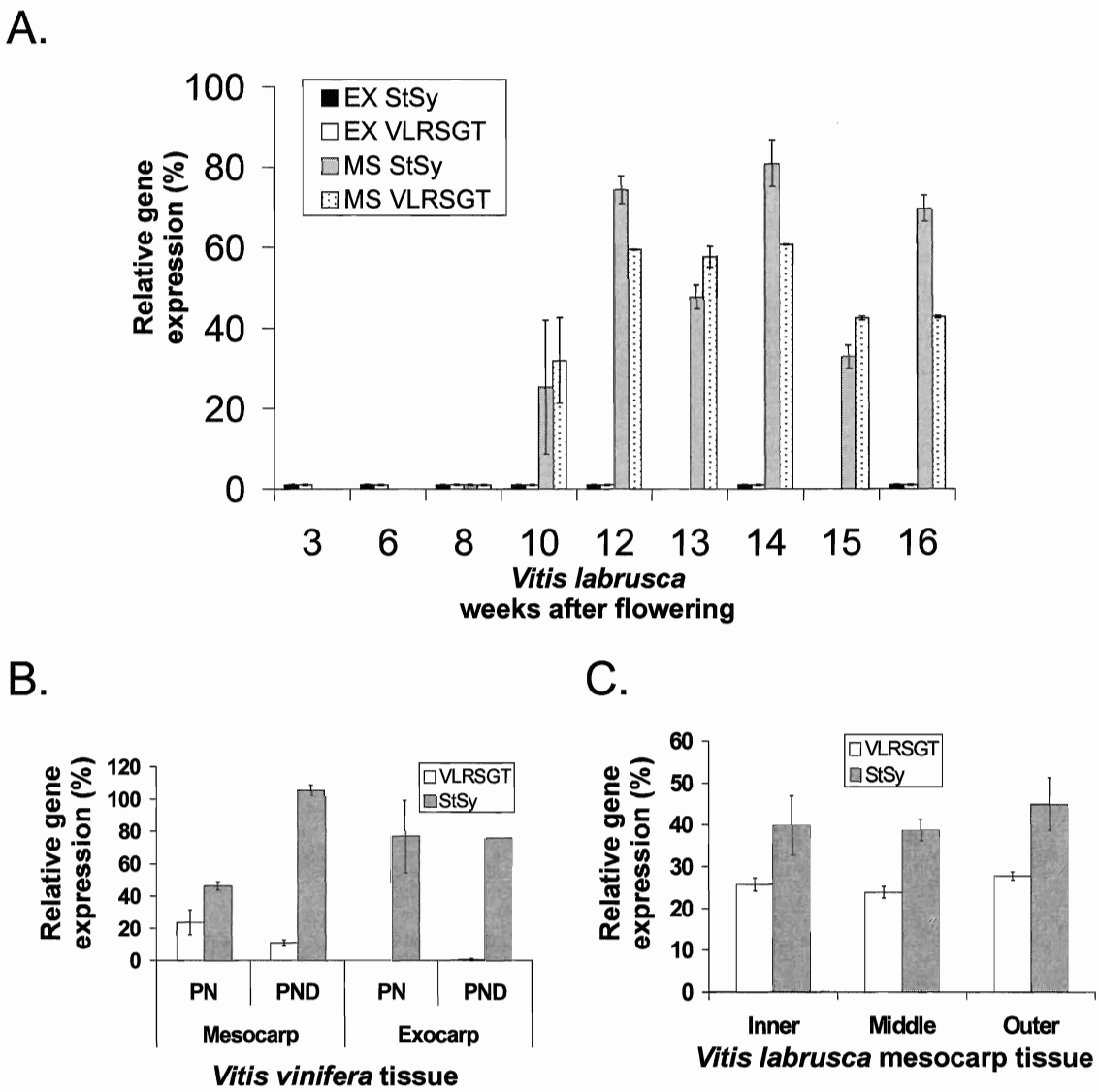


Figure 5. Relative gene expression of *VLRSGT* compared to *VLActin* expression. Developmental gene expression profile of *VLRSGT* and *StSy* in relation to *ACTIN* gene expression in (A) *Vitis labrusca* (*VL*) cv. Concord berry mesocarp (MS) and berry exocarp (EX) tissues and in (B) *Vitis vinifera* cvs. Pinot Noir (PN) and Pinot Noir Droit (PND) berry mesocarp and exocarp . C. Relative gene expression of *VLRSGT* and *StSy* in relation to *ACTIN* gene expression in *Vitis labrusca* inner, middle and outer berry mesocarp tissue. All values represent the mean of at least two trials ± standard deviation.

could be detected in grape berry exocarp tissue devoid of mesocarp by using nested PCR (25 cycles, 1:1000-fold dilution of template; 32 cycles; data not shown).

The week 12 Concord grapes were separated into outer, middle and inner mesocarp to show that *StSy* and *VLRSGT* transcripts appear to be uniformly distributed throughout this tissue (Figure 5C), and this was also consistent with the uniform distribution of sinapic acid/*trans*-resveratrol glucosyltransferase activity that was observed (data not shown). The mesocarp localization of *VLRSGT* is quite remarkable, as the enzyme was initially purified using exocarp-enriched grape tissue (Table 1) (Experimental procedures).

In order to verify whether *VLRSGT* is associated with mesocarp, grape exocarp tissues devoid of any visible mesocarp as well as mesocarp tissues were harvested and extracted separately as described in the VLRSGT purification protocol. Each extract was partially purified by Q Sepharose anion exchange chromatography, and the activity profile of resveratrol glucosyltransferase shows that eight times more VLRSGT activity is associated with grape mesocarp rather than the exocarp (Figure 6). It is also interesting to note that grape exocarp contains a second very different resveratrol-glucosylating enzyme that elutes from the column before the beginning of the salt gradient, and this corroborates the earlier suggestion that grape exocarp contains a second resveratrol glucosyltransferase activity compared with grape mesocarp.

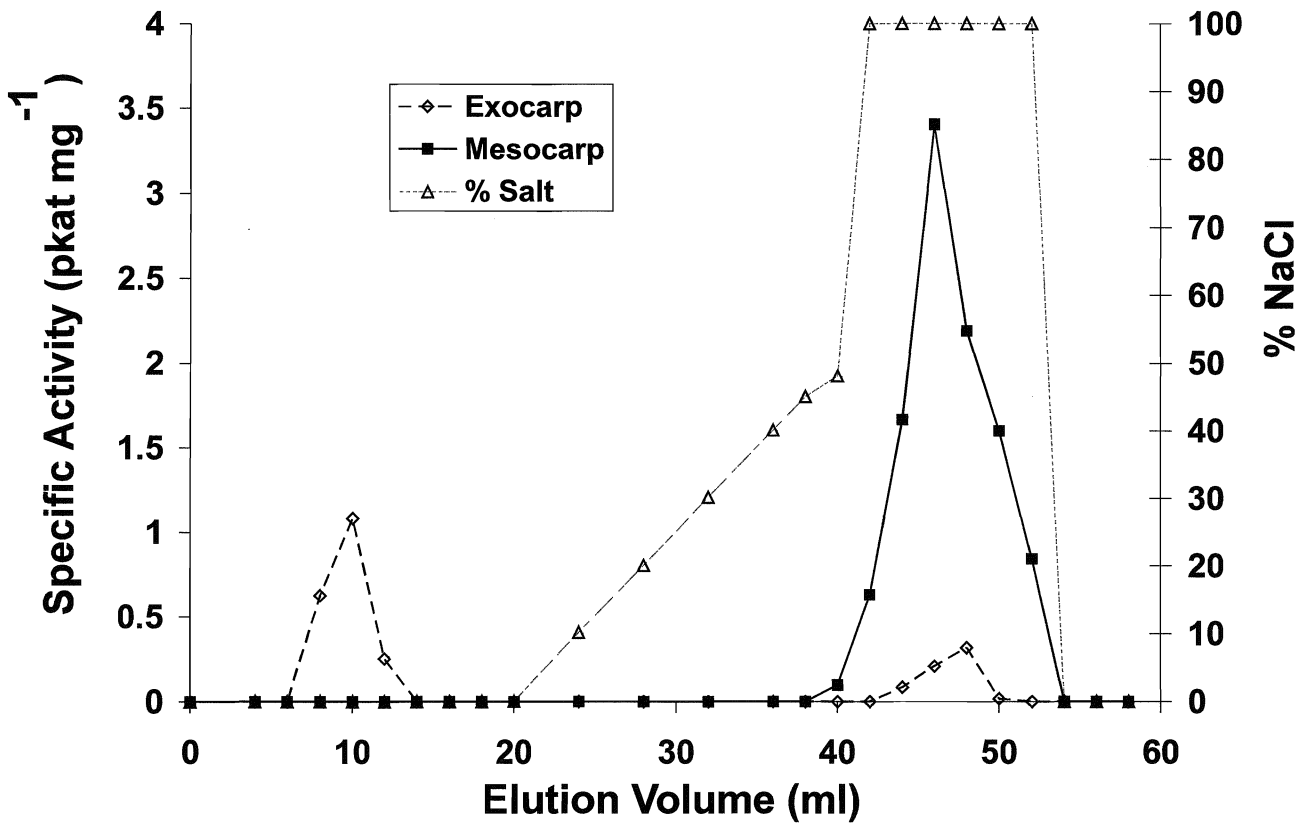


Figure 6. Q Sepharose anion exchange chromatography of crude desalted protein extracts obtained from grape (*Vitis labrusca*) exocarp and mesocarp, respectively. Exocarp-based and mesocarp-based fractions were assayed for resveratrol-*O*-glucosyltransferase activity.

3.4.7 - Metabolite profiling of week 12 and 14 Vitis labrusca berry exocarp and mesocarp tissue

Metabolites were extracted in triplicate from week 12 and 14 Concord grape berry exocarp and mesocarp tissue with 80% methanol. Extracts were filtered and analyzed directly by HPLC to identify grape metabolites related to the VLRSGT activities found *in vitro*. *Trans*- and *cis*-resveratrol, together with their respective monoglucosides (*cis*- and *trans*-piceid), but not the 4'-*O*-glucoside, were detected in both berry exocarp and mesocarp, with many-fold higher levels of these glucosides being detected compared with aglycone in both tissues (Figure 7). While sinapic acid, caffeic acid and their respective glucose esters were not detected in exocarp or mesocarp tissue, low levels of *p*-coumaric acid could be detected in berry mesocarp tissues but not in exocarp tissue that contained anthocyanins (Figure 7). While the levels of resveratrol and *p*-coumaric acid aglycones remained fairly constant at weeks 12 and 14, their respective glucosides and glucose esters tended to increase with ripening in both grape tissues.

The concentrations of *p*-coumaric acid glucose ester were similar to those of resveratrol glucosides (Figure 7), but these metabolites only represented 1 – 5 % of the total phenols found in various tissues in week 12 and 14 grapes. The tartaric acid esters of *p*-coumaric (coutaric acid), caffeic (caftaric) and ferulic (fertaric) acid have been documented as the most prevalent phenolic metabolites to accumulate in ripening Concord grape berries (Oszmianski and Lee, 1990). HPLC analysis (data not shown) of both Concord berry mesocarp and exocarp extracts showed the presence of high levels of caftaric acid (approximately 40 % and 7.5 – 14 % of the total phenol pool in berry mesocarp and exocarp, respectively), as identified by its retention time and UV spectral

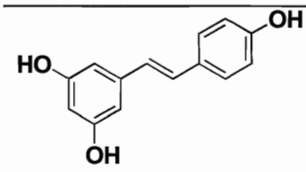
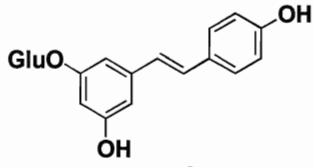
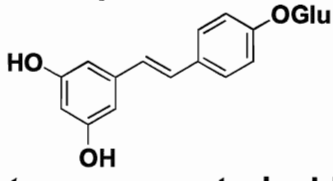
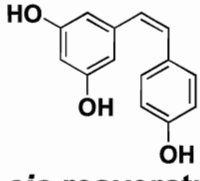
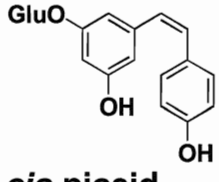
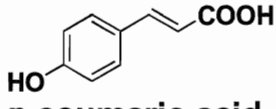
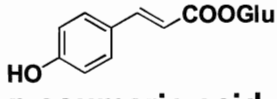
Metabolite	µg berry exocarp^{-1}		µg berry mesocarp^{-1}	
	Wk 12	Wk 14	Wk 12	Wk 14
 *trans*-resveratrol	0.31 ± 0.01	0.35 ± 0.09	0.23 ± 0.03	0.22 ± 0.02
 *trans*-piceid	7.7 ± 1.4	8.3 ± 3.3	2.9 ± 0.43	2.6 ± 0.15
 *trans*-resveratroloside	3.3 ± 0.35	2.2 ± 1.1	0.18 ± 0.03	0.27 ± 0.09
 *cis*-resveratrol	0.05 ± 0.01	0.04 ± 0.01	0	0
 *cis*-piceid	1.4 ± 0.2	1.4 ± 0.2	0.55 ± 0.2	1.0 ± 0.09
 *p*-coumaric acid	ND	ND	2.5 ± 0.9	1.4 ± 0.2
 *p*-coumaric acid glucose ester	8.0 ± 1.0	11.1 ± 1.5	1.1 ± 0.4	13.3 ± 4.1

Figure 7. Metabolite profile of *Vitis labrusca* cv. Concord grape week 12 and week 14 after flowering exocarp and mesocarp tissue. Each value represents the mean of three independent trials ± standard deviation. ND represents values which were not detected by the HPLC methods used.

analysis (UV_{max} = 329.9 nm; Mozetič et al., 2006), but neither coutaric or fertaric acid were detected.

3.4.8 - Phylogenetic analysis of VLRSGT with other functionally characterized glucosyltransferases

A neighbor-joining phylogenetic tree with other functionally characterized glucosyltransferases places VLRSGT with other glucose ester-forming enzymes and with the *Citrus unshiu* limonoid glucosyltransferase (Figure 8). VLRSGT has 54–76% sequence identity to previously characterized limonoid glucosyltransferase from *Citrus unshiu* (AB033758; Kita et al., 2000) and glucose ester-forming glucosyltransferases from *Fragaria ananassa* (AY663785), *Brassica napus* (AF287143) and *Arabidopsis thaliana* (UGT84A1-A4) that produce glucose esters of cinnamic acids (Lim et al., 2001; Lunkenbein et al., 2006; Milkowski et al., 2000a,b).

The dual functionality of VLRSGT, which forms glucose esters with aromatic carboxylic acids and hydroxycinnamic acids, and glucosides with stilbenes, flavonoids and coumarins, was not reported for the previously characterized glucose ester-forming enzymes of this group. The *Vitis vinifera* *p*HBA glucosyltransferase is reported to form aromatic carboxylic glucosides with *p*HBA at low levels at pH > 6.5 (Meyer et al., 2003). Similarly, a salicylic acid glucosyltransferase forms primarily the glucose ester, but also the glucoside, of salicylic acid at low levels (Lee and Raskin, 1999). However, the substrate specificities and activities of these enzymes remain to be investigated at higher pH.

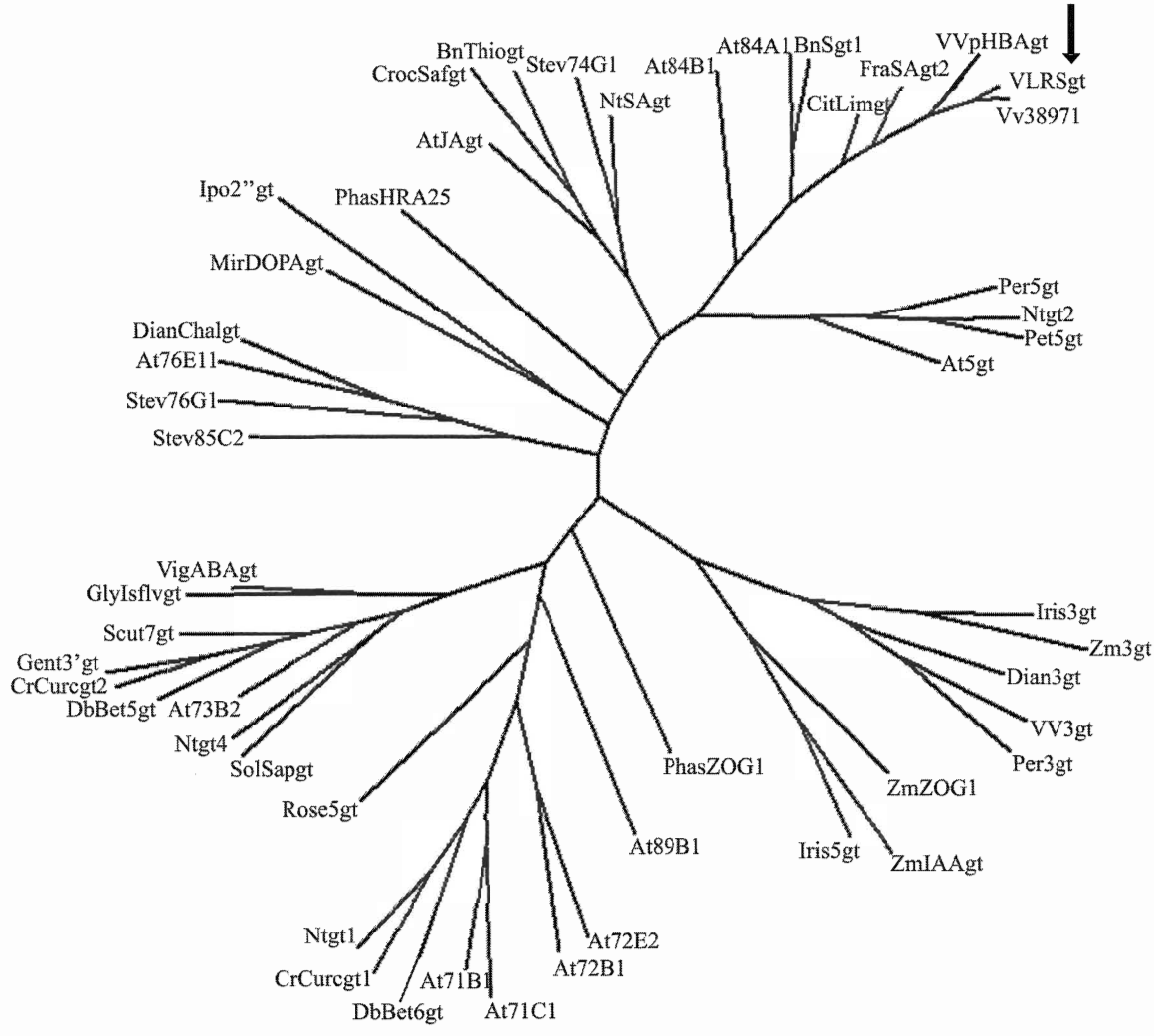


Figure 8. Neighbour joining phylogenetic tree of some functionally characterized glucosyltransferases and VLRSgt characterized in this study (indicated with arrow). **Abbreviations:** Zm3gt - *Zea mays* flavonol 3-*O*-glucosyltransferase (X13502); ZmIAAgt- *Zea mays* indole-3-acetic acid glucosyltransferase (MZEIAGLU); Iris5gt - *Iris hollandica* anthocyanin 5-*O* glucosyltransferase (AB113664); AtJAgt *Arabidopsis thaliana* jasmonic acid glucosyltransferase (DQ158907); CrocSafgt -*Crocus sativus* glucosyltransferase 2 (AY262037); BnThiogt - *Brassica napus* thiohydroximate *S*-glucosyltransferase (AF304430); Stev74G1 - *Stevia rebaudiana* steviol glucosyltransferase (AY345982); NtSAgt - Nicotiana tabacum salicylic acid glucosyltransferase (AF190634); At84B1 - *Arabidopsis thaliana* UGT84B1 indole-3-acetic acid glucosyltransferase (NM127890); At84A1 - *Arabidopsis thaliana* UGT84A1 hydroxycinnamic acid glucosyltransferase (BT015796); BnSgt1 - *Brassica napus* sinapate glucosyltransferase (AF287143); CitLimgt - *Citrus unshiu* liminoid glucosyltransferase (AB033758); FraSAgt2 - *Fragaria* x *ananassa* cinnamate glucosyltransferase (AY663785); VvpHBAgt - *Vitis vinifera p*-hydroxybenzoic acid glucosyltransferase (Meyer et al. 2003); VLRSgt - *Vitis labrusca* resveratrol-sinapic acid *O*-glucosyltransferase (this study); Vv38971 - *Vitis vinifera* EST TC38971 (TIGR grape gene index); Per5gt-*Perilla frutescens* anthocyanin 5-*O*-glucosyltransferase (AB013597); Pet5gt - *Petunia* x *hybrida* anthocyanin 5-*O*- glucosyltransferase

Figure 8. Abbreviations cont.: NtGt2-*Nicotiana tabacum* flavonol 7-*O*-glucosyltransferase (AB072919); At5gt -*Arabidopsis thaliana* anthocyanin 5-*O*-glucosyltransferase (AT4G14090); DianChalcgt - *Dianthus caryophyllus* chalcone glucosyltransferase (AB191249); At76E11 - *Arabidopsis thaliana* UGT 76E11 flavonol glucosyltransferase (NM114534); At72E2 *Arabidopsis thaliana* UGT72E2 TCP glucosyltransferase (NM126067); At72B1 - *Arabidopsis thaliana* UGT72B1 hydroxybenzoic acid glucosyltransferase (NM116337); At71C1 - *Arabidopsis thaliana* UGT71C1 phenolic alcohol glucosyltransferase (NM128529); At71B1 *Arabidopsis thaliana* UGT71B 1 hydroxybenzoic acid glucosyltransferase (NM113070); DbBet6gt - *Dorotheanthus bellidiformis* betanidin 6-*O*-glucosyltransferase (AF374004); CrCurcgt1 - *Catharanthus roseus* curcumin glucosyltransferase1 (AB159212); NtGt1 *Nicotiana tabacum* glucosyltransferase 1 (AB052557); Rose5gt - *Rosa hybrida* anthocyanin 5-*O*-glucosyltransferase (AB201050); SolSapgt - *Solanum tuberosum* solanidine glucosyltransferase (DQ218277); At73B2 *Arabidopsis thaliana* UGT73B2 flavonol 7-*O*-glucosyltransferase (AY339370); NtGt4 - *Nicotiana tabacum* flavonoid glucosyltransferase (AB176522); DbBet5gt - *Dorotheanthus bellidiformis* betanidin 5-*O*-glucosyltransferase (Y18871); CrCurcgt2 - *Catharanthus roseus* curcumin glucosyltransferase2 (AB159213); Gent3'gt - *Gentiana triflora* anthocyanin 3'-*O*-glucosyltransferase (AB076697); Scut7gt - *Scutellaria baicalensis* flavonoid 7-*O*-glucosyltransferase (AB031274); GlyIsoflavgt - *Glycine max* isoflavonoid glucosyltransferase (DQ278439); VigABAgt - *Vigna angularis* abscisic acid glucosyltransferase (AB065190); PhasZOG1 - *Phaseolus lunatis* zeatin-*O*-glucosyltransferase (AF101972); At89B1 - *Arabidopsis thaliana* UGT89B1 hydroxybenzoic acid glucosyltransferase (NM106048); MirDOPAgt - *Mirabilis jalapa* cyclo-DOPA 5-*O*- glucosyltransferase (AB182643); PhasHRA25 -*Phaseolus vulgaris* glucosyltransferase HRA25 (AF303396); Stev85C2 - *Stevia rebaudiana* steviol glucosyltransferase (AY345978); Stev76G1*Stevia rebaudiana* steviol glucosyltransferase (AY345974); Ipo2"gt - *Ipomoea purpurea* anthocyanin 2"-*O*-glucosyltransferase (AB192318); Dian3gt - *Dianthus caryophyllus* flavonoid 3-*O*-glucosyltransferase (AB191247); Vv3gt - *Vitis vinifera* flavonoid 3-*O*-glucosyltransferase (AF000372); Per3gt - *Perilla frutescens* flavonoid 3-*O*-glucosyltransferase (AB002818); Pet3gt - *Petunia hybrida* flavonoid 3-*O*-glucosyltransferase (AB027454); ZmZOG1 - *Zea mays* zeatin *O*-glucosyltransferase (AF318075); Iris3gt -*Iris hollandica* anthocyanidin 3-*O*-glucosyltransferase (Ab161175).

3.5 - Discussion

The present report describes the purification to apparent homogeneity of the resveratrol/hydroxycinnamic acid *O*-glucosyltransferase that appears to be expressed during later ripening stages of Concord grape berry mesocarp (Table 1). The pure protein was sequenced to yield eight individual peptides that were 100% identical to those from a putative open reading frame of a full-length EST 38971 from the *Vitis vinifera* TIGR grape gene index. Based on this information, an almost identical full-length *Vitis labrusca* clone was obtained by RT-PCR using primers based on EST 38971 (Figure 2). The Concord grape *VLRSGT* clone was 98% and 92% identical at the nucleotide level to EST 38971 and hydroxybenzoate glucosyltransferase (Meyer et al., 2003), respectively, from *Vitis vinifera*. Further DNA sequence comparisons also showed that VLRSGT had high sequence identity (54–74%) to a number of previously characterized glucosyltransferases that form glucose esters of hydroxycinnamic acid and other phenylpropanoids (Lim et al., 2001; Lunkenbein et al., 2006; Meyer et al., 2003; Milkowski et al., 2000a). Similarly, phylogenetic analysis groups VLRSGT with glucose ester-forming enzymes (Lim et al., 2001; Lunkenbein et al., 2006; Meyer et al., 2003; Milkowski et al., 2000a) and with a limonoid glucosyltransferase from *Citrus unshiu* (Kita et al., 2000; Figure 8). Initial inspection of the biochemical properties of the purified plant protein (Table 2), together with the sequence identity and the phylogenetic grouping obtained) suggested that VLRSGT was likely to be a glucose ester-forming enzyme.

3.5.1 - VLRSGT forms glucose esters and glucosides with various substrates in vitro

Initial substrate specificity studies with rVLRSGT showed that sinapic acid, as well as kaempferol and *trans*-resveratrol, could be used as substrates. Determination of the pH optima for these three substrates indicates that rVLRSGT can glucosylate different substrates and functional groups in a pH-dependent manner (Figure 3). While the pH optima of rVLRSGT (pH 5.5–6.5) for sinapic acid as the acceptor substrate is consistent with results obtained for other glucose ester-forming enzymes (pH 6.0; Lim et al., 2002; Milkowski et al., 2000a, b), the optimal catalysis of *trans*-resveratrol and kaempferol glucoside formation between pH 7.5 and 10 is a unique feature of VLRSGT and emphasizes the importance of assay pH in characterization of the substrate specificity of this class of enzymes.

Generally, glucosyltransferases exhibit a strict regio-specificity for the acceptor site (Vogt and Jones, 2000), but two separate reports have shown that glucose ester-forming enzymes can also catalyze a low level of formation of *O*-glucosides of the same substrate (Lee and Raskin, 1999; Meyer et al., 2003), and another described several *Arabidopsis thaliana* glucosyltransferases that were capable of glucosylating several hydroxyl groups of quercetin (Lim et al., 2004). Additionally, a glucosyltransferase cloned from *Eucalyptus perriniana* cell suspension cultures catalyzes the formation of cinnamic acid glucose esters and glucosides of terpenes and flavonoids at pH 7.5 (Nagashima et al., 2004). However, VLRSGT is the first enzyme from this class that catalyzes the pH-dependent *in vitro* formation of glucose esters for one set of substrates and glucosides for a second set of substrates.

3.5.2 - Kinetic analyses are consistent with a dual role for VLRSGT

The kinetic properties of rVLRSGT with sinapic acid as acceptor substrate (K_m = 27 μM; K_{cat} = 4.68 sec^{-1}; K_{cat} K_m^{-1} = 172.7 mM^{-1} sec^{-1}) is consistent with the properties of previously characterized glucose ester-forming enzymes from *Fragaria ananassa* (K_m = 108–708 μM; K_{cat} K_m^{-1} = 30–420 mM^{-1} sec^{-1}) and *Vitis vinifera* (K_m = 700 μM; K_{cat} = 10.9 sec^{-1}; K_{cat} K_m^{-1} = 15.6 mM^{-1} sec^{-1}), both of which form glucose esters *in vivo* (Lunkenbein et al., 2006; Meyer et al., 2003). The affinity of the rVLRSGT for *trans*-resveratrol glucoside formation (K_m = 8.6 μM) was at least seven times higher than that obtained using a crude protein extract from *Vitis vinifera* cv. Gamay Freaux cell suspension cultures (K_m = 60 μM), which also catalyzed the formation of *trans*-piceid and a second uncharacterized glucoside at more basic pH values (Krasnow and Murphy, 2004). The similarity of the rVLRSGT kinetic parameters to previously characterized glucosyltransferases suggests that both phenylpropanoids and stilbenes are potential substrates for this enzyme *in vivo*.

3.5.3 - Gene expression and metabolite profiling identifies the berry mesocarp as a site of production and accumulation of stilbenes in Concord grapes

As a result of the health benefits attributed to human consumption of the stilbene resveratrol (Fremont, 2000; Kris-Etherton et al., 2002), research efforts to characterize its regulation and the location of its biosynthesis and accumulation has increased. It is generally accepted that *trans*- and *cis*-resveratrol and their respective 3-*O*-monoglucosides (*trans*- and *cis*-piceid) are the major stilbenes that accumulate in grape exocarp tissue (Ali and Strommer, 2003; Versari et al., 2001). *Vitis vinifera* accumulates

the majority of its stilbenes in the berry exocarp (1.69–8.69 μg per berry skin), with the amount and type of stilbene (glucosylated or not) that accumulates being cultivar-specific (Ali and Strommer, 2003). In addition, it has been shown that UV irradiation of *Vitis vinifera* (cv. Pinot Noir) or *Vitis labrusca* grape berries increases many-fold the accumulation of resveratrol in exocarp tissue (60 and 70–100 μg g FW^{-1}, respectively), while under these conditions <1 and 3 μg g FW^{-1}, respectively, were detected in irradiated mesocarp (Jeandet et al., 1991). To obtain a realistic determination of stilbene content in Concord grape tissues, we calculated that an average berry exocarp (0.12 g FW^{-1}) and mesocarp (1.06 g FW^{-1}) contain stilbene concentrations of 8.4–12 μg per berry in the exocarp and 3.18 μg per berry in the mesocarp, suggesting that Concord berry mesocarp is a significant site of stilbene synthesis and accumulation.

Analysis of Concord grape berry exocarp and mesocarp tissues for *trans*- and *cis*-resveratrol contents and their respective glucosides suggest that, on a per berry basis, exocarp and mesocarp tissues accumulate similar amounts of stilbene glucosides, that increase in both tissues between weeks 12 and 14 (Figure 7). Previous developmental studies with *Vitis vinifera* cv. Corvina berries suggested that *StSy* gene expression is restricted to grape exocarp and increases with grape maturation (Versari et al., 2001). The accumulation of stilbenes in maturing berry mesocarp tissue of Concord grape (Figure 7) prompted the investigation of *StSy* and *VLRSGT* gene expression throughout development by RT-PCR using RNA extracted from Concord berry mesocarp and exocarp tissues (Figure 5). Concord grape mesocarp but not exocarp appears to express both *StSy* and *VLRSGT* in a coordinated development-specific fashion, consistent with the accumulation of stilbenes within this ripening tissue (Figure 7). This raises the question of how

Concord grape berry exocarp tissues can accumulate stilbenes despite the fact that *StSy* is not detected in this tissue throughout grape development. Conversely, in *Vitis vinifera* cv. Pinot Noir, where stilbenes accumulate predominantly in berry exocarp, the *StSy* transcript is easily detected in mRNA from both berry exocarp and mesocarp tissues (Figure 5B). Together, these data support a unique pattern of stilbene accumulation and *StSy* gene expression in Concord grapes, when compared with the pattern of *Vitis vinifera*.

3.5.4 - An in vivo role for VLRSGT in stilbene glucosylation?

Enzyme assays with rVLRSGT showed that it could catalyze the efficient *in vitro* formation of glucose esters with various phenylpropanoids and of glucosides with some stilbenes, flavonoids and coumarins (Figure 4). However, the observed pH optima (Figure 3) for making glucose esters (pH 5.5–6.5) and glucosides (8.5–9.5) raise the issue of the biological relevance of this enzyme in catalyzing these reactions under *in vivo* conditions. Grapes, together with other fruits, are well known for their high acidity (Figure 1), because vacuoles accumulate large amounts of organic acids. While nothing is known about the cytoplasmic pH of grape cells, there is a consensus from measurements in other plant species that cytoplasmic pH may vary between 7.0 and 7.5 (Felle, 2005; Schulte et al., 2006). It is interesting that the cytoplasmic pH falls within the trough where the VLRSGT enzyme (Figure 3) could form either glucose esters or glucosides depending on which substrate is supplied by cell metabolic processes. It is important to note that, in addition to pH and substrate, other cellular factors might be relevant in determining whether the cell makes glucose esters or glucosides (Winkel, 2004). The *in*

vivo activity of VLRSGT will depend on its developmentally regulated expression within the ripening grape mesocarp cells, the local micro-environment conditions that supply particular acceptable substrates, the local cellular conditions that modulate substrate specificity, and the possible association of VLRSGT with particular enzymes of one or another pathway. The *in vivo* role of a strawberry (*Fragaria ananassa*) glucosyltransferase (SaGT2) was elucidated by over-expression of this gene in strawberry, and by monitoring the altered metabolite profile observed in transgenic plants. The transgenic plants accumulated increased levels of cinnamic acid and *p*-coumaric acid glucose esters compared with untransformed control plants, whereas no change occurred for any other glucose esters, including those that can be formed by SaGT2 *in vitro* (Lunkenbein et al., 2006).

Expression of the *Vitis vinifera p*HBA glucosyltransferase in transgenic *p*HBA-accumulating tobacco (*Nicotiana tabacum*) and *Arabidopsis* greatly increased the levels of the related glucose esters (Meyer et al., 2003), but the biological role of this enzyme in grape has not been elucidated. The high sequence identity of the *Vitis vinifera p*HBA glucosyltransferase to VLRSGT could suggest a common biological function of these two enzymes. However, their *in vitro* substrate specificities were considerably different from one another; with the *Vitis vinifera* r*p*HBAgt producing *p*HBA and sinapic acid glucose esters at a tenfold higher rate and a nine-fold lower rate, respectively, than the *Vitis labrusca* rVLRSGT (Figure 4). The *in vitro* results imply that a unique and different *in vivo* function exists for *Vitis vinifera p*HBAgt that may be related to its substrate specificity. It also remains to be established whether the *Vitis vinifera* TC38971 clone

that is 98% identical to *Vitis labrusca* VLRSGT has the same dual biochemical role in phenylpropanoid glucose ester and stilbene glucoside biosynthesis.

In Concord grape berries, the mesocarp-specific gene expression profile of *VLRSGT* positively correlates with the mesocarp-localized expression of *StSy*, both of which increase with berry maturation (Figure 5). Similarly, the increased accumulation of resveratrol glucosides from weeks 12 to 14 in berry mesocarp and exocarp is consistent with a role for VLRSGT in glucosylating stilbenes. Within the grape berry, resveratrol and its glucosides are known phytoalexins. Glucosylation protects the aglycone from enzymatic oxidation by polyphenol oxidases within the plant cell, increasing its half-life and correspondingly decreasing its turnover rate (Regev-Shoshani et al., 2003).

3.5.5 - A second in vivo role for VLRSGT in the production of glucose ester intermediates?

The *trans*- and *cis*-tartaric esters of caffeic acid (caftaric acid), *p*-coumaric acid (coutaric acid) and ferulic acid (fertaric acid) accumulate as major phenols in the vacuoles of *Vitis vinifera* berry mesocarp and exocarp tissues. Additionally, the glucose esters of ferulic and *p*-coumaric acid are detected at comparatively low levels within these tissues (reviewed in Monagas et al., 2005). The major phenolics found in Concord berry tissue are *trans*-caftaric acid (1821.5 $\mu g\ g\ FW^{-1}$) and *trans*-coutaric acid (338.4 $\mu g\ g\ FW^{-1}$; Oszmianski and Lee, 1990). Similarly, metabolite analysis of Concord grape berry exocarp and mesocarp tissue extracts identified caftaric acid as the predominant phenol (data not shown), with low levels of the *p*-coumaric acid glucose ester also accumulating (Figure 7). The biological pathway leading to synthesis of the

tartaric acid esters within the grape berry is not clear, but the glucose esters of phenylpropanoids have well-known roles as activated intermediates in the production of fatty acid esters (Li and Steffens, 2000) and phenylpropanoid ester derivatives, such as sinapoylmalate (Lehfeldt et al., 2000). The formation of sinapoylmalate, the predominant sinapate ester in *Arabidopsis thaliana*, is catalyzed by a serine carboxypeptidase-like sinapoyl glucose:malate sinapoyltransferase that uses the activated sinapic acid glucose ester to acylate sinapic acid with malate (Lehfeldt et al., 2000). A BLAST search of the TIGR grape gene EST database identified a full-length EST (TC39894) with 70% sequence identity to the previously characterized sinapoyltransferase (Lehfeldt et al., 2000), and we performed enzyme assays with crude desalted grape extracts to detect caffeoylglucose:tartrate caffeoyltransferase activity that converted caffeoyl glucose and tartaric acid to caftaric acid (data not shown).

The presence of caffeoyl-glucose:tartrate caffeoyltransferase in *Vitis labrusca* provides a plausible *in vivo* role for VLRSGT in the biosynthesis of caftaric acid. In this pathway, similar enzymes would exist to produce coutaric and fertaric acids, and VLRSGT would produce the glucose ester intermediates of phenylpropanoid substrates for these transferases. Similarly, grapes are known to accumulate chlorogenic acid (caffeoyl quinic acid). The pathway leading to the production of chlorogenic acid is unknown in grape berry; however, a similar pathway, that requires the activated caffeic acid glucose ester as a high-energy intermediate to transfer caffeic acid to quinic acid, suggests another possible role for VLRSGT in caffeic acid glucose ester formation.

3.6 - Conclusion

In conclusion, we have purified, cloned and characterized a unique glucosyltransferase that converts phenylpropanoids into glucose esters and stilbenes, flavonoids and coumarins into glucosides in a pH-dependent manner when assayed *in vitro*. A dual *in vivo* role for VLRSGT is proposed in the formation of phenylpropanoid glucose esters and in the formation of stilbene glucosides during ripening of Concord grape mesocarp. The evidence for this is based on (i) the kinetic properties of VLRSGT, which suggest that phenylpropanoids and stilbenes are both good substrates (Table 2, Figure 4) for this enzyme, (ii) the coordinated mesocarp-specific (Figure 5A) expression of *VLRSGT* and *StSy* that has been observed, (iii) the expression of these genes throughout the mesocarp (Figure 5C), and (iv) the accumulation of *p*-coumaric acid glucose esters and stilbene glucosides within the grape mesocarp (Figure 7). While no glucose esters of caffeic and ferulic acid could be found within grape mesocarp, substrate specificity studies make it plausible that VLRSGT catalyzes the production of activated *p*-coumaric, caffeic and ferulic acid glucose ester intermediates that may be involved in the biosynthesis of coutaric, caftaric and fertaric acids that are among the most abundant phenols in grape berry.

Chapter 4.

Molecular cloning and biochemical characterization of members of the 5GT-like gene family from Concord grape (*Vitis labrusca*) which glucosylate flavonols and a xenobiotic, but not anthocyanidins.

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Molecular cloning and biochemical characterization of members of the *5GT*-like gene family from Concord grape (*Vitis labrusca*) which glucosylate flavonols and a xenobiotic, but not anthocyanidins.

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4.1 - Abstract

Grapes accumulate a myriad of reactive secondary metabolites (including flavonoids) that have health-promoting effects in humans, that are important for the organoleptic attributes of wine and that contribute to the UV-protection, pigmentation and stress responses of the plant. In addition to the natural products they produce, plants encounter a wide-range of pathogen- and human-derived reactive xenobiotic compounds. The enzymatic glucosylation of both sets of metabolites by plants change their reactivity, stability and subcellular location. Anthocyanins are the flavonoids responsible for the red/blue colour in fruits and flowers, and glucosylation changes the biochemical and spectral properties of these compounds. In grapes, accumulation of anthocyanin 3,5-*O* diglucosides in the berry exocarp produces a blue colour, which is undesirable for viticulture and breeding. The enzyme anthocyanin-5-*O*-glucosyltransferase (5GT) glucosylates the 5-*O* position of anthocyanin 3-*O* monoglucosides, and has been cloned and biochemically characterized in several plant species. Three glucosyltransferases were identified from *Vitis labrusca* cv. Concord grape berry exocarp tissue, with high levels of sequence identity to 5GTs. Phylogenetic analysis of these genes places them in a clade with the previously characterized 5GTs and with several flavonoid 7-*O*

glucosyltransferases. *In vitro*, these enzymes do not glucosylate anthocyanins, instead glucosylating flavonols and the xenobiotic 2,4,5-trichlorophenol (TCP). Kinetic analysis indicates that TCP is the preferred substrate for these enzymes, and RT-PCR analysis reveals variable transcription of these genes in the grape berry mesocarp and exocarp tissue. The role of the *Vitis labrusca* 5GT-like family of genes from in the modification of flavonols and xenobiotics is discussed.

4.2 - Introduction

Plants encounter a wide range of external stimuli and environmental conditions that trigger the production of a diverse array of secondary metabolites. In the plant these compounds accumulate within the vacuole, in specialized cell types or outside the plant in cell walls, on the leaf or in the rhizosphere surrounding plant roots. Enzymatic modification by hydroxylation, methylation, acylation and glycosylation, changes the physiological and biochemical properties of these molecules and alters their role and location within the plant.

Grapes have been cultivated for thousands of years for the production of wine, and remain culturally, economically and agronomically important worldwide. The grape berry can be divided into the seed, mesocarp (flesh), and exocarp (skin) tissues, which differ in their enzymatic and metabolite profiles throughout development. Veraison is a viticultural term which describes the stage of grape maturity at which the berry softens, rapidly accumulates total soluble solids (°Brix) and the exocarp accumulates anthocyanins to produce red/blue colours (Jackson, 2000).

Flavonoids are aromatic secondary metabolites that contribute to the pigmentation of plants, protect them from harmful UV rays, stimulate pollen growth to enhance male fertility and enhance the plant defense response (Winkel-Shirley, 2001). The flavonoids that accumulate within grape berries include the colourful anthocyanins, the UV-protectant flavonols, and the plant defensive proanthocyanidins (condensed tannins) (van de Wiel et al., 2001; Peters and Constabel, 2002; Kennedy et al., 2006). These compounds are important to the organoleptic characteristics (colour, flavour, astringency) of wine (Monagas et al., 2005), and their regular consumption is associated with myriad health benefits to humans (Soleas et al. 1997; Croft, 1998; Kris-Etherton et al., 2002).

Anthocyanins are the flavonoids responsible for the red, purple and blue colouring of grape berries. Glucosylation and acylation of anthocyanidin aglycones changes their spectral properties, stability and solubility (Vogt and Jones, 2000). Anthocyanidin aglycones are highly reactive and do not accumulate within the grape berry, whereas glucosylation at the 3-*O*-position produces the stable anthocyanins that accumulate within the vacuoles of the grape berry exocarp (Moskowitz and Hrazdina, 1981). Grape berry anthocyanin profiles are species-, variety- and cultivar-specific and the unique ratios of anthocyanin mono- and di-glucosides (and their acylated derivatives) can be used to identify the genetic origin of the berry (Núñez et al., 2004). Traditional "elite" cultivars of European grapes (*Vitis vinifera*) accumulate simple anthocyanin profiles consisting mainly of anthocyanidin 3-*O*-monoglucosides and small amounts of anthocyanin 3,5-*O*-diglucosides (Mazza and Miniati, 1993; Tian et al., 2005). Conversely, North American grapes (including *Vitis labrusca* and *Vitis riparia*) produce complex anthocyanin profiles consisting of more than 25 unique anthocyanin 3-*O*-monoglucosides; 3,5-*O*-diglucosides

and 6''-*O* acylated monoglucosides (Mazza and Miniati, 1993; Ali and Strommer, 2003; Wu and Prior, 2005). North American grape varieties including *Vitis labrusca* cv. Concord possess the desirable traits of disease resistance and cold tolerance that "elite" European varieties lack, however the complexity of the native North American anthocyanin profiles produces blue wine which makes these grapes undesirable for use in viticulture and breeding.

Glucosyltransferases (GTs) catalyze the transfer of glucose from uridine 5'-diphosphoglucose (UDPG) to an acceptor molecule, including plant-derived (flavonoids) and xenobiotic (pathogen- or human-derived) metabolites (Vogt and Jones, 2000; Schröder et al., 2006). In plants, GTs exist as large, multi-gene families (Bowles, 2002), and exhibit broad substrate specificity, but strict regio-selectivity for their acceptor substrate (Vogt and Jones, 2000). Flavonoid GTs have been cloned and functionally characterized from a number of plant species, and catalytic function can often be elucidated by nucleotide and amino acid sequence comparison.

Anthocyanin 5-*O*-GTs (5GT) catalyze the biosynthesis of anthocyanin 3,5-*O*-diglucosides by transferring a glucose moiety to the 5-*O*-position of anthocyanin 3-*O* monoglucosides. These enzymes have been cloned and biochemically characterized from several plant species where they display 50-70 % protein sequence identity to each other and they cluster together on a phylogenetic tree (Yamazaki et al., 1999; Yamazaki et al., 2002, Lorenc-Kukula et al., 2005; Tohge et al., 2005). Interestingly, this cluster also contains five enzymes (with 45-85 % protein sequence identity to 5GTs) that *O*-glucosylate terpenes, hydroxycinnamic acids, hydroxybenzoic acids or the 7-*O*-position

of several other flavonoid substrates (Lim et al., 2002; Meβner et al., 2003; Taguchi et al., 2003; Nagashima et al., 2004; Tian et al., 2006b), but not anthocyanins. Remarkably, two additional gene products from iris (Imayama et al., 2004) and rose (Ogata et al. 2005) do catalyze the 5GT reaction, but do not group phylogenetically within the 5GT clade.

The present study details the homology-based molecular cloning and biochemical characterization of three full-length members of the 5GT-family from *Vitis labrusca* cv. Concord that will *O*-glucosylate flavonols, but not anthocyanins *in vitro*. Substrate specificity and kinetic analysis, RT-PCR gene expression studies, and *in vivo* enzyme activity profiles suggest putative roles for these GTs in the modification of xenobiotics and flavonols.

4.3 - Experimental Procedures

4.3.1 - Plant material

Grape berries (*Vitis labrusca* cv. Concord) were harvested weekly from June until October, 2003 to 2006 at the 3rd St. site of the G & H Wiley vineyard (St. Catharines, ON). *Vitis vinifera* cv. Pinot Noir and *Vitis vinifera* cv. Pinot Noir Droit were harvested from the Château des Charmes Vineyard (Niagara on the Lake, ON) in September of 2004. All stages of grape berry maturity are reported as weeks after flowering (AF), and physiological parameters of grape berry development were determined as reported in Chapter 3. Concord pre-bloom flower buds were harvested June 12, 2006, grape flowers were harvested June 16, 2006 (week 4 AF) and grapevine leaves and stems were harvested June 21, 2006 (week 5 AF). All plant material was stored at -80°C until future

use. To separate grape berry exocarp from mesocarp tissue, berries were briefly thawed; the exocarp was removed by hand, scraped to remove residual mesocarp tissue, and was quick frozen in liquid nitrogen until future use.

4.3.2 - Chemicals

All chemicals were purchased from Sigma (http://www.sigmaaldrich.com) (Oakville, ON. Canada) or Indofine Chemical (http://www.indofinechemical.com) (Hillsborough, NJ. USA) and were prepared as described in Chapter 3, except that UDP-glucose (UDPG), UDP-galactose (UDP-Gal) and UDP-glucuronic acid were diluted to 180 mM in water. Kaempferol, quercetin, and isorhamnetin 3-*O*-glucosides and quercetin 7-*O*-glucoside were prepared enzymatically using the recombinant UDP-glucose: flavonoid 3-*O* glucosyltransferase (VL3GT) (Chapter 5) or the recombinant UDP-glucose: resveratrol/hydroxycinnamic acid glucosyltransferase (VLRSGT) respectively (Chapter 3).

4.3.3 - Crude protein extraction and profiling of GT enzyme activity in grape tissue throughout development

Vitis labrusca cv. Concord pre-bloom flower bud (1 g), leaf (0.5 g), flower (1 g), week 1 whole berry (1 g), weeks 3, 6, 8, 10, 12, 14, and 16 AF and post-veraison (week 14 AF) *Vitis vinifera* cv. Pinot Noir and cv. Pinot Noir Droit exocarp (1 g) and mesocarp (2 g) tissue was ground to a fine powder in a mortar and pestle with liquid nitrogen and extracted in 4 ml of grape extraction buffer (500 mM Tris-HCl, pH 8.0, 0.1% β-mercaptoethanol, 1% PVP-10 (polyvinylpyrrolidone), 5mM sodium metabisulfite

($Na_2S_2O_5$), 10% glycerol (v/v), 1 mM phenylmethyl sulfonyl fluoride (PMSF), and 10% insoluble polyvinylpyrrolidine (PVPP). The extracts were centrifuged at 21000 *g* for 10 min at 4°C, and the supernatant was desalted on a PD-10 Sephadex G-25 column (GE Healthcare; http://www.gehealthcare.com/caen), as per the manufacturer's instructions. For each enzyme assay, 100 µl of protein was used with 100 µM quercetin or cyanidin 3-*O*-glucoside (kuromanin) and 9 mM UDPG in a final reaction volume of 125 µl. The assays were incubated for 90 min at 30°C, stopped with 100% methanol, and processed prior to HPLC analysis as described below. The amount of protein in each extract was determined using a protein assay kit (Bio-Rad Laboratories, http://www.bio-rad.com). All specific activities are reported as picomoles of glucoside produced in 1 second by 1 mg of total protein.

4.3.4 - Acid hydrolysis and ethyl acetate extraction of grape berry metabolites

Metabolites were extracted from week 6, 8, 12 and 14 AF *Vitis labrusca* mesocarp and exocarp tissues as detailed in Chapter 3. Acid hydrolysis of metabolites was adapted from a previously published method (McNally et al., 2003) with the following changes. Crude methanol extracts (1.5 ml) were evaporated to dryness, re-suspended in water, acidified and extracted 3 times with ethyl acetate. The organic phase was dried, resuspended in 50% aqueous methanol and incubated with an equal volume of 4N HCl for 1 h at 78°C. Hydrolysis was terminated with the addition of 3 x volume of ethyl acetate, and the organic phase after extraction was evaporated to dryness, re-suspended in 50 µl of methanol and stored at -80°C for future use.

4.3.5 - High Performance Liquid Chromatography (HPLC) analysis of enzyme assays

Enzyme assays were performed and analyzed by HPLC as described previously (Chapter 3). Additionally, the *O*-glucosides of luteolin (350 nm), eriodictyol (290 nm), myricetin (370 nm), isorhamnetin (370 nm), dihydroquercetin (290 nm) and 3,6,2',3'-tetrahydroxyflavone (320 nm) were monitored using a 2996 photodiode array detector (Waters, Milford MA, USA; www.waters.com). HPLC detection limits for kuromanin and kaempferol were calculated at 10 pmol (5 ng) and 5 pmol (1.5 ng) respectively.

4.3.6 - RNA extraction

RNA was extracted from grape berry exocarp and mesocarp tissue as described in Chapter 3 with the following changes. RNA was extracted using the Plant RNA Extraction reagent (Invitrogen, http://www.invitrogen.com/) as per the manufacturer's instructions with the exceptions that 1 µl of linear acrylamide (5 µg/µl) was added with the RNA as a co-precipitant and the extracted RNA was resuspended on ice. RNA (0.5 - 1 µg) was reverse transcribed using either Superscript III reverse transcriptase (Invitrogen) or the Takara RNA PCR kit version 3.0 (Fisher Scientific, http://www.fishersci.ca/) as per the manufacturer's instructions, and were diluted 5-fold with water. For RACE-ready cDNA, 5 µg of *Vitis labrusca* week 12 AF berry exocarp RNA was extracted as above, and prepared using the GeneRacer Kit (Invitrogen) as per the manufacturer's instructions.

4.3.7 - Cloning of anthocyanin 5-O-glucosyltransferase (5GT) -like genes from Vitis labrusca

A BLAST search of The Institute for Genomic Research (TIGR) *Vitis vinifera* grape gene index with the previously characterized *Petunia* x *hybrida* anthocyanin 5GT (Yamazaki et al., 2002) as a query identified 93 expressed sequence tags (ESTs) with sequence identity to GTs, two of which were identified as having at least 70% sequence identity (TC17383 – 890 bp and TC31413 - 728 bp) to the *Petunia* 5GT.

a) Isolation of full length VLOGT1,2

Gene specific primers were designed to amplify TC17383 from the *Vitis vinifera* TIGR grape gene database at the conserved GT amino acid sequences NTFN/DALE (FORWARD PRIMER 1 – 5' AACACGTTTAATGCGTTGGAA 3'; REVERSE PRIMER 1 5' GGTTGGTAACAGAATGGAAGGTAGG 3') and RAEEGVVERE (REVERSE PRIMER 2 – 5' CTCTCTCCACAACCCCTTCTT 3') and PCR with forward primer 1 and reverse primer 2 amplified a 550 bp product from *Vitis labrusca* week 12 AF exocarp cDNA. Additional primers for RACE PCR amplification were designed based on the conserved GT amino acid sequence LPSILLPT (FORWARD PRIMER 2 - 5' CTACCTTCCATTCTGTTACCAACCA 3'; REVERSE PRIMER 3 - 5' CTTCCAACGCATTAAACGTGTT 3'). Nested PCR with FORWARD PRIMER 2 and the GeneRacer 3' primer was run for 25 cycles with *Vitis labrusca* RACE ready cDNA (Round 1), was diluted 100 fold and 1 µl was used as a template in PCR using FORWARD PRIMER 1 and the GeneRacer 3' primer (Round 2). For 5' RACE, nested

PCR with REVERSE PRIMER 3, the GeneRacer 5' primer and *Vitis labrusca* RACE ready cDNA (Round 1) was diluted 100 fold and 1 µl was used as a template with REVERSE PRIMER 1 and the GeneRacer 5' nested primer (Round 2), which generated a nearly full-length gene product; and primers were designed with an added start codon at the N-terminus to amplify the putative open reading frame (ORF). PCR with *Vitis labrusca* week 12 AF exocarp-specific cDNA as a template and FORWARD PRIMER 6 (5'ATGCCCACCTTTCATCTGGTTAAGCT 3') and REVERSE PRIMER 11 (5'TCAGGCCCAAGCCCACTAAAGATG 3') (Round 1) followed by FORWARD PRIMER 6 and REVERSE PRIMER 12 (5' ATGAGTCCTGACTTCTAAGCTTTCCA 3') (Round 2) yielded a PCR product of 1296 bp (*trVLOGT4*; EF533707) which translated to a protein of 431 amino acids.

A second 5'RACE nested PCR with *Vitis labrusca* RACE ready cDNA as a template, REVERSE PRIMER 4 (5'CCAAGGGAGGACGAGGCCGTAGAT 3') and the GeneRacer 5' primer (Round 1) followed by REVERSE PRIMER 5 (5'GACCGCGCTCGTTGGATAGGCTTAA 3') and the GeneRacer 5' nested primer (Round 2) was performed as described above, and resulted in the amplification of the 5' terminus of VL17383. Primers were designed to amplify the full-length gene (*VLOGT*) using FORWARD PRIMER 3 (5' ATGGACAAACATCACTTCCTCTTAC 3') and REVERSE PRIMER 6 (5' CTCAATGAGTCCTGACTTCTAAGC 3'), and *Vitis labrusca* week 12 AF exocarp-specific cDNA, the product of which was cloned into the pGEM-T easy TA-vector (Promega, www.promega.com). Restriction enzyme analysis identified two unique digestion patterns and two different nucleotide sequences corresponding to full-length clones (*VLOGT1*; EF533704 and *VLOGT2*; EF 533705).

b) Isolation of full-length VLOGT3

Gene specific primers were designed for clone TC31413 from the *Vitis vinifera* TIGR grape gene index. Nested PCR with *Vitis labrusca* week 12 AF exocarp cDNA, FORWARD PRIMER 4 (5' GATCAGCACCCGGAAACGGAGAA 3') and REVERSE PRIMER 7 (5' TTCAAATAAAAGCAAGCACTCTAA 3') (Round 1), followed by FORWARD PRIMER 4 and REVERSE PRIMER 8 (5' TACACTTTCATATCAACACAAGTT 3') (Round 2) as described above generated a product of 505 bp with 98 % sequence identity to the *Vitis vinifera* clone TC31413. The 5' terminus was amplified by 5' RACE nested PCR using *Vitis labrusca* RACE ready cDNA with the Gene Racer 5' primer and REVERSE PRIMER 8 (Round 1) followed by Gene Racer 5' Nested primer and REVERSE PRIMER 9 (5' GCTACAACTGGAACCCCAGA 3') (Round 2) as described above. Primers were designed to amplify the full-length gene (*VLOGT3*; EF533606) from *Vitis labrusca* week 12 AF exocarp-specific cDNA as described above with the primers FORWARD PRIMER 5 (5' ATGGGGCAGCACCACTTCCTCA 3') and REVERSE PRIMER 10 (Round 1) followed by FORWARD PRIMER 4 and REVERSE PRIMER 14 (5' CTAAAAAATACTGCCTTGTCCCA 3') (Round 2).

4.3.8 - Expression of recombinant VLOGT1, VLOGT2, VLOGT3 and trVLOGT4

Oligonucleotide primers were designed to amplify full-length *VLOGT1*, *VLOGT2* and *VLOGT3* with restriction enzyme sites to facilitate cloning into the pGEX-4T-1 GST-fusion protein expression vector (GE Healthcare). *Vitis labrusca* week 12 AF exocarp-specific cDNA was used as a template with the primers FORWARD PRIMER 7 (5'

GCAAGAATTCATGGGGCAGCACCACTTCC 3') and REVERSE PRIMER 13 (5' GAATTGGGACAAGGCAGTCTCGAGAACG 3') to amplify *VLOGT3*; the primers FORWARD PRIMER 8 (5' CGAAGAATTCATGGACAAACATCACTTCC 3') and REVERSE PRIMER 15 (5' GAAGTCAGGACTCATTGACTCGAGAACG 3') to amplify *VLOGT1* and *VLOGT2*. The products of these reactions were purified from agarose gel (Toyobo, www.toyobo.co.jp/e/), digested with the restriction enzymes *Eco*RI and *Xho*I as per the manufacturer's instructions (Promega) and cloned into the corresponding restriction sites in the pGEX-4T-1 vector.

Restriction enzyme digestion identified *VLOGT1*, *VLOGT2* and *VLOGT3*, which were sequenced, transformed into *Escherichia coli* (*E. coli*) cells for recombinant protein expression. Several conditions were tested to increase the production of soluble protein that included the testing of several *E. coli* strains, using different inducing conditions (0.1 mM or 1 mM isopropyl-1-thio-β-D-galactoside (IPTG)), growing cultures at 16-18°C, 25°C, or 37°C, and concurrently expressing the groES-groEL chaperone proteins to assist in GT folding. Soluble protein was extracted, assayed for GT activity and subjected to SDS-PAGE analysis to determine the optimal growth conditions for these enzymes (data not shown). Expression in the pGEX-4T-1 N-terminal GST-fusion vector in DE3 *E. coli* cells expressing chaperone protein, induced with 0.1 mM IPTG and grown for 20 h at 16-18°C was determined to yield the most active, soluble protein (data not shown) and these conditions were used for all future analysis. Briefly, a 3 ml culture in 2 x yeast tryptone medium containing 50 μg ml^{-1} ampicillin and 34 μg ml^{-1} chloramphenicol (2YTAC) was inoculated and grown overnight at 37°C. Five hundred μl of the 3 ml culture was inoculated to 50 ml of 2YTAC and was induced to express the chaperone proteins with L-

arabinose. The culture was grown to an OD_{600} of 1.0, was induced for recombinant GT protein expression with 0.1 mM of isopropyl-1-thio-β-D-galactoside (IPTG) (Bioshop, www.bioshopcanada.com), was grown for 20 h at 16°C and was harvested by 5 min centrifugation at 3800 *g* and stored at -20˚C as a cell pellet until future use.

trVLOGT4 was cloned into a pCR-T7 / NT-TOPO expression vector as per the manufacturer's instructions (Invitrogen) and was transformed into XLI Blue *E. coli* cells for protein expression. Briefly, 3 ml of Luria broth with 50 µg/ml ampicillin (LBA) was inoculated with the bacterial clone and grown overnight at 37°C. One ml of the 3 ml culture was inoculated into a 50 ml LBA culture, grown to OD_{600} of 0.5, induced with 1 mM IPTG, grown for 20 h at 16°C, and were harvested and stored as described above. Generally, cell pellets were resuspended in 3 ml of 100 mM Tris-HCl pH 8.5, and were centrifuged for 5 min at 3500 g to remove cell debris. The supernatant was sonicated (3 times, 20 sec pulses) and was centrifuged at 21000 *g* for 10 min at 4°C to obtain soluble (supernatant) and insoluble (pellet) protein fractions. For SDS-PAGE analysis, 5-10 µg of protein was subjected to SDS-PAGE on 12.5% acrylamide and proteins were visualized with Coomassie blue.

4.3.9 - RT-PCR analysis of VLOGT gene expression.

Gene expression was monitored using the gene specific primers: *VLOGT1,2* – forward primer: 5' GCCCTTGGACACTATATGAATG 3', reverse primer: 5' GGCGATGACTTTCCATCAGTC 3'; *VLOGT3* – forward primer: 5' GGCGCTACAA GCTGTGGATAAGG 3', reverse primer: 5' CTGCTGCTGTGTAGCAAAGC 3'; *VL3GT* – forward primer : 5'TGCAGGGCCTAACTCACTCT 3', reverse primer: 5'

GCAGTCGCCTTAGGTAGCAC 3'; as well as the gene specific primers used in RT-PCR analysis for *VLRSGT* and *VLActin* as reported in Chapter 3. *VLOGT1,2*; *VLOGT3*; and *VLActin* gene expression was repeated in duplicate, was quantified using Multigauge ver 3.0 (Fujifilm, Tokyo, Japan; www.fujifilm.ca), and the mean values were divided by the mean actin gene expression value to obtain a relative value for gene expression (% expression) in these tissues.

4.3.10 - In vivo biocatalysis of GT reactions

E. coli cultures expressing *Vitis labrusca* putative GT protein were grown as described above. Concurrent with the induction of GT gene expression, substrates were added to a final concentration of 100 µM and the induced bacterial culture was grown for 20 h at 16°C, at which time the cultures were harvested by centrifugation at 3600 *g* for 5 min. Following centrifugation, the supernatant was decanted, acidified and extracted three times with ethyl acetate. The ethyl acetate fractions were pooled, evaporated to dryness, dissolved in 1 ml of methanol, and subjected to HPLC analysis as described in Chapter 3.

4.3.11 - Recombinant GT enzyme activity assays

Recombinant protein (rVLOGT1, rVLOGT2, rVLOGT3) was isolated and rVLOGT2 was further purified by Glutathione sepharose 4B (GE Healthcare) affinity chromatography (as described in Chapter 3). Typically, the enzymes were assayed in 100 mM Tris, pH 8.0 with 0.1% β-mercaptoethanol in a final reaction volume of 125 µl, with 9 mM UDPG, 100 µM acceptor substrate and 2 – 8 µg of purified rVLOGT2, 140 – 283

μg of total desalted total soluble rVLOGT1 protein or 119 – 242 μg of desalted total soluble rVLOGT3 protein. Assays were incubated for 2 h at 30°C, were stopped with an equal volume of methanol or 0.12 N HCl in methanol (anthocyanidin substrates), were centrifuged, filtered and subjected to HPLC analysis as described in Chapter 3. Enzyme assays with acid hydrolyzed grape berry metabolites (5 μl) contained 9 mM UDPG and either 4.52 μg or 7.43 μg of desalted rVLOGT2 and rVLOGT3 respectively in a final assay volume of 100 μl. The assays were incubated for 2 h at 30°C, stopped with methanol, and prepared for HPLC analysis as described above.

The acceptor substrates quercetin, 2,4,5-tricholorphenol (TCP), phenol, benzyl alcohol and phenylethanol were assayed radioactively in a 100 μl reaction volume. A typical assay contained 100 mM Tris-HCl, pH 8.0 with 0.1% β-mercaptoethanol, 100 μM (quercetin, TCP, phenol) to 20 mM (benzyl alcohol, phenylethanol) acceptor substrate, 25mM gluconic acid lactone, 196 μM ^{14}C-UDPG (specific activity = 304 mCi/mmol), 804 μM cold UDPG and protein as described above. The assays were incubated for 30 - 120 min at 30°C, stopped with 10 μl of 1N HCl, extracted with 500 μl of ethyl acetate, centrifuged at 21000 *g* for 10 min and the organic phase was evaporated to dryness. The products were resuspended in 10 μl of methanol, spotted on a Polygram Sil/UV$_{254}$ (Machery-Nagel; Fisher Scientific) TLC plate and developed in the solvent systems ethyl acetate:glacial acetic acid:formic acid: water (100:11:11:20) (quercetin); ethyl acetate:methanol:water (60:35:8) (TCP, phenol) or ethyl acetate:acetone: dichloromenthane:methanol:water (40:30:12:10:8) (benzyl alcohol, phenethyl alcohol) (Jones et al., 1999). The TLC plate was dried, and exposed to a storage phosphor screen

(GE Healthcare) for 16-48 h prior to analysis with a Phosporimager FLA-3000 (Fujifilm) and MultiGuage ver 3.0.

4.3.12 - Characterization of rVLOGT1, rVLOGT2 and rVLOGT3

Determination of pH optima, substrate specificities and kinetic analysis were completed as described in Chapter 3 with the exceptions that the typical buffer system was 100 mM Tris-HCl pH 8.0, 0.1% β-mercaptoethanol, and that the assay incubation time was 2 h. For the determination of kinetic parameters with TCP, a 50 µl final reaction volume (100 mM Tris-HCl, pH 8.0, 0.1% β-mercaptoethanol), 50 µM C^{14}UDPG, 250µM of cold UDPG 0.04 µg of purified rVLOGT2 and varying concentrations of TCP were incubated for 90 min at 30°C, were stopped with the addition of 5 µl of 0.12 N HCl and extracted with 500 µl ethyl acetate, which was dried and processed as described above. The glucosylated products of the GT reaction were analyzed and identified by HPLC–mass spectrometry (HPLC-ESI/MS) on an Agilent 1100 HPLC system equipped with a DAD detector and a Bruker HCT+ ESI/MS.

4.3.13 - Sequence alignment and Phylogenetic Analysis

The protein sequences of the *Vitis labrusca* GTs with *Nicotiana tabacum* NtGT2 (Taguchi et al., 2003; BAB88985) and *Petunia* x *hybrida* 5GT (Yamazaki et al., 2002; BAA89009) were aligned using ClustalW 2 (http://align.genome.jp/) and representatively shaded using Boxshade (http://www.ch.embnet.org/software/BOX_form.html). Nucleotide sequence alignment and phylogenetic analysis of trVLOGT4, VLOGT1, *VLOGT2*, and *VLOGT3* with other previously characterized GTs was analyzed using

BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the DNA distance neighbour joining phylogenetic analysis was visualized using Phylodraw (http://pearl.cs.pusan.ac.kr/phylodraw/).

4.4 - Results

4.4.1 - Anthocyanin 5-O-glucosyltransferase (5GT) activity is detected in Vitis labrusca berry exocarp but not mesocarp tissue

Crude, desalted protein extracts were prepared from *Vitis labrusca* grape mesocarp and exocarp tissue harvested at different stages of berry development; post-veraison *Vitis vinifera* cv. Pinot Noir and cv. Pinot Noir Droit mesocarp and exocarp tissue; and *Vitis labrusca* leaf, flower and pre-bloom bud tissue and were assayed for 5GT activity with cyanidin 3-*O* glucoside (kuromanin) as a substrate. Enzyme activity was first detected in *Vitis labrusca* grape berry exocarp at week 8 AF (veraison) and increased to maximal activity at weeks 10 and 12 AF where it was maintained until harvest at week 16 AF. Low levels of 5GT activity were detected in Concord grape leaves, flowers and pre-bloom flower buds (Figure 1), but not in *Vitis labrusca* berry mesocarp tissue, nor in week 14 AF *Vitis vinifera* cv. Pinot Noir and Pinot Noir Droit berries (data not shown).

4.4.2 - Identification of 5GT-like genes in grape

A BLAST search of The Institute for Genomic Research (TIGR) *Vitis vinifera* gene index identified more than 90 expressed sequence tags (ESTs) with sequence identity to the previously characterized *Petunia* x *hybrida* 5GT (Yamazaki et al., 2002;

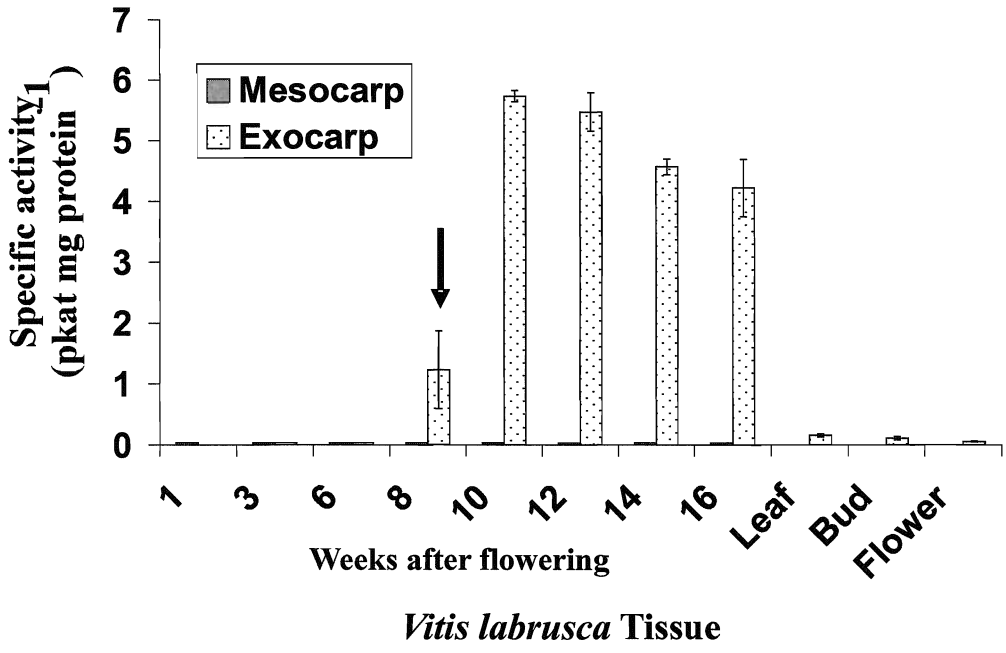


Figure 1. Anthocyanin 5-*O*-glucosyltransferase (5GT) activity in *Vitis labrusca* leaf, flower bud, flower, week 1 whole berry and week 3,6, 8, 10, 12, 14 and 16 after flowering grape berry exocarp and mesocarp crude protein extracts throughout development. The arrow indicates the onset of veraison at wk 8 after flowering. Each bar represents the mean of three independent trials ± standard deviation

AB027455). One (TC38971) of the five candidate ESTs with the highest sequence identity to the *Petunia* 5GT corresponded to full-length *VLRSGT* (Chapter 3; DQ832169). Four additional candidate ESTs (TC44031; TC17383; TC31413; TC42104) were not full-length, but they aligned with the C-terminus of the *Petunia* 5GT protein. Two of these ESTs were derived from leaf (TC42104) and flower (TC44031) cDNA libraries, respectively, tissues that do not typically accumulate anthocyanins. Conversely, TC17383 and TC31413 ESTs were both derived from a grape berry cDNA library. Since grape berry tissues are the site of 5GT activity (Figure 1), the corresponding full-length clones were isolated by 5' and 3' RACE.

4.4.3 - Cloning of 5GT-like genes from Vitis labrusca

Gene specific primers were designed based on TC17383, and the products from 5' and 3' RACE, to amplify a truncated gene with an artificial start codon (ATG) which generated a product of 1302 bp (*trVLOGT4*) with an ORF of 431 amino acids and a calculated molecular weight of 47.7 kDa. A second round of 5' RACE yielded a putative full-length gene of 1350 bp in length and an ORF of 448 amino acids. Sequencing revealed two unique full-length clones with 88% sequence identity to each other. *VLOGT1* (1347 bp) and *VLOGT2* (1344 bp), which encode putative proteins of 447 aa and 448 aa respectively, had corresponding theoretical molecular weights of 49.8 kDa and 49.7 kDa; and 99% and 92% nucleotide sequence identity and 96% and 85% protein sequence identity to *trVLOGT4* (Figure 2). Additional gene specific primers were designed based on TC31413 and the product of 5' RACE to amplify a putative full-length

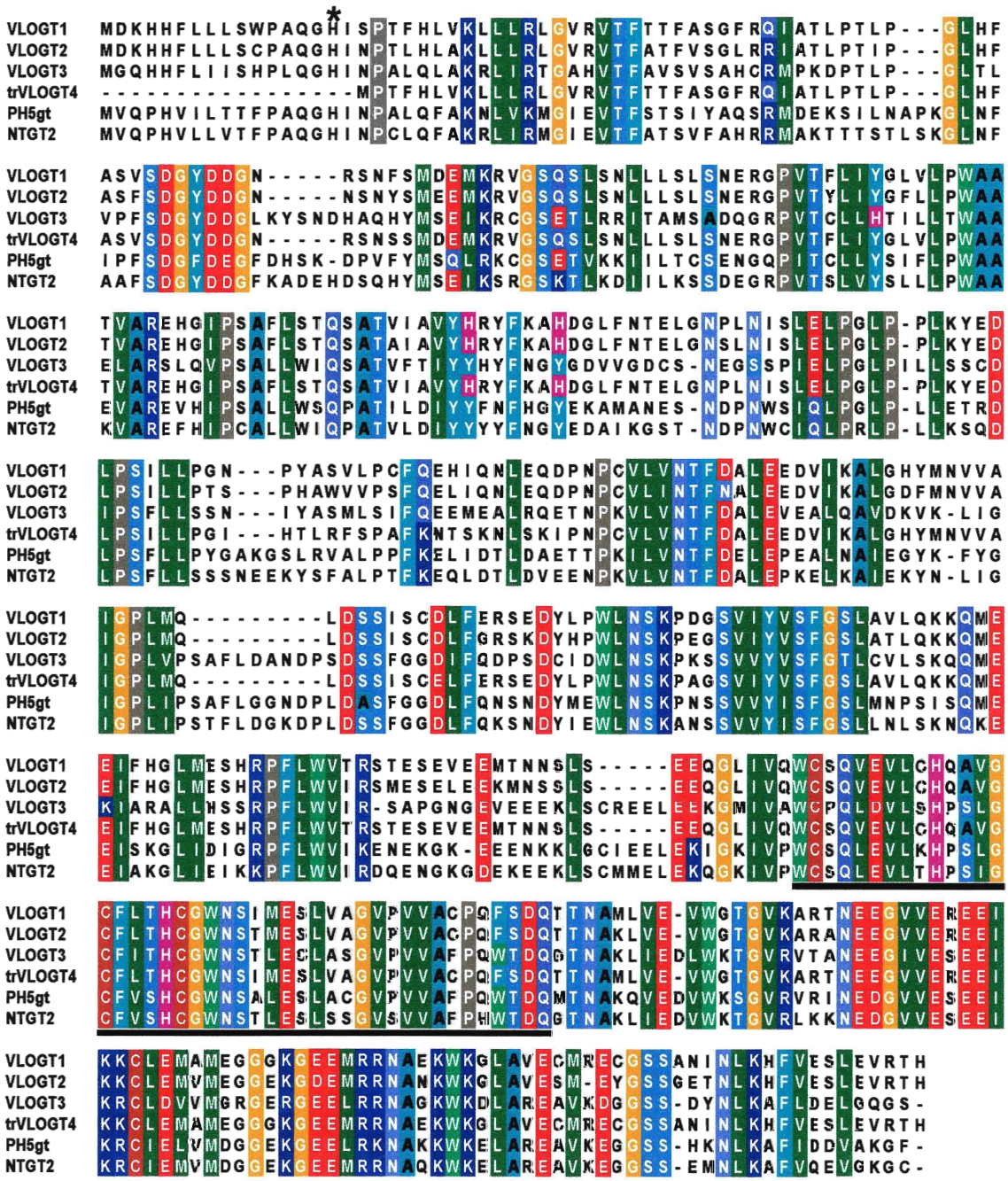


Figure 2. Protein sequence alignment of the *Vitis labrusca* 5GT-like genes (VLOGT1, VLOGT2, VLOGT3,and trVLOGT4) from this study, the *Nicotiana tabacum* 3-*O* coumarin/7-*O* flavonoid GT (Nt_NTGT2, BAB88935) and the *Petunia* x *hybrida* anthocyanin 5-*O* glucosyltransferase (Ph_5GT, BAA89009). The plant secondary product glucosyltransferase (PSPG) consensus sequence is underlined and the asterisk indicates the catalytic histidine present in all plant type 1 plant glucosyltransferases. Coloured blocks indicate amino acids with similar properties.

GT from *Vitis labrusca* with an ORF of 1395 bp (*VLOGT3*) which translates to a protein of 464 amino acids with a calculated molecular weight of 51.3 kDa.

The C-terminus of the four *Vitis labrusca* clones contained the Plant Secondary Product GT (PSPG) sequence (Vogt and Jones, 2000), placing these proteins with more than 90 other functionally characterized family 1 GTs. A search of the NCBI conserved domain database with *VLOGT1*, *VLOGT2*, *VLOGT3* and *trVLOGT4* identified two putative conserved domains characteristic of GTs. A BLAST search with the four ORF sequences showed highest protein sequence identity to several anthocyanin 5GTs (Yamazaki et al., 1999 (BAA36423; BAA36422, BAA36421); Yamazaki et al., 2002 (BAA89009); Lorenc-Kukula et al., 2005) (Figure 2). The grape GTs also showed significant identity to a *Nicotiana tabacum* GT (NtGT2) that glucosylates the 3-*O* position of coumarins and the 7-*O* position of flavonols (Taguchi et al., 2003; BAB88935), to a *Eucalyptus perriniana* monoterpene *O*-GT (Nagashima et al, 2004; BAD90934) and to a *Maclura pomifera* dihydroflavonol 7-*O* GT (7GT) (Tian et al., 2006b; ABL85474).

4.4.4 - Recombinant expression of VLOGT1, VLOGT2, VLOGT3 and truncated VLOGT4

Escherichia coli (*E. coli*) cells expressing VLOGT1, VLOGT2, VLOGT3 and trVLOGT4 were grown, expressed and harvested as described in Experimental Procedures. Protein was extracted and separated into soluble and insoluble protein fractions. SDS-PAGE analysis indicated that the majority (approximately 95%) of the GST-fusion recombinant proteins (80 kDa) was insoluble, except for trVLOGT4, which

produced 90% soluble protein (50 kDa). Attempts to solubilize and refold an active protein from the insoluble protein fraction were unsuccessful (data not shown) and further purification of the proteins by removal of the GST tag did not change the activity of these proteins.

The soluble protein fraction was either affinity purified by glutathione sepharose 4B, or was desalted prior to use in enzyme assays. One 50 ml bacterial culture produced 25-150 µg of purified recombinant VLOGT2 (rVLOGT2), 1-5 µg of purified recombinant VLOGT1 (rVLOGT1) or 1-10 µg of purified recombinant VLOGT3 (rVLOGT3). For this reason, only rVLOGT2 was purified prior to enzyme assays, while the desalted soluble fraction was used in enzyme assays for rVLOGT3 and rVLOGT1 with the exception that rVLOGT3 was purified prior to performing kinetic analyses.

Preliminary screening of the recombinant enzymes with anthocyanin substrates indicated that these proteins did not glucosylate cyanidin, or kuromanin the putative substrates for the 5GT reaction.

4.4.5 - Biofermentation of kaempferol by rVLOGT1, rVLOGT2, and rVLOGT3

Bacterial cultures (50 ml) expressing rVLOGT1, rVLOGT2, rVLOGT3, trVLOGT4, or the pGEX empty vector (EV) were incubated with kuromanin (anthocyanin), kaempferol (flavonol) and sapogenin (saponin) as potential substrates for glucosylation. Cultures expressing EV or trVLOGT4 did not glucosylate any of the substrates; whereas cultures expressing rVLOGT1, rVLOGT2, and rVLOGT3 glucosylated kaempferol, producing one product in the ratio of 2:3:8 respectively, but did

not glucosylate sapogenin or kuromanin. The product of kaempferol glucosylation was identified as kaempferol 7-*O* glucoside (Table 1) by co-chromatography and UV spectral analysis (Chapter 3).

4.4.6 - Preliminary characterization of recombinant GTs in vitro

Screening of rVLOGT1, rVLOGT2, rVLOGT3 and trVLOGT4 desalted soluble protein with the substrates quercetin, isorhamnetin, dihydroquercetin, sinapic acid, *para*-hydroxybenzoic acid, *trans*-resveratrol, and esculetin identified quercetin and isorhamnetin as substrates for rVLOGT1 and rVLOGT3 that both yielded a single reaction product. In contrast, rVLOGT2 produced single products with kaempferol, isorhamnetin and dihydroquercetin as substrates and three separate products with quercetin as substrate. No reaction products were formed with soluble protein extracted from trVLOGT4, with boiled enzymes or with bacterial extracts expressing EV.

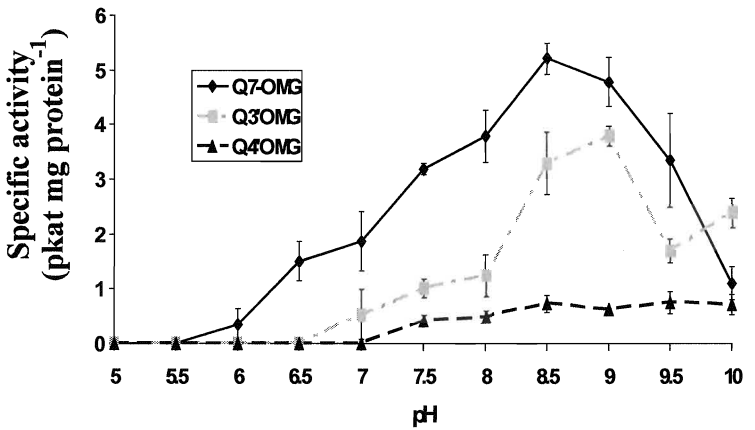
4.4.7 - Properties of rVLOGT1, rVLOGT2 and rVLOGT3

Recombinant VLOGT2 and rVLOGT3 were assayed for GT activity with quercetin and isorhamnetin respectively, between pH 5.0 and 10.0 with MES (pH 5.0 – 6.5), Tris (pH 7.0 – 9.0), and CAPS (pH 9.0 – 10.0) as described previously (Chapter 3). Recombinant VLOGT2 produced three products identified as monoglucosides by mass spectrometry (data not shown), while rVLOGT3 produced one product which co-chromatographed on HPLC with the major glucosylated product of rVLOGT2 (MG1). At pH 7.5 and 8.0 the three quercetin monoglucosides (MG1:MG2:MG3) were produced, whereas maximal rVLOGT3 activity was detected at pH 8.5 (Figure 3).

Table 1. Biocatalysis of kaempferol, kuromanin and sapogenin by *E. Coli* cells expressing different members of the recombinant *Vitis labrusca* 5GT-like gene family (rVLOGT1, rVLOGT2, rVLOGT3, trVLOGT4) compared to *E. Coli* expressing the pGEX empty vector (pGEX EV). ND represents values which were not detected by the HPLC methods used (see Experimental Procedures)

Enzyme	Substrate	Product formed	Culture medium (ng/ml in supernatant)
rVLOGT1	Kaempferol	Kaempferol 7-*O* glucoside	40.5 ± 11.6
	Sapogenin	ND	ND
	Kuromanin	ND	ND
rVLOGT2	Kaempferol	Kaempferol 7-*O* glucoside	112.5 ± 14.0
	Sapogenin	ND	ND
	Kuromanin	ND	ND
rVLOGT3	Kaempferol	Kaempferol 7-*O* glucoside	26.8 ± 2.6
	Sapogenin	ND	ND
	Kuromanin	ND	ND
trVLOGT4	Kaempferol	ND	ND
pGEX EV	Kaempferol	ND	ND

A.



B.

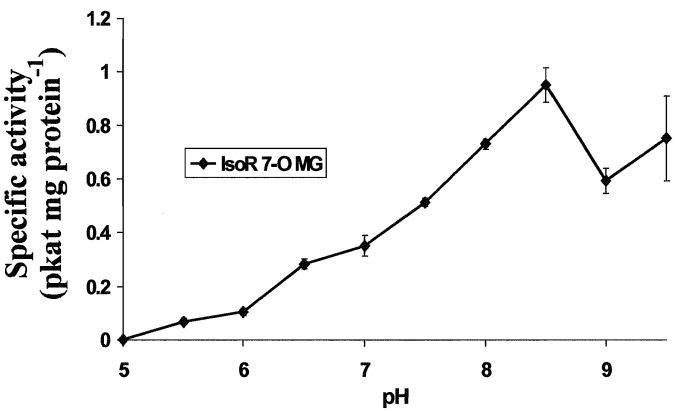


Figure 3. Effect of assay pH on rVLOGT2 and rVLOGT3 *in vitro*. A. Effect of assay pH on the production of quercetin 7-*O* monoglucoside (Q 7-O MG), quercetin 3'or 5-*O* monoglucoside (Q 3'-O MG), and quercetin 4'-*O* monoglucoside (Q 4'-O MG) by rVLOGT2 *in vitro*.
B. Effect of assay pH on the production of isorhamnetin 7-*O* monoglucoside (IsoR 7-O MG) by rVLOGT3 *in vitro*. Each point represents the mean of three independent trials ± standard deviation.

HPLC co-chromatography with standards and UV spectral analysis indicate that quercetin 3-*O*-glucoside is not produced by any of the enzymes assayed, whereas UV spectral analysis and co-chromatography with quercetin 7-*O*-glucoside identified MG1 as quercetin 7-*O*-glucoside (no hypsochromic shift of UV max from 370 nm) (Harborne, 1967; Vogt et al., 1997; Kurioka and Yamazaki, 2002; Kramer et al., 2003; Chapter 3). Spectral analysis identified MG2 as quercetin 3'- or 5- *O*-glucoside (Jurd, 1962; Kurioka and Yamazaki; 2002) and MG3 as quercetin 4'*O*-glucoside (hypsochromic shift of UV max to 365 nm) (Jurd, 1962; Vogt et al., 1997; Kurioka and Yamazaki, 2002; Kramer et al., 2003).

4.4.8 – Substrate specificity of rVLOGT1, rVLOGT2 and rVLOGT3

Substrate specificity of the recombinant GT proteins was determined *in vitro* at pH 8.0 using 45 different substrates for rVLOGT3 and rVLOGT2 and 34 different substrates for rVLOGT1. The three recombinant enzymes did not glucosylate anthocyanins and primarily glucosylated the 7-*O*-position of flavonoid substrates, and the non-flavonoid model xenobiotic substrate 2,4,5-trichlorophenol (TCP) (Figure 4, Figure 5).

Purified rVLOGT2 produced the 7-*O*-glucoside of several flavonoids with diverse hydroxylation and methylation patterns, glucosylated TCP, and glucosylated luteolin and eriodictyol, with the highest specific activities (Figure 4). The 3'*O*- glucosides of luteolin and 3,6,2',3'-tetrahydroxyflavone and the 4'*O*-glucosides of luteolin and

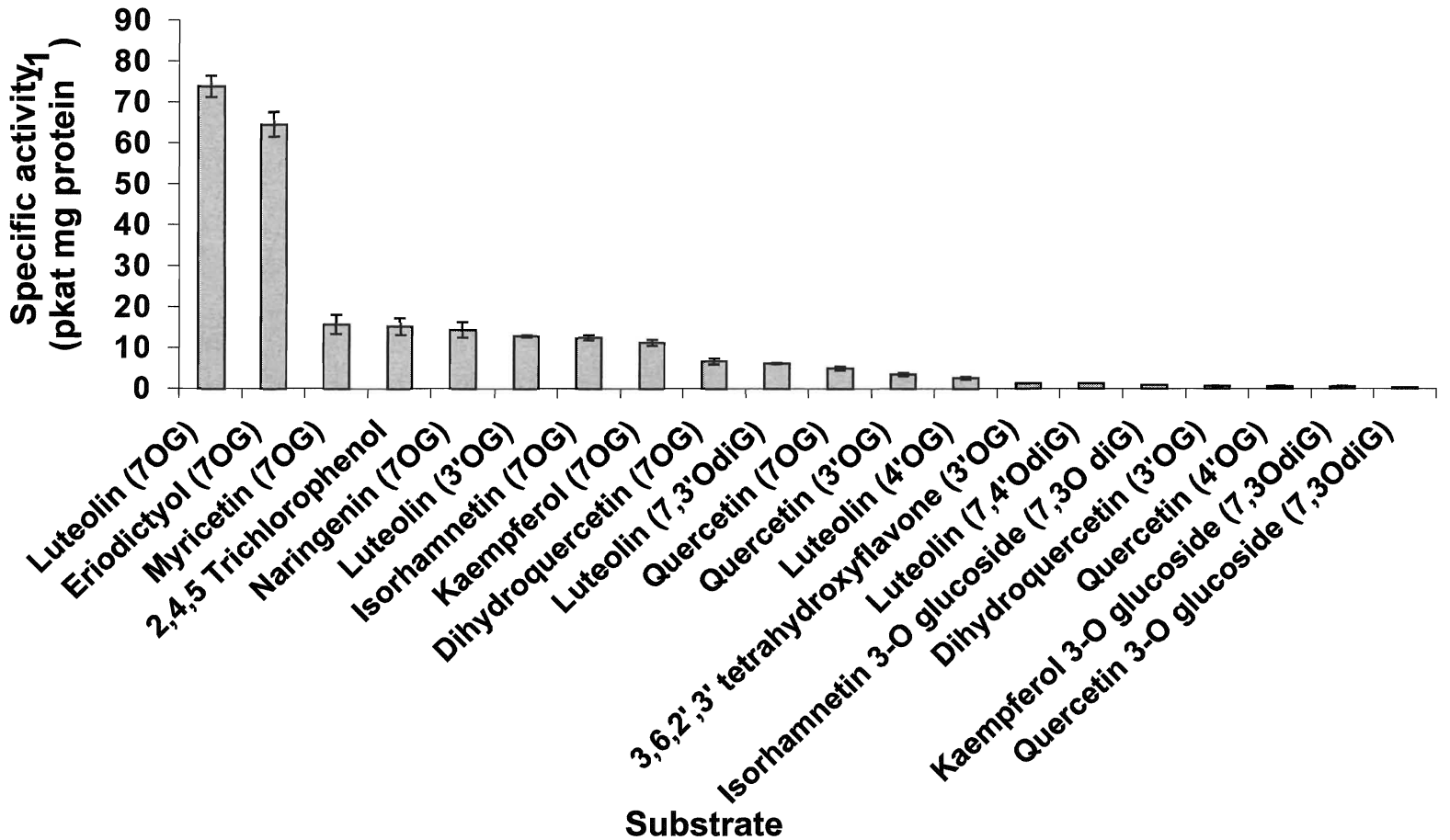


Figure 4. *In vitro* substrate specificity of rVLOGT2.
A. Substrate specificity of rVLOGT2. The substrate in each assay is identified, followed by the product formed in brackets (7OG - 7-*O* glucoside; 3'OG - 3' or 5-*O* glucoside; 4'OG - 4'-*O* glucoside; 7,4'OdiG - 7,4'-*O* diglucoside; 7,3-*O* diG - 7, 3 *O*-diglucoside; 7,3'OdiG - 7, 3'- or 5-*O* glucoside; Each bar represents the mean of at least three trials ± standard deviation.

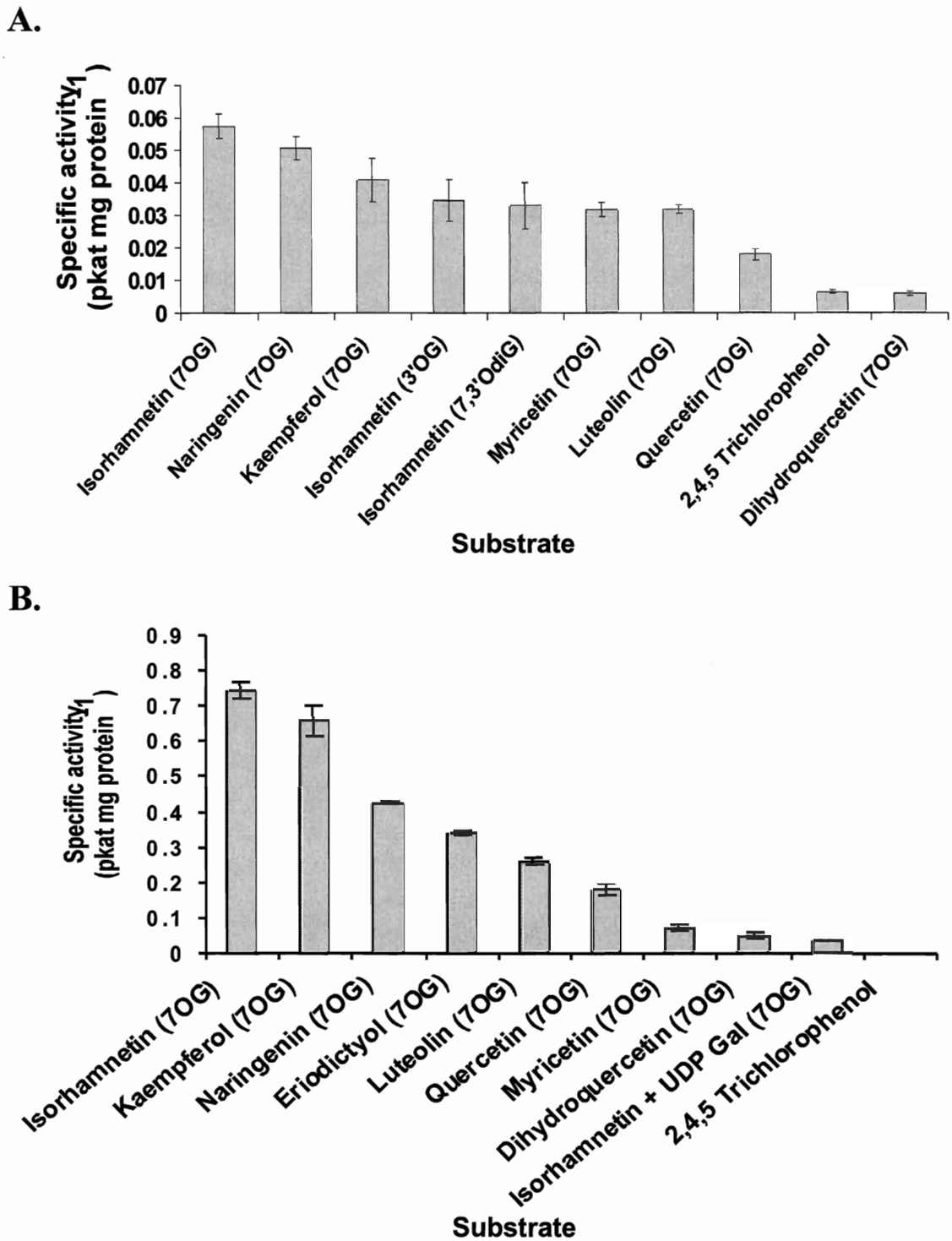


Figure 5. *In vitro* substrate specificity of rVLOGT1 and rVLOGT3.
A. Substrate specificity of rVLOGT1. B. Substrate specificity of rVLOGT3. The substrate in each assay is identified, followed by the product formed in brackets (7OG - 7-*O* glucoside; 3'OG - 3' or 5-*O* glucoside; 4'OG - 4'-*O* glucoside; 7,4'OdiG - 7,4'-*O* diglucoside; 7,3-*O* diG - 7, 3 *O*-diglucoside; 7,3'OdiG - 7, 3'- or 5-*O* glucoside. Each bar represents the mean of at least three trials ± standard deviation.

quercetin were also produced, at low levels as identified by UV spectral analysis (Harborne, 1967; Schweiger et al., 2006; Ko et al., 2007). The 3-*O*-glucosides of isorhamnetin, kaempferol and quercetin were glucosylated producing one product, identified as quercetin 3, 7-*O* diglucoside by UV spectral analysis (hypsochromic shift of UV max to 354 nm) (Mabry et al., 1970; Kramer et al., 2003). The 7,3'-*O*-diglucoside and 7, 4'-*O*-diglucoside of luteolin were also produced under these assay conditions and were identified by UV spectra analysis (Harborne, 1967; Ficarra et al., 1990; Kramer et al., 2003; Schweiger et al., 2006), whereas no triglucosides were formed with any substrate (Figure 4).

Incubation of rVLOGT1 with isorhamnetin and UDP-glucose (UDPG) produced three new products, isorhamnetin 7-*O*-glucoside (major) isorhamnetin 4'-*O*- or 5-*O*-glucoside (minor) and isorhamnetin 7, 4'-*O*-diglucoside (minor) (Ross, 1984; Litvinencko and Bubenchikova, 1989). rVLOGT1 also glucosylated the 7-*O* position of the flavonoids naringenin, kaempferol, myricetin, luteolin, quercetin and dihydroqueretin as well as glucosylating TCP at low levels (Figure 5A).

rVLOGT3 produced the 7-*O*-glucosides of isorhamnetin, kaempferol, naringenin, eriodictyol, luteolin, quercetin, myricetin and dihydroquercetin, in addition to glucosylating TCP (Figure 5B). Interestingly, VLOGT3 was able to use UDP-galactose (UDP-Gal) as a donor with isorhamnetin as an acceptor at low levels (Figure 5B).

The anthocyanin substrates cyanidin, kuromanin, cyanidin 3,5-*O* diglucoside, peonidin, peonidin 3-*O* glucoside, delphinidin, and malvidin; the stilbenes *trans*-resveratrol, *cis*-resveratrol and *trans*-piceatannol; the saponin, sapogenin; the

hydroxycinnamic acids coumaric acid, sinapic acid, caffeic acid, cinnamic acid, ferulic acid, and chlorogenic acid; the hydroxybenzoic acids *para*-hydroxybenzoic acid, benzoic acid and gallic acid; the alcohols vanillin, benzyl alcohol and phenylethanol; the coumarin esculetin; the catechins catechin and epicatechin; phenol, abscisic acid, indole-3-acetic acid, salicylic acid and UDP-glucuronic acid were not accepted by any of the recombinant enzymes. UDP-galactose was not accepted by rVLOGT2 with luteolin as an acceptor substrate, eriodictyol was not accepted by rVLOGT1 and 3, 6, 2', 3' tetrahydroxyflavone was not accepted by rVLOGT3 (data not shown).

4.4.9 – Kinetic analysis of rVLOGT2 and rVLOGT3

rVLOGT2 and rVLOGT3 were purified and used in kinetic analysis with isorhamnetin and UDPG as substrates for rVLOGT3 and with myricetin, TCP and UDPG as substrates for rVLOGT2. Kinetic analysis of rVLOGT2, with 9 mM UDPG and varying concentrations of myricetin was linear for 2 h; whereas varying concentrations of TCP with 300 µM UDPG (in a ratio of 1:5 ^{14}C UDPG:UDPG) were linear for 90 min. Michaelis-Menton kinetic analysis of rVLOGT2 calculated 3.8 fold higher affinity for myricetin than for TCP (48.4 and 184.5 µM) (Table 2). The turnover number (K_{cat}) with TCP as a substrate for rVLOGT2 (K_{cat} = 105.7 s^{-1}) is 10^4 fold higher than with myricetin (K_{cat} = 2.4 x E^{-4} s^{-1}) and the calculated catalytic efficiency (K_{cat}/K_m) is 10^5 fold higher with TCP (K_{cat}/K_m = 0.573 μM^{-1} s^{-1}) than with myricetin (K_{cat}/K_m = 4.96 E^{-6} μM^{-1} s^{-1}), suggesting that TCP is the best substrate for rVLOGT2.

Kinetic analysis of rVLOGT3 with 9 mM UDPG and varying concentrations of isorhamnetin followed by Michaelis-Menton kinetic calculations yielded a K_m of 30.2

Table 2. Kinetic parameters of rVLOGT2 and rVLOGT3.

A. Kinetic data for rVLOGT2 with myricetin, 2,4,5-trichlorophenol and UDPG as substrates.

A.

	rVLOGT2		
	K_m (μM)	**K_{cat} (s^{-1})**	**K_{cat}/K_m ($\mu M^{-1} s^{-1}$)**
Myricetin	48.4 ± 5.0	$2.4\ E^{-4} \pm 1.1\ E^{-5}$	$4.96\ E^{-6}$
2,4,5-trichlorophenol	184.5 ± 29.4	105.7 ± 18.1	0.573
UDPG (Myricetin)	940 ± 109	$2.0\ E^{-4} \pm 3.5\ E^{-5}$	$2.13\ E^{-7}$

B.

	rVLOGT3		
	K_m (μM)	**K_{cat} (s^{-1})**	**K_{cat}/K_m ($\mu M^{-1} s^{-1}$)**
Isorhamnetin	30.2 ± 2.6	$8.11\ E^{-7} \pm 6.96\ E^{-8}$	$2.69\ E^{-8}$
UDPG (Isorhamnetin)	886 ± 26.8	$7.67\ E^{-6} \pm 3.85\ E^{-8}$	$8.66\ E^{-9}$

Mean values from at least three independent experiments ± standard deviation are shown.

μM, a K_{cat} of 8.11 E^{-7} s^{-1}, and a K_{cat}/K_m of 2.69 E^{-8} μM^{-1} s^{-1}, similar to the kinetic analysis of myricetin as a substrate for rVLOGT2.

Kinetic analysis of UDPG for rVLOGT2 (100 μM myricetin) and rVLOGT3 (100 μM isorhamnetin) yielded K_ms of 940 μM, and 886 μM respectively; K_{cat}s of 2.0 E^{-4} s^{-1} and 7.67 E^{-6} s^{-1} respectively and K_{cat}/K_ms of 2.13 E^{-7} μM^{-1} s^{-1} and 8.66 E^{-9} μM^{-1} s^{-1} respectively (Table 2).

4.4.10 - Gene expression profiling of VLOGT1 and VLOGT2 (VLOGT1,2) and VLOGT3 in grape berry, vegetative and reproductive tissues

RNA was extracted from several grape tissues as listed in Experimental Procedures. Template generated from reverse transcription without reverse transcriptase was checked for genomic DNA contamination (data not shown). Primers designed to amplify *Vitis vinifera* actin were used with the cDNA templates to determine baseline expression between samples. Glucosyltransferase gene expression was quantified and divided by the mean *Actin* gene expression values to obtain the relative % expression of *VLOGT1,2* and *VLOGT3* compared to those of *Actin* in these tissues (Figure 6). *Vitis labrusca* UDP-glucose: flavonoid 3-*O*-GT (*VL3GT*) primers amplified a product from exocarp tissue, but not mesocarp tissue (Chapter 5); while *VLRSGT* primers amplified a product from mesocarp tissue but not exocarp tissue (Chapter 3), to verify the tissue-specificity of the RNA extracts. *VLOGT1,2* gene specific primers amplified a product from plasmids containing *VLOGT1* and *VLOGT2*, but not from plasmids containing *VLOGT3*, *VLRSGT* nor *VL3GT*. Similarly *VLOGT3* and *VL3GT* gene specific primers amplified a product from plasmids containing *VLOGT3* and *VL3GT* respectively (but not

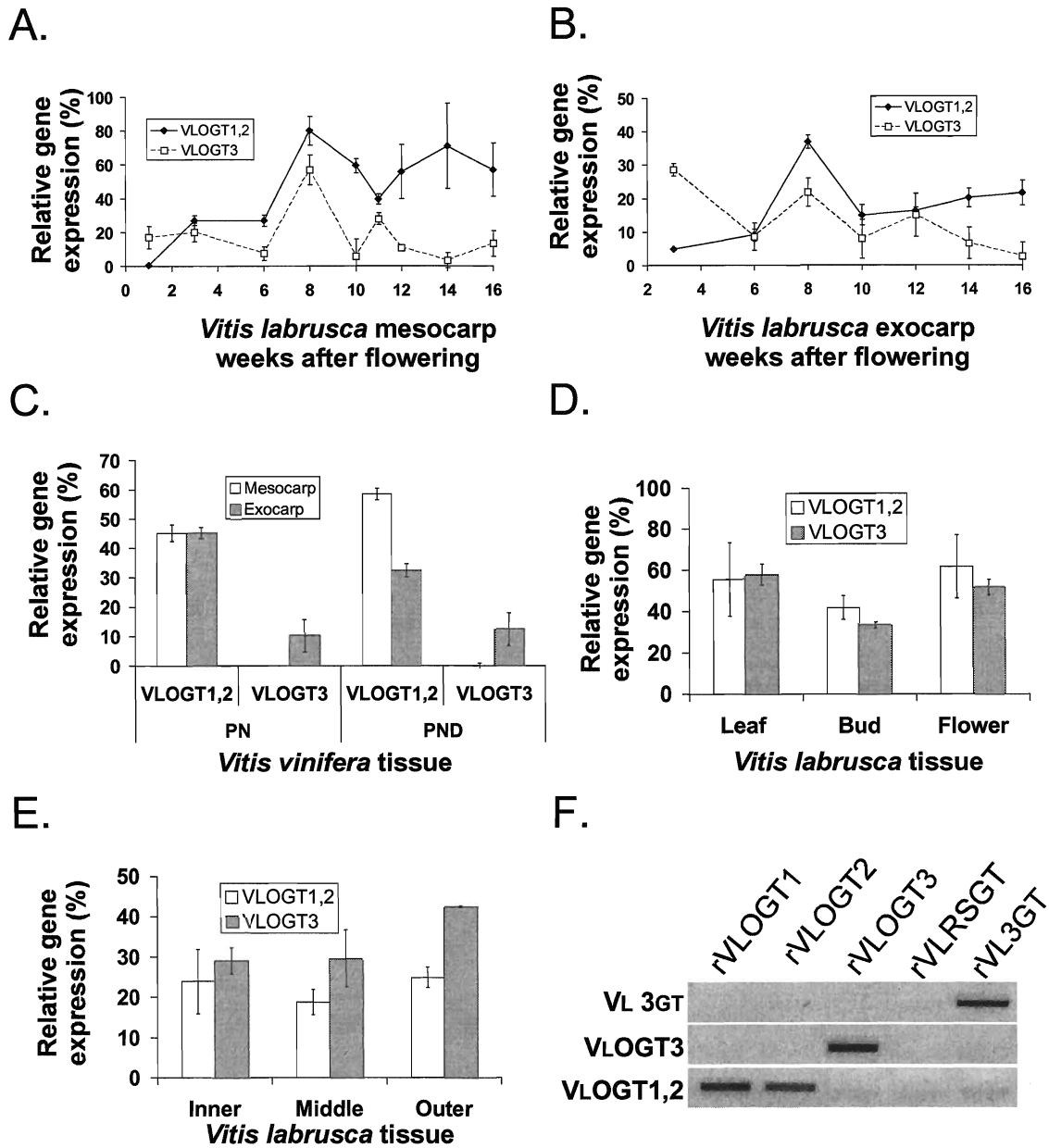


Figure 6. Relative gene expression of *VLOGT1,2*, and *VLOGT3* compared to *VLActin* expression. Gene expression of *VLOGT1,2,* and *VLOGT3* in relation to *ACTIN* gene expression in *Vitis labrusca* (*VL*) cv. Concord berry mesocarp (A) and berry exocarp (B) tissues and in *Vitis vinifera* cvs. Pinot Noir (PN) and Pinot Noir Droit (PND) berry mesocarp and exocarp (C). D. Relative gene expression of *VLOGT1,2,* and *VLOGT3* in relation to *ACTIN* gene expression in *Vitis labrusca* leaf, flower bud and flower tissue. E. Relative gene expression of *VLOGT1,2,* and *VLOGT3* in *VL* inner, middle and outer berry mesocarp tissue (week 12 AF) in relation to *Actin* gene expression. F. Specificity of VL3GT, VLOGT1,2 and VLOGT3 primers to expression plasmids containing the full-length rVLOGT1, rVLOGT2, rVLOGT3, rVLRSGT and rVL3GT. All values represent the mean of two trials ± standard deviation.

reciprocally), but did not amplify PCR products from plasmids containing *VLOGT1*, *VLOGT2* or *VLRSGT* (Figure 6F). PCR using *VLOGT1,2* and *VLOGT3* gene specific primers with identical amounts of plasmid template and identical PCR cycling conditions generated nearly identical amounts of amplified product (data not shown).

With the exception of week 1 whole berry tissue and week 3 AF mesocarp and exocarp tissues, *VLOGT1,2* was expressed at higher levels than *VLOGT3* throughout development (Figure 6A,B; Appendix I), and generally, the expression of these genes in berry mesocarp tissue was 2-fold higher than in berry exocarp tissue. Interestingly, neither *VLOGT1,2* nor *VLOGT3* gene expression was linearly related to berry developmental stage in berry exocarp ($R^2_{VLOGT1,2}$ = 0.1698, R^2_{VLOGT3} = 0.5725) or mesocarp ($R^2_{VLOGT1,2}$ = 0.5408; R^2_{VLOGT3} = 0.0403) tissues.

VLOGT3 transcript was detected in Concord week 1 AF whole berry tissue and in pre-veraison berry mesocarp and exocarp tissues, reaching maximal values at week 8 AF, and again at week 11 AF in mesocarp tissue and week 12 AF in exocarp tissue (Figure 6 A,B). *VLOGT1,2* transcript was maximally expressed at week 8 AF, and increased again at week 12 and 14 AF (Figure 6A, Appendix I). In *Vitis labrusca* berry exocarp tissue *VLOGT1,2* transcript was detected at maximal levels 8 weeks AF, followed by a decrease in gene expression at week 10 and a small increase in gene expression until harvest (week 16 AF; Figure 6B, Appendix I).

VLOGT1,2 was expressed at high levels in *Vitis vinifera* cv. Pinot Noir and Pinot cv. Noir Droit week 14 AF berry exocarp and mesocarp tissues, whereas *VLOGT3* transcript was detected at low levels in berry exocarp tissue and was absent in berry

mesocarp tissue (Figure 6C). Conversely, both *VLOGT1,2* and *VLOGT3* transcripts were detected in *Vitis labrusca* leaf, flower bud and flower tissues (Figure 6D), and in *Vitis labrusca* week 12 AF inner, middle and outer grape berry mesocarp (Figure 6E).

4.4.11 - Flavonoid glucosyltransferase activity is detected in several grape tissues

Crude, desalted grape protein extracts were assayed for 5GT activity (see above, Figure 1), and for flavonoid *O*-GT activity with quercetin as a substrate (Figure 7). In all tissues, the predominant product was quercetin 3-*O*-glucoside (Q3G) (data not shown), with secondary and tertiary products being quercetin 7-*O* glucoside (Q7G) and quercetin 3'- or 5-*O*-glucoside (Q3'G). Quercetin 7-*O*-GT (Q7GT) activity is first detected in week 12 AF in Concord mesocarp tissue and increases to maximal levels at week 14 AF before decreasing at week 16 AF, while Q7GT activity is seen at low levels in Concord week 14 and 16 AF exocarp tissue (Figure 7A). Quercetin 3' or 5-*O* GT (Q3'5GT) activity is biphasic in *Vitis labrusca* berry mesocarp, occurring at week 1 AF in whole berries and week 3 AF mesocarp tissue, and re-appearing post-veraison at week 10 AF, reaching maximal levels at week 14 AF. In *Vitis labrusca* exocarp tissue Q3'5GT activity is first detected at week 8 AF that increases and is maintained throughout ripening (week 10-16 AF) (Figure 7B). Pinot Noir and Pinot Noir Droit post-veraison berry mesocarp tissues catalyzes the Q7GT reaction, but not the Q3'GT reaction; whereas both berry exocarp tissues catalyze the Q7GT and Q3'GT reactions at low levels (Figure 7A, B). Concord flower bud, leaf and flower tissues catalyze the Q7GT and the Q3'GT reactions, with maximal Q7GT activity detected in flower tissue, and maximal Q3'GT activity detected in leaf tissue (Figure 7C).

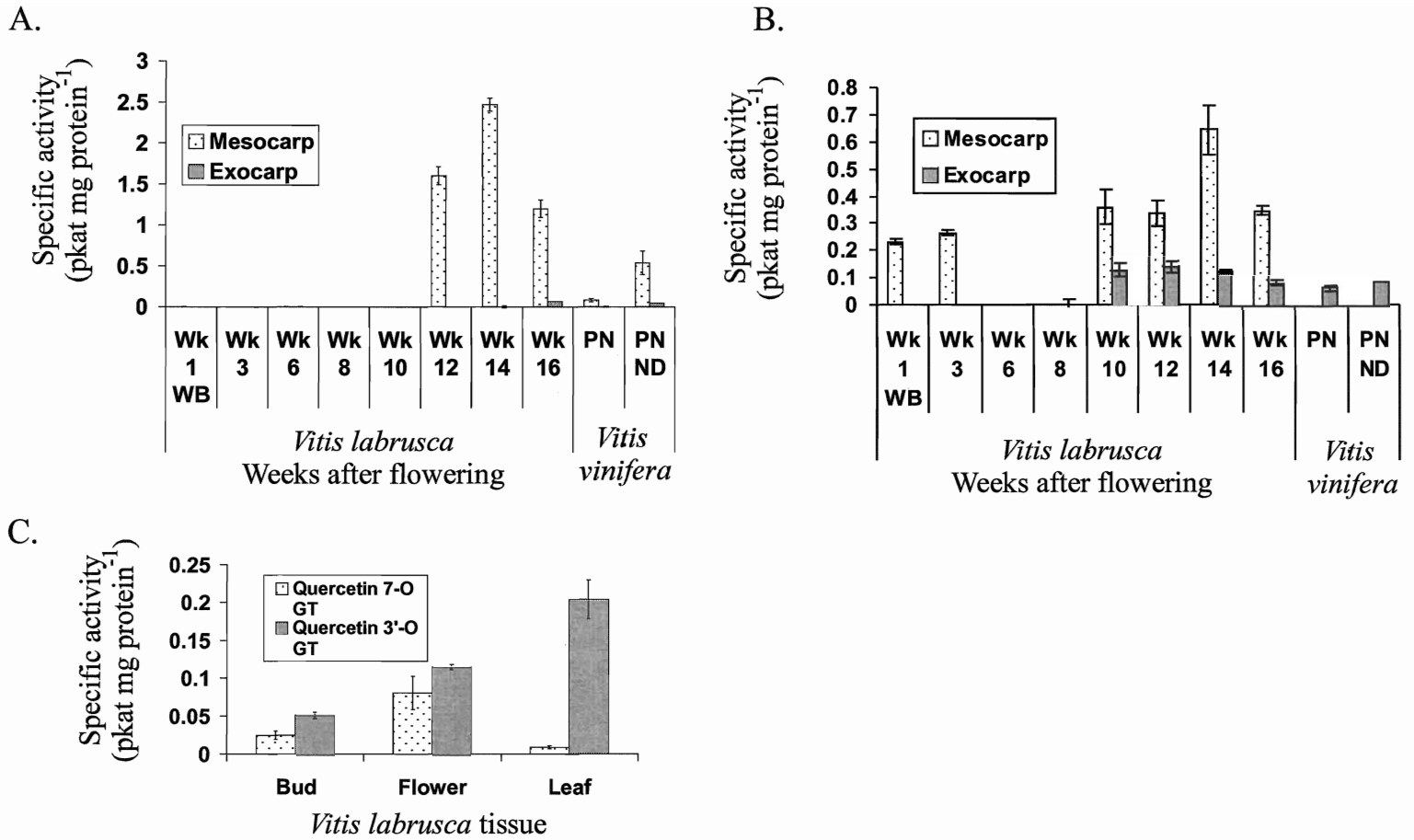


Figure 7. Developmental quercetin 7-*O*- and 3'- or 5-*O* glucosyltransferase activity profile in grape berry tissues. A. Quercetin 7-*O* glucosyltransferase (Quercetin 7-*O* GT) activity in *Vitis labrusca* grape berry exocarp and mesocarp tissues and *Vitis vinifera* cv. Pinot Noir (PN) and Pinot Noir Droit (PND) post-veraison (week 14 after flowering) mesocarp and exocarp tissues. B. Quercetin 3'- or 5- *O* glucosyltransferase (Quercetin 3'-*O* GT) activity in *Vitis labrusca* grape berry exocarp and mesocarp tissue and *Vitis vinifera* cv. Pinot Noir (PN) and Pinot Noir Droit (PND) post-veraison (week 14 after flowering) mesocarp and exocarp tissues. C. Quercetin 3'-*O* GT and quercetin 7-*O* GT activity in *Vitis labrusca* leaf, flower and pre-bloom bud tissue. Bars represent the mean of three independent trials ± standard deviation

4.4.12 - Ethyl acetate extracted metabolite profiles are consistent with a putative role for VLOGT3 and VLOGT2 in flavonoid modification.

Aqueous metabolite preparations from *Vitis labrusca* week 8 and 12 AF exocarp and mesocarp tissue were extracted with ethyl acetate and analyzed by HPLC. In all tissues analyzed quercetin 3-*O* glucoside (Q3G) was the only flavonoid detected, with maximal amounts present in weeks 12 and 8 AF exocarp tissues respectively. Acid hydrolysis of the ethyl acetate fractions released the quercetin aglycone and decreased the amount of Q3G present in these extracts. Enzyme assays with rVLOGT2 or rVLOGT3 and the acid hydrolyzed metabolites from week 12 AF *Vitis labrusca* exocarp tissue produced Q7G as the only product, whereas enzyme assays with EV did not catalyze this reaction (Figure 8). These results show that grape metabolites harvested from grapes, such as quercetin, could be glucosylated with rVLOGT2 or rVLOGT3 at the 7-*O* position and are consistent with the substrate specificity studies with the recombinant protein (Figure 4; Figure 5) and a role for these enzymes in flavonols modification.

4.4.13 - Phylogenetic analysis places VLOGT1, VLOGT2, VLOGT3 and trVLOGT4 in a clade with 5GTs and flavonoid 7GTs.

A neighbour-joining phylogenetic tree places the *Vitis labrusca* GTs from this study in a clade with enzymes catalyzing 5GT and 7GT reactions (Figure 9). *VLOGT1*, *VLOGT2* and *trVLOGT4* are most related to the *Arabidopsis thaliana UGT75B1* and *UGT75D1* that glucosylates xenobiotics, forms hydroxybenzoic acid glucose esters and flavonol-7-*O*-glucosides, respectively (Lim et al., 2002; Meβner et al., 2003). VLOGT3 is most closely related to the *Petunia* x *hybrida* 5GT (Yamazaki et al., 2002) and the

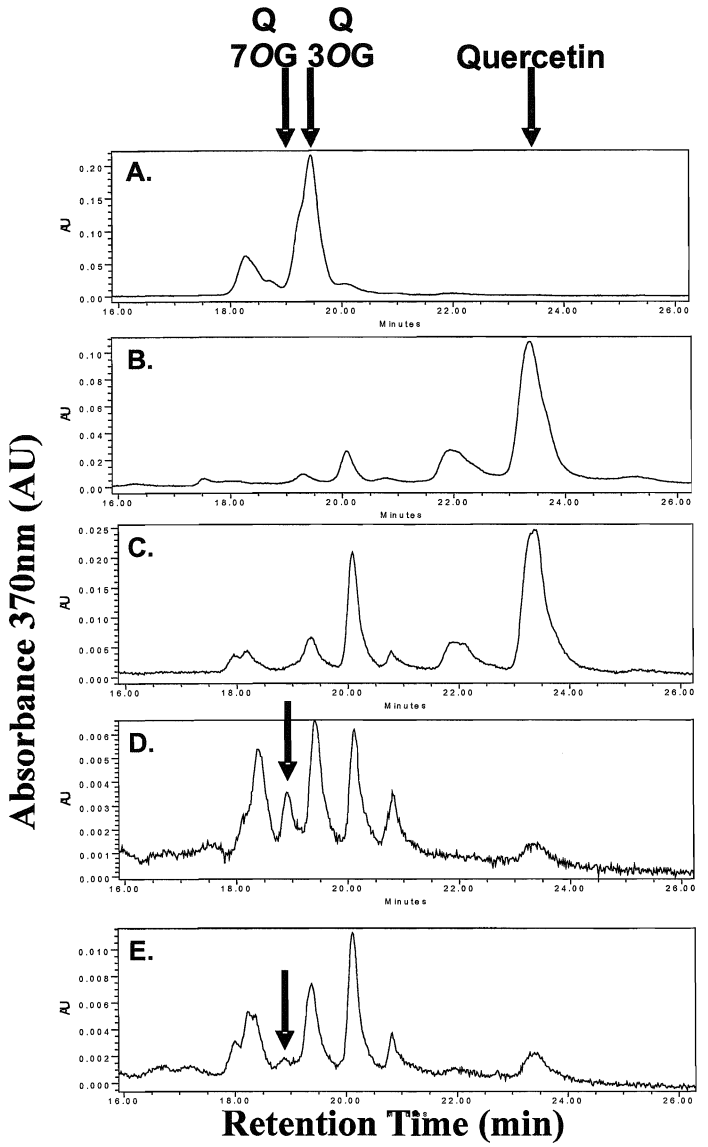


Figure 8. Representative HPLC profile of *Vitis labrusca* week 12 after flowering exocarp metabolites at OD 370nm; following ethyl acetate extraction, acid hydrolysis and enzyme assays. A. HPLC profile of *Vitis labrusca* week 12 AF exocarp metabolites after ethyl acetate extraction. B. HPLC profile of *Vitis labrusca* ethyl acetate extracted week 12 AF exocarp metabolites after acid hydrolysis. C. HPLC profile of *Vitis labrusca* week 12 AF ethyl acetate extracted, acid hydrolyzed metabolites after an enzyme assay with an *E. Coli* extract expressing pGEX empty vector protein. D. HPLC profile of *Vitis labrusca* week 12 AF ethyl acetate extracted, acid hydrolyzed metabolites after an enzyme assay with rVLOGT2. E. HPLC profile of *Vitis labrusca* week 12 AF ethyl acetate extracted, acid hydrolyzed metabolites after an enzyme assay with rVLOGT3. Arrows indicate elution of quercetin, quercetin 3-*O* glucoside (Q3*O*G) and quercetin 7-*O* glucoside (Q7*O*G).

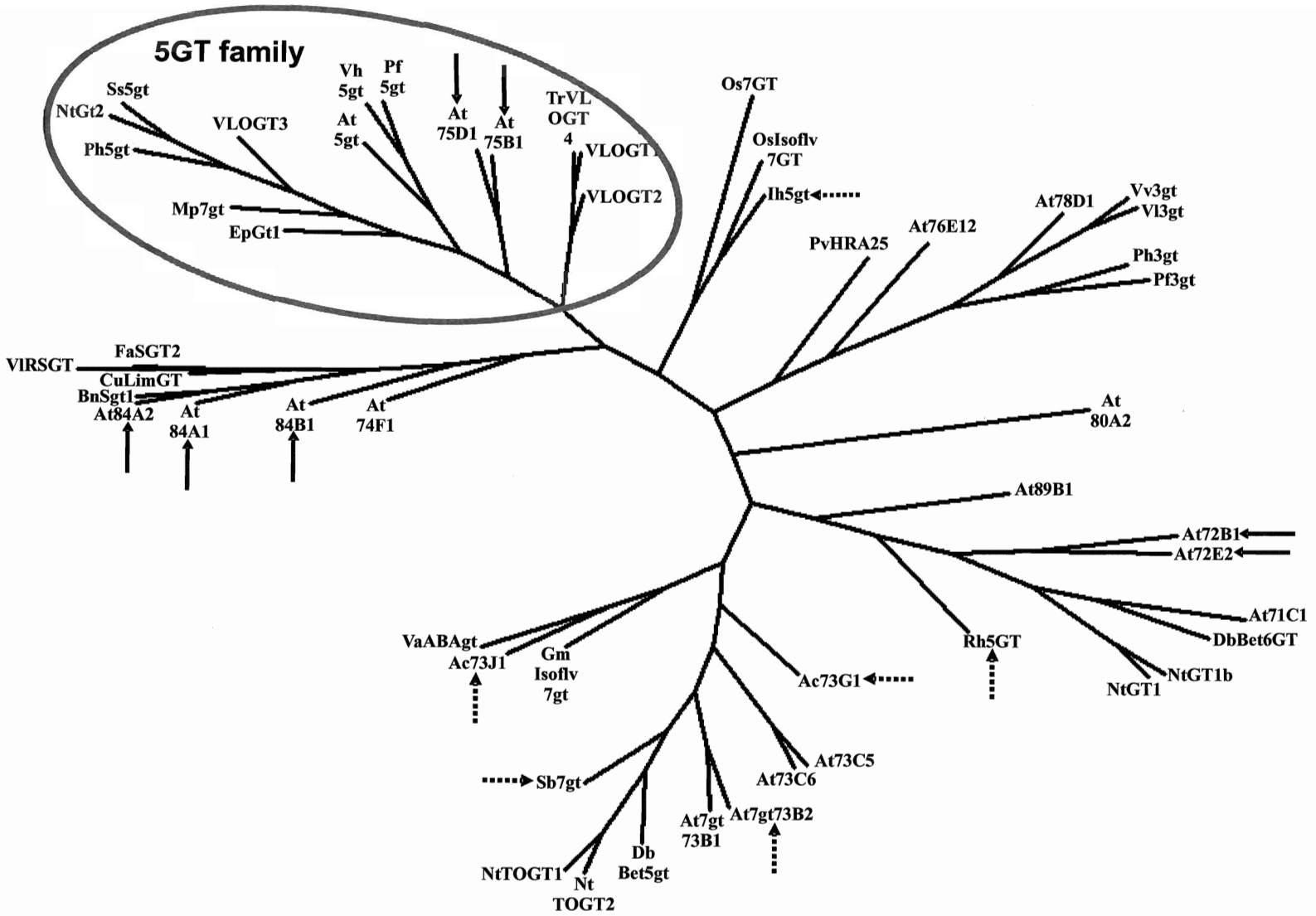


Figure 9. Neighbour joining phylogenetic tree of the *Vitis labrusca* anthocyanin 5-*O* glucosyltransferase (5gt)-like gene family and other previously characterized glucosyltransferases. Solid arrows indicate enzymes which glucosylate the xenobiotic TCP in addition to their natural substrates, and broken arrows indicate flavonoid 7-*O*-glucosyltransferases and anthocyanin 5-*O*-glucosyltransferases which are not members of the 5GT gene family. Abbreviations on following page.

Figure 9 Abbreviations:
DbBet5gt - *Dorotheanthus bellidiformis* betanidin 5-*O* glucosyltransferase (AF374004), NtTOGT2 - *Nicotiana tabacum* phenylpropanoid glucosyltransferase 2 (AF346432); NtTOGT1 - *Nicotiana tabacum* phenylpropanoid glucosyltransferase 1 (AF346431); Sb7gt - *Scutellaria baicalensis* flavonoid 7-*O* glucosyltransferase (AB031274); At7gt73B2 - *Arabidopsis thaliana* UGT73B2 flavonol 7-*O*-glucosyltransferase (AY339370); At7gt73B1 - *Arabidopsis thaliana* UGT73B1 flavonol 7-*O*- glucosyltransferase; At73C6 - *Arabidopsis thaliana* UGT73C6 flavonoid 7-*O*-glucosyltransferase; At73C5 - *Arabidopsis thaliana* UGT73C5 deoxynivalenol glucosyltransferase; Ac73G1 - *Allium cepa* 7GT UGT73G1 (Ay262062); GmIsoflv7gt - *Glycine max* isoflavonoid glucosyltransferase (DQ278439); Ac73J1 - *Allium cepa* 7GT UGT73J1 (AY262063); VaABAgt - *Vigna angularis* abscisic acid glucosyltransferase (AB065190); At89B1 - *Arabidopsis thaliana* UGT89B1 hydroxybenzoic acid glucosyltransferase (Nm106048); At72B1 - *Arabidopsis thaliana* UGT72B1 hydroxybenzoic acid glucosyltransferase (NM116337); At72E2 *Arabidopsis thaliana* UGT72E2 TCP glucosyltransferase (NM126067); At71C1 - *Arabidopsis thaliana* UGT71C1 phenolic alcohol glucosyltransferase (NM128529); DbBet6gt - *Dorotheanthus bellidiformis* betanidin 6-*O* glucosyltransferase (AF374004); NtGt1b - *Nicotiana tabacum* glucosyltransferase 1b (AB052558); NtGt1 - *Nicotiana tabacum* glucosyltransferase 1 (AB052557); At80A2 - *Arabidopsis thaliana* UGT80A2 sterol glucosyltransferase; Rh5GT - *Rosa hybrida* anthocyanidin 5-*O* glucosyltransferase (AB201050); At76E12 - *Arabidopsis thaliana* UGT76E12 flavonol *O*-glucosyltransferase; PvHRA25 - *Phaseolus vulgaris* glucosyltransferase HRA25 (AF303396); At78D1 - *Arabidopsis thaliana* UGT 78D1 UDP-rhamnose flavonol 3-*O*-glucosyltransferase; Vv3gt - *Vitis vinifera* flavonoid 3-*O*-glucosyltransferase (AF000372); VL3gt - *Vitis labrusca* flavonoid 3-*O*-glucosyltransferase (EF630356); Pet3gt - *Petunia hybrida* flavonoid 3-*O*-glucosyltransferase (AB027454); Per3gt - *Perilla frutescens* flavonoid 3-*O*-glucosyltransferase (AB002818); At74F1 - *Arabidopsis thaliana* UGT74F1 anthranilate glucosyltransferase; At75D1 - *Arabidopsis thaliana* UGT75D1 xenobiotic and flavonol glucosyltransferase; At75B1 - *Arabidopsis thaliana* UGT75B1 hydroxybenzoic acid glucosyltransferase; Mp7gt - *Maclura pomifera* flavonoid 7GT (DQ985179); EpEPGT1 - *Eucalyptus perriniana* monoterpene glucosyltransferase 1 (AB166765); Ph5gt - *Petunia* x *hybrida* anthocyanin 5-*O* glucosyltransferase (AB027455); NtGt2 - *Nicotiana tabacum* flavonol 7-*O* glucosyltransferase (AB072919); St5gt - *Solanum sogarandinum* anthocyanin 5-*O*-glucosyltransferase (AY033489); VLOGT3 - *Vitis labrusca* *O*-glucosyltransferase 3 (Ef533706); Ih5gt - *Iris hollandica* anthocyanin 5-*O*-glucosyltransferase (AB113664); trVLOGT4 - truncated *Vitis labrusca* *O*-glucosyltransferase 4 (EF533707); VLOGT1 - *Vitis labrusca* *O*-glucosyltransferase 1 (EF533704); VLOGT2 - *Vitis labrusca* *O*-glucosyltransferase 2 (EF533705); At84B1 - *Arabidopsis thaliana* UGT84B1 indole-3-acetic acid glucosyltransferase (NM127890); At84A1 - *Arabidopsis thaliana* UGT84A1 hydroxycinnamic acid glucosyltransferase (BT015796); At84A2 - *Arabidopsis thaliana* UGT84A2 hydroxycinnamic acid glucosyltransferase; BnSgt1 - *Brassica napus* sinapate glucosyltransferase (AF287143); CuLimgt - *Citrus unshiu* liminoid glucosyltransferase (AB033758); FaSGT2 - *Fragaria* x *ananassa* cinnamate glucosyltransferase (AY663785); VLRSGT - *Vitis labrusca* resveratrol/hydroxycinnamic acid *O*-glucosyltransferase (DQ832169); OsIsoFlv7GT - *Oryza sativa* isoflavonoid 7-*O*-glucosyltransferase (BAC80066); Os7gt - *Oryza sativa* flavonoid 7-*O*-glucosyltransferase (BAB68093). At5GT - *Arabidopsis thaliana* UGT75C1 anthocyanin 5-*O*-glucosyltransferase; Pf5GT - *Perilla frutescens* anthocyanin 5-*O*-glucosyltransferase (Ab013596); Vh5GT - *Verbena* x *hybrida* anthocyanin 5-*O*-glucosyltransferase (AB013598).

Maclura pomifera 7GT (Tian et al., 2006b). GTs which accept TCP as a substrate (Loutre et al., 2003; Meβner et al., 2003), other 7GTs (Hirotani et al., 2000; Kramer et al., 2003; Jones et al., 2003; Willits et al., 2004; Ko et al., 2006; Ko et al., 2007) and other 5GTs (Imayama et al., 2004; Ogata et al., 2005; Tohge et al., 2005) are located throughout the phylogentic tree, including in the 5GT/7GT clade of glucoslytransferases.

Although expressed in grape exocarp tissue, the location of anthocyanin 5-*O* glucosylation *in vivo*, and grouping within the same clade as the other previously characterized 5GTs, the three full-length *Vitis labrusca* 5GT-like enzymes (rVLOGT1, rVLOGT2, rVLOGT3) do not catalyze this reaction *in vitro*.

4.5 - Discussion

A BLAST search of the *Vitis vinifera* TIGR grape gene database with the *Petunia* x *hybrida* 5GT as a query identified more than 90 putative GT sequences, including 5 sequences with at least 70% protein sequence identity to several previously characterized 5GTs. The current study describes the homology-based cloning and biochemical characterization of three full-length (*VLOGT1, VLOGT2, VLOGT3*) and one truncated (*trVLOGT4*) members of the *Vitis labrusca* cv. Concord anthocyanin 5GT-like gene family.

The deduced protein sequences of these genes are highly identical to the 5GTs from other plant species (Yamazaki et al., 2002), and group phylogenetically with the 5GTs and with several 5GT-like genes that glucosylate monoterpenes, phenolic acids, flavonols and dihydroflavonols *in vitro* (Figure 9; Lim et al., 2002; Taguchi et al., 2003;

Nagashima et al., 2004; Tian et al., 2006b). The full-length *Vitis labrusca* genes were cloned from week 12 after flowering exocarp-specific cDNA (the site of 5GT activity, Figure 1) and the corresponding proteins were heterologously expressed. *In vitro*, the *Vitis labrusca* GTs do not glucosylate anthocyanins, instead glucosylating the xenobiotic TCP and flavonols. Gene expression profiling and enzyme assays with crude plant extracts suggest that this family of genes has potential roles in the glucosylation of xenobiotics and in the low level glucosylation of flavonols.

4.5.1 - Vitis labrusca has multiple genes with sequence identity to 5GTs

In plants, GTs exist as large multigene families (Li et al., 2001; Bowles, 2002; Lim et al., 2003) and have broad, but regio-selective substrate specificity (Vogt and Jones, 2000), allowing the plant to glucosylate a wide range of natural products and xenobiotics. More than 90 GTs have been cloned and functionally characterized from plants with techniques including homology-based molecular cloning, expression in heterologous systems, and genetic analysis of the *in vivo* system with gene overexpression or repression studies to elucidate the *in planta* role of these enzymes. Identification of 5GTs have been elucidated by overexpression of the 5GT (Tohge et al., 2005), or 5GT-activating regulatory genes (Lorenc-Kukula et al., 2005). Alternatively, 5GT genes could be screened with 5GT probes from previously characterized 5GT genes (Yamazaki et al., 1999; Yamazaki et al., 2002) of cDNA libraries selected by metabolite analysis, transcriptional changes *in planta* or by differential display analysis of cDNA libraries derived from anthocyanin accumulating and non-accumulating tissues. Southern blot analysis of *Petunia* x *hybrida* (Yamazaki et al., 2002) and *Perilla frutescens*

(Yamazaki et al., 1999) identified only two 5GT genes per genome, suggesting that homology-based cloning could be a successful strategy to identify novel 5GT genes.

4.5.2 - rVLOGT1, rVLOGT2, and rVLOGT3 glucosylate flavonoids and the xenobiotic TCP, but not anthocyanins in vitro

Biocatalysis with bacterial cultures expressing rVLOGT1, rVLOGT2, and rVLOGT3 converted kaempferol to the 7-*O*-glucoside (Table 1). These enzymes also glucosylated the model xenobiotic TCP and other flavonoids (Figure 4; Figure 5), whereas rVLOGT2 also produced several flavonoid mono- and di-glucosides (Figure 4). The rVLOGT2 predominantly glucosylated the 7-*O*-position *in vitro*, with the flavone luteolin and the flavanone eriodictyol (differing only in B-ring stereochemistry) yielding the two highest specific activities. Whereas enzyme assays with luteolin yielded 3 mono- and 2 di-glucosylated products, only the 7-*O* glucoside was detected with eriodictyol as a substrate, suggesting that B-ring orientation is essential for secondary and tertiary glucosylations. The secondary glucosylated product of luteolin was identified as luteolin 3'-*O*-glucoside by UV spectral analysis (Ko et al., 2007), and was consistent with substrate specificity studies (substrates lacking a free 3'-OH; kaempferol, isorhamnetin and naringenin produced only the 7-*O* glucoside). Interestingly, only one glucosylated product was observed with myricetin as a substrate, suggesting that a free 5'OH may hinder secondary and tertiary glucosylation. Small amounts of the third monoglucoside, and two diglucosides were detected with luteolin as a substrate which were identified as the 4'*O* glucoside, and the 7, 3'-*O* and 7,4'*O* diglucosides by UV spectral analysis.

Anthocyanins were not glucosylated by any of the *Vitis labrusca* 5GT-like enzymes, indicating that despite high levels of sequence identity these enzymes are not 5GTs, and emphasizing the importance of functional characterization when using homology-based approaches for gene cloning. Whereas the 5GTs are generally substrate- and regio-specific, flavonoid 7GTs can often glucosylate multiple positions on several flavonoid substrates *in vitro* (Kramer et al., 2003; Tian et al., 2006b; Ko et al., 2007), a result that is consistent with the activities of rVLOGT1 and rVLOGT2.

4.5.3 - Developmental gene expression analysis corresponds with GT activity profiling

The polyphenols produced and accumulated within the grape berry are dependent on the grape cultivar and variety (Cantos et al., 2002). Within Concord grape berry, the predominant flavonoids in exocarp and mesocarp tissue are the anthocyanidin 3-*O*-glucoside (Wu and Prior, 2005) and the quercetin 3-*O* glucoside respectively (Figure 8). Anthocyanin 3,5-*O*-diglucosides, anthocyanin 3-(6''-acetoyl) glucosides, anthocyanin 3-(6''-coumaroyl) glucosides (Wu and Prior, 2005), quercetin 3-*O* galactoside, and the 3-*O* glycosides of myricetin, kaempferol, isorhamnetin (Park and Cha, 2003) are detected at low levels in metabolite extracts from berry exocarp of many grape cultivars including Concord. Consistent with the prevalence of 3-*O*-glucosylated metabolites and lack of 7-*O*-; 3'-*O*-; or 4'-*O*- glucosides in Concord berries, Q3GT activity was 100 fold higher than Q3'GT or Q7GT activity in all grape berry tissues (data not shown) and quercetin 3-*O* glucoside was the abundant flavonoid in these tissues. Low levels of Q3'GT activity were detected biphasically in Concord berry mesocarp tissue, whereas both Q3'GT and Q7GT activity were detected during veraison in *Vitis labrusca* and *Vitis vinifera* exocarp

tissue (Figure 7A,B), a result consistent with the biphasic *VLOGT1,2* and *VLOGT3* gene expression in pre- and post- veraison mesocarp and exocarp (Figure 6A,B). The low reaction rates observed *in vitro* are consistent with the low accumulation of these metabolites within the grape berry and these observations are consistent with a role for these genes in flavonol modification *in planta*. RT-PCR analysis of *Vitis labrusca* mesocarp tissue suggests that both *VLOGT1,2* and *VLOGT3* are expressed before veraison (Figure 6); however neither Q7GT activity nor Q7G is detected in these tissues, suggesting additional and unique roles for these enzymes *in vivo*. Within Concord leaf tissue, Q7GT activity is detected (Figure 7C), and *VLOGT1,2* and *VLOGT3* are expressed (Figure 6D), consistent with the accumulation of kaempferol 3,7-*O*-diglycoside (Park and Cha; 2003) and luteolin 7-*O* glucoside (Moore and Giannasi, 1994) in these tissues (Figure 6C).

4.5.4 - Biphasic gene expression suggests a biological role for VLOGT1,2 and VLOGT3 in flavonoid modification

Grape is a nonclimacteric fruit, whose growth involves two phases of rapid berry expansion, separated by a lag phase (Coombe and McCarthy, 2000). Biphasic gene expression is characteristic of genes involved in the flavonoid biosynthetic pathway in developing pigmented fruit including grapes, bilberry and strawberry (Boss et al., 1996a, b; Chen et al., 2006a; Jaakola et al; 2002; Halbwirth et al., 2006). In grapes, temporal- and tissue- specific gene expression allows the accumulation of specific metabolites at the corresponding developmental stages (Bogs et al., 2006; Chen et al., 2006a). In grape pre-bloom flower buds and berries, flavonols are synthesized and accumulate in the berry

exocarp until two weeks before veraison, acting as UV-protectants; during veraison, the berry exocarp synthesizes and accumulates the colourful anthocyanins, where they become the major product of flavonoid biosynthesis (Coombe and McCarthy, 2000; Robinson and Davies, 2000). Microarray and gene expression profiling in grape berries show biphasic gene expression and are consistent with the metabolite biosynthesis and accumulation in exocarp tissue, (Boss et al., 1996a,b; Waters et al., 2005; Bogs et al., 2006), and with the biphasic expression of *VLOGT1,2* and *VLOGT3* in *Vitis labrusca* mesocarp and exocarp tissue (Figure 6A,B) where they have putative roles in flavonoid modification.

4.5.5 - Broad substrate specificity and kinetic analysis suggests a biological role for VLOGT1, VLOGT2 and VLOGT3 in the plant stress response to xenobiotics

Plant GTs have broad substrate specificity but high regiospecificity, glucosylating one position on many substrates (Vogt and Jones, 2000; Jones and Vogt, 2001). Although *in vitro* characterization of GTs may not reflect their *in vivo* function, overexpression in heterologous systems facilitates the discovery of several catalytic roles. Recently, the glucosylation of multiple positions and functional groups on many metabolites in addition to glucosylation of the endogenous substrate has been demonstrated (Lee and Raskin, 1999; Vogt et al., 1999; Meyer et al., 2003; Kramer et al., 2003; Nagashima et al., 2004; Tian et al., 2006b; Ko et al., 2007, Chapter 3). Previously characterized 7GTs often produce a single 7-*O* monoglucoside with flavonol substrates (Hirotani et al., 2000; Taguchi et al., 2003; Kramer et al., 2003; Kim et al, 2006a), whereas 7GTs from *Maclura pomifera, Oryza sativa, Allium cepa* and *Scutellaria*

baicalensis are not regio-selective, producing multiple mono- and di-glucosides with flavonoid substrates (Kramer et al., 2003; Jones et al., 2003; Tian et al., 2006b; Ko et al., 2006; Ko et al., 2007; Masada et al., 2007a). Similarly, rVLOGT1, rVLOGT2 and rVLOGT3 exhibit broad substrate specificity and VLOGT2 also demonstrates broad regio-specificity with flavonol substrates (Figure 4, Figure 5).

Broad substrate specificity of GTs is a proposed mechanism that plants use to respond to their constantly changing environment, and serves as a mechanism to detoxify and compartmentalize xenobiotics (Jones and Vogt, 2001; Schäffner et al., 2002; Lim and Bowles, 2004; Schröeder, 2006; Brazier-Hicks et al., 2007). The recombinant *Vitis labrusca* GTs characterized in this study glucosylate the xenobiotic TCP *in vitro* (Figure 4, Figure 5), and kinetic analysis of rVLOGT2 reveals that TCP is the preferred substrate for this enzyme (K_{cat}/K_m for TCP is 10^5 fold higher than with myricetin) (Table 2), consistent with a possible role for these enzymes in xenobiotic detoxification. Detoxification of TCP by glucosylation is evident throughout the plant kingdom (Pflugmacher and Sandermann, 1998). Many *Arabidopsis thaliana* GTs glucosylate plant metabolites and are also active in glucosylating xenobiotics with varying affinities and catalytic efficiencies (Meβner et al., 2003).

Similarly, many GTs with broad substrate specificity (including 7GTs) are often rapidly induced by the application of herbicide safeners (Brazier et al., 2002; Loutre et al., 2003) and by wounding (Hirotani et al., 2000). The induction of GTs by treatment with methyl jasmonate (Imanishi et al., 1998, Taguchi et al., 2003) or with salicylic acid (Hirotani et al., 2000; Taguchi et al., 2001, Taguchi et al., 2003) suggests a proposed

downstream role in systemic acquired resistance and the plant defense response (Kunkel and Brooks, 2002, Dong, 2004). Simultaneous initiation of the plant defense response and an increase of GT gene expression would prepare the plant for the concomitant increase of secondary metabolites produced by the plant (which are glucosylated and stored in vacuoles) to combat the pathogen, as well as to prepare the plant for exposure to pathogen-derived metabolites that can be detoxified by glucosylation. The 5GT-like gene family of *Vitis labrusca* likely contributes to this model of plant defense, since these genes are expressed spatially and temporally in tissues frequently subjected to pathogen attack, such as post-veraison berry exocarp, leaf, flower and pre-bloom bud tissue (Figure 6).

4.5.6 - Truncation of the first 18 amino acids of VLOGT4 may be responsible for the inactive enzyme.

No activity was detected in biocatalysis or enzyme assays with trVLOGT4, which is missing 18 amino acids at the N-terminus (Figure 2, Table 1), suggesting that at least one of the missing amino acids is required to produce a catalytically active protein. The amino acid residue most likely responsible for the loss of activity is amino acid HIS16, which is conserved in all family 1 GTs and has previously been identified by site-directed mutagenesis as essential for catalytic activity (Hans et al., 2004). The importance of HIS16 has been further characterized by homology-based modelling, site-directed mutagenesis, and substrate docking studies (Hans et al., 2004; Thorsøe et al., 2005) while crystal structure analysis identifies this residue as a catalytic base (Shao et al., 2005). Site-directed mutagenesis of this residue in the *Vitis vinifera* anthocyanin 3-*O* GT

abolishes enzyme activity, and confirms the importance this residue in GTs from *Vitis* spp. (Offen et al., 2006).

4.6 - Conclusion

This study has used homology-based cloning to identify three full-length members of the *Vitis labrusca* cv. Concord 5GT-like gene family. Sequence alignment and phylogenetic analysis place the *Vitis labrusca* clones with members of the 5GT clade containing several members that have 7GT activity *in vitro*. Biocatalysis and substrate specificity studies indicate that the *Vitis labrusca* GTs glucosylate the xenobiotic TCP and flavonols *in vitro*. Biphasic gene expression analysis in grape berry exocarp throughout development is consistent with a putative role for these GTs in flavonoid modification. Kinetic analysis indicates that TCP is the preferred substrate for this class of enzymes, and gene expression of *VLOGT1,2* and *VLOGT3* in tissues which are commonly exposed to pathogen attack and xenobiotics suggests a second role for the 5GT-like gene family of *Vitis labrusca* in plant defense.

Chapter 5

Molecular cloning and biochemical characterization of the UDP-glucose: flavonoid 3-*O*-glucosyltransferase from Concord grape (*Vitis labrusca*)

Dawn Hall, Xiao Xin Yuan, Jun Murata and Vincenzo De Luca

This manuscript describes the molecular cloning, heterologous expression in *E. coli*, protein purification by affinity chromatography, and biochemical characterization of the *Vitis labrusca* UDP-glucose: flavonoid 3-*O*-glucosyltransferase (VL3GT).

Four authors assisted in the preparation of this manuscript, and as first author my contribution is described below.

1. Molecular cloning of *VL3GT* from *Vitis labrusca* exocarp tissue
    a. Primer design, RNA extraction from *Vitis labrusca* exocarp tissue, and PCR amplification
    b. Sub-cloning of *VL3GT* into sequencing and expression vectors
2. Development of conditions for recombinant VL3GT (rVL3GT) expression
    a. Optimization of protein expression (two vectors, three bacterial strains; three temperature conditions and two concentrations of IPTG)
3. Development Glutathione-Sepharose 4B-mediated affinity purification protocol
4. RT-PCR analysis of *VL3GT* gene expression
    a. RNA extraction from several grape berry developmental stages and grapevine tissues
    b. VL3GT primer design for RT-PCR
5. Supervision of Honour's thesis student Xiao Xin Yuan throughout the biochemical characterization (kinetics; substrate specificity) of rVL3GT.
    a. Collaborated on the extraction of crude protein from several grape berry developmental and tissues stages
6. Manuscript preparation and editing

Molecular cloning and biochemical characterization of the UDP-glucose: flavonoid 3-*O*-glucosyltransferase from Concord grape (*Vitis labrusca*)

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5.1 - Abstract

Glucosylation of anthocyanidin substrates at the 3-*O*-position is crucial for the red colour in grape berries and wine. The enzyme which catalyzes this reaction (3GT) has been cloned from *Vitis labrusca* cv. Concord, heterologously expressed, and the recombinant enzyme (rVL3GT) was characterized *in vitro*. VL3GT has 96 % amino acid sequence identity with the previously characterized *Vitis vinifera* 3GT (VV3GT) and groups phylogenetically with several other flavonoid 3-*O*-glycosyltransferases. *In vitro* substrate specificity studies and kinetic analyses of rVL3GT indicate that this enzyme preferentially glucosylates cyanidin as compared with quercetin. Crude protein extracts from several Concord grape tissues were assayed for glucosyltransferase activity with cyanidin and quercetin as acceptor substrates. A comparison of the VL3GT activities toward with these substrates showed that the 3GT enzyme activity is consistent with the expression of VL3GT in these tissues and is coincident with the biosynthesis of anthocyanins in both location and developmental stages. Enzyme activities in grape mesocarp, pre-veraison exocarp, leaf, flower bud, and flower tissues glucosylated quercetin but not cyanidin at high rates, suggesting the presence of additional enzymes

which are able to glucosylate the 3-*O*-position of flavonols with higher specificity than anthocyanidins.

5.2 - Introduction

The cultivation of grapes (viticulture) and the production of wine are of cultural and economic importance worldwide. The wine industry relies on several traditional European (*Vitis vinifera*) cultivars of grape which are recognized as the "elite" viticulturally-important varieties. These traditional cultivars can be classified based by their organoleptic attributes including the degree of colouration of red wines to the non-coloured white wines. Red wine is produced from red grapes that accumulate anthocyanin pigments mostly in the berry exocarp. In grapes, the anthocyanin profile is highly dependent on the species, cultivar, environmental conditions and developmental stage of the berry.

Anthocyanins are members of a large and diverse group of phenylalanine-derived plant secondary metabolites, the flavonoids (Winkel-Shirley, 2001). Within the plant, secondary metabolites mediate the plant's interaction with its environment and have important roles attracting pollinators and seed dispersers, as volatile aroma compounds, as structural components in cell wall biosynthesis, and in the plant's stress response (Koes et al., 1994; Wink, 2003; Winkel-Shirley, 2001). The diversity of plant natural products is increased by enzymatic modification including hydroxylation, methylation, glucosylation and acylation, changing the biochemical characteristics of these compounds.

Glucosylation is often the terminal reaction in flavonoid biosynthesis, and is catalyzed by a large, multi-gene family of cytosolic enzymes, the glucosyltransferases (Li et al., 2001; Bowles, 2002; Lim et al., 2003). These enzymes generally have broad but regio-selective substrate specificity and transfer glucose from uridine 5'-diphosphoglucose (UDPG) to low molecular weight molecules including plant-derived or exogenous metabolites encountered by the plant (Vogt and Jones, 2000; Jones and Vogt, 2001). Glucosylation changes the toxicity, stability, complexity, spectral characteristics, and solubility of these compounds (Vogt and Jones, 2000). Within the plant, glucosylation is often required for metabolite transport and storage, and glucosylated metabolites are frequently recognized by signal receptors within the same plant, bacteria and other plants (Jones and Vogt, 2001).

Grapes contain more than 200 glucosylated metabolites including flavonoids, hormones, stilbenes, terpenes and hydroxycinnamic and hydroxybenzoic acids (Sefton, 1993; Sefton, 1994; Monagas et al., 2005). Grapes accumulate cyanidin, delphinidin, peonidin, petunidin and malvidin-derived anthocyanins which have unique patterns of B-ring hydroxylation and methylation (Mazza and Miniati, 1993). The anthocyanidin aglycone is highly reactive, and is greatly affected by pH, temperature, solvents, light, enzymes and the presence of other molecules which can function as co-pigments (Prior and Wu, 2006). Glucosylation of the anthocyanidin at the 3-*O*-position, increases its stability, decreases its reactivity, and changes its spectral characteristics. The enzyme which catalyzes this reaction, UDP-glucose: flavonoid 3-*O*-glucosyltransferase (3GT), has been identified and functionally characterized in several plant species (Tanaka et al.,

1996; Gong et al., 1997; Ford et al., 1998; Yamazaki et al., 2002; Yoshihara et al., 2005; Kim et al., 2006b; Offen et al., 2006).

In *Vitis vinifera* cv. Shiraz, the 3GT has been cloned and characterized (VV3GT; Ford et al., 1998; Offen et al., 2006), and *in vitro*, glucosylates the 3-*O*-position of flavonols and anthocyanidins (Ford et al., 1998). In the plant, *VV3GT* is expressed coincident with anthocyanidin but not flavonol biosynthesis and kinetic analysis reveals that VV3GT glucosylates anthocyanidins at 48-fold higher rates than their corresponding flavonols; suggesting that the *in planta* the VV3GT is involved in the glucosylation of anthocyanidins, but not flavonols (Ford et al., 1998). Consistent with a role in anthocyanin biosynthesis, *VV3GT* gene expression is crucial for anthocyanin accumulation in the berry exocarp of red grapes; whereas this gene is not expressed in tissues which do not accumulate anthocyanins (berry mesocarp, white grapes, vegetative tissues) (Boss et al., 1996a,b; Kobayashi et al., 2001). Unlike several other plant species which exert transcriptional control of anthocyanin biosynthesis at earlier steps in the enzymatic pathway (Mol et al., 1998; Koes et al., 2005)), it appears that anthocyanin biosynthesis in grapes is controlled by expression of the 3GT (Boss et al., 1996a,b; Kobayashi et al., 2002). Recently, the crystal structure of VV3GT has been resolved and several amino acid residues which are essential for catalytic activity for both donor and acceptor substrate binding have been identified (Offen et al., 2006).

This report describes the molecular cloning and preliminary biochemical characterization of a 3GT from the North American grape *Vitis labrusca* (VL) cv. Concord, a grape which is frequently used in the production of grape juices and jams, but has limited value in viticultural applications. VL3GT has 96% protein sequence identity

to the previously characterized VV3GT, and glucosylates flavonols and anthocyanidins *in vitro*. Substrate specificity and kinetic analysis indicate that this enzyme preferentially glucosylates anthocyanidins *in vitro,* which corresponds with the expression of this gene in tissues which are producing and accumulating anthocyanins. Enzyme assays with crude VL protein extracts reveal high levels of anthocyanidin 3GT activity in post-veraison grape berry exocarp, whereas enzyme activities in grape flesh and other tissues showed higher activity towards quercetin.

5.3 - Experimental Procedures

5.3.1 - Plant material

Vitis labrusca cv. Concord grape berries were harvested weekly from June until October, 2003 to 2006 at the 3rd St. site of the G & H Wiley vineyard (St. Catharines, ON). *Vitis vinifera* cv. Pinot Noir and cv. Pinot Noir Droit berries were harvested from the Château des Charmes Vineyard (Niagara on the Lake, ON) in September of 2004. All stages of grape berry maturity are reported as weeks after flowering (AF), and physiological parameters of grape berry development were determined as reported in Chapter 3. Concord pre-bloom flower buds were harvested June 12, 2006, grape flowers were harvested June 16, 2006 and grapevine leaves and stem were harvested June 21, 2006. Grapes were thawed briefly, the exocarp and mesocarp were separated by hand, and the seeds were removed. The exocarp was scraped to remove residual mesocarp tissue and was quick frozen in liquid nitrogen. All plant material was stored at -80˚C for future use.

5.3.2 - Chemicals

All chemicals were purchased from Sigma (http://www.sigmaaldrich.com) (Oakville, ON. Canada) or Indofine Chemical (http://www.indofinechemical.com) (Hillsborough, NJ. USA) and were prepared as described in Chapter 3, except that 5'-diphosphoglucose (UDPG) and 5'- diphosphogalactose (UDPGal) were diluted to 180 mM in water.

5.3.3 - Extraction of crude protein and GT enzyme activity profiling in grape tissue throughout development

Concord pre-bloom flower bud (1 g), leaf (0.5 g), flower (1 g), week 1 AF whole berry (1 g), weeks 3, 6, 8, 10, 12, 14, and 16 AF and post-veraison (week 14 AF) *Vitis vinifera* cv. Pinot noir and cv. Pinot Noir Droit exocarp (1 g) and mesocarp (2 g) was ground to a fine powder in a mortar and pestle with liquid nitrogen, and was extracted in 4 ml of grape extraction buffer (500 mM Tris-HCl, pH 8.0, 0.1% β-mercaptoethanol, 1% PVP-10 (polyvinylpyrrolidone), 5 mM sodium metabisulfite ($Na_2S_2O_5$), 10% glycerol (v/v), 1 mM phenylmethyl sulfonyl fluoride (PMSF)), and 10% insoluble polyvinylpyrrolidine (PVPP). The extracts were centrifuged at 21000 *g* for 10 min at 4°C, and the supernatant was desalted on a PD-10 Sephadex G-25 column (GE Healthcare; http://www.gehealthcare.com/caen), pre-equilibrated in desalting buffer (100mM Tris-HCl; pH 8.0 + 0.1% β-mercaptoethanol). Each assay contained 100 µl of protein with 16 µM quercetin or 6 µM cyanidin and 9 mM UDPG in a final volume of 125 µl. The assays were incubated for 3 min (cyanidin) or 5 min (quercetin) at 30°C, and were stopped with the addition of equal volume 0.1% (0.12 N) HCl in methanol (MeOH)

or 100% MeOH respectively. The amount of protein in each extract was determined using a protein assay kit (Bio-Rad Laboratories, http://www.bio-rad.com). All specific activities are reported as picomoles of glucoside produced in 1 second by 1 mg of total protein.

5.3.4 - HPLC analysis of enzyme assays

Enzyme assays were processed and analyzed by HPLC as described previously (Chapter 3). Assays with anthocyanidin substrates were monitored at 520 nm while assays with kaempferol, isorhamnetin, quercetin and dihydroquercetin were monitored at 365 nm; 370 nm; 370nm and 290 nm respectively, using a 2996 photodiode array detector (Waters, Milford MA, USA; www.waters.com).

5.3.5 - RNA extraction

RNA was extracted from all grape tissues using the Plant RNA Extraction reagent (Invitrogen, http://www.invitrogen.com/) as described in Chapter 3 with the exceptions that 1 µl of linear acrylamide (5 µg/µl) was added with to the RNA as a co-precipitant and the extracted RNA was resuspended on ice. RNA (0.5 -1 µg) was reverse transcribed using either Superscript III reverse transcriptase (Invitrogen) or the Takara RNA PCR kit version 3.0 (Fisher Scientific, http://www.fishersci.ca/) as per the manufacturer's instructions, and was diluted with 5 volumes of water.

5.3.6 - Molecular cloning of VL3GT

The previously characterized full-length *Vitis vinifera* (Vv) *3GT* (AF000372; Ford et al., 1998) was used to design forward primer 1 (F1 - 5'ATGTCTCAAACCACCACCAAC 3') and reverse primer 1 (R1 - 5'CTAGACATCCTTTGGTTTTGAC 3'). PCR with F1, F2 and 1 µl of Concord week 12 AF exocarp-specific cDNA amplified a product of approximately 1400 bp, which was subcloned into a TA-vector for sequencing. The vector containing the desired sequence was isolated, diluted 1000-fold and used as a template with forward primer 2 (F2 – 5' CGAAGAATTCATGTCTCAAACCACCACC 3') and reverse primer 2 (R2 - 5'AACCAAAGGATGTCTAGCTCGAGAACG 3') to introduce suitable restriction sites for cloning into the pGEX 4T-1 GST fusion expression vector (GE Healthcare).

The vector containing the full-length gene-GST fusion (VL3GT) was transformed by electroporation into *Escherichia coli* (*E. coli*) DE3 pLys S cells (which express the groES-groEL chaperone protein; CHAP2; Fisher Scientific), and the recombinant protein was expressed. Briefly, a 3 ml culture in 2 x yeast tryptone medium containing 50 µg ml^{-1} ampicillin and 25 µg ml^{-1} chloramphenicol (2YTAC) was inoculated and grown to saturation at 37°C. Five hundred µl of saturated culture was inoculated to 50 ml 2YTAC culture and was induced to express the chaperone proteins with L-arabinose. The culture was grown to an OD of 1.0 at 37°C, was induced for recombinant GT protein expression with 0.1 mM of isopropyl-1-thio-β-D-galactoside (IPTG) and was grown for 20-24 h at 16-18°C. The cell pellets containing recombinant VL3GT (rVL3GT) fusion protein were centrifuged for 5 min at 2800 *g* and were stored at -20°C.

Recombinant protein was extracted from bacterial cell pellets and purified by Glutathione Sepharose 4B (GE Healthcare) affinity chromatography as per the manufacturer's instructions, with specific conditions as described in Chapter 3. The eluted rVL3GT protein was desalted by MicroSpin G-25 columns (GE Healthcare) as per the manufacturer's instructions and was stored as a 50 % glycerol stock at -20 °C.

5.3.7 - Recombinant VL3GT enzyme activity assays

rVL3GT was assayed with 3.6 mM donor substrate (UDPG or UDPGal) and 32 µM flavonol-related (quercetin, isorhamnetin, dihydroquercetin, kaempferol) or 6 µM anthocyanidin (cyanidin, kuromanin, delphinidin, peonidin, malvidin) acceptor substrates. Each assay contained 0.875 µg of rVL3GT for anthocyanidin substrates and 1.75 µg rVL3GT for flavonol-related substrates in 100 mM Tris-HCl pH 8.0, 0.1% β-mercaptoethanol buffer to a final reaction volume of 125 µl. All assays were repeated in at least triplicate and were incubated and stopped as described above prior to processing and analysis by HPLC (Chapter 3), with the exception that assays were incubated for 1-180 min to determine reaction linearity.

For the determination of kinetic parameters, all assays were incubated for 3 min at 30°C to a final reaction volume of 125 µl with 100 mM Tris-HCl, 0.1 % β-mercaptoethanol. For acceptor substrates, each assay contained 9 mM UDPG with varying concentrations of quercetin and cyanidin. UDPG kinetics were determined with 6 µM cyanidin as the acceptor substrate. The reactions were stopped and analyzed as described above.

5.3.8 - RT-PCR analysis of VL3GT gene expression

VL3GT gene expression was monitored using the gene specific primers: VL3GT – forward primer 3 (F3 - 5'TGCAGGGCCTAACTCACTCT 3') and VL3GT reverse primer 3 (R3 - 5' GCAGTCGCCTTAGGTAGCAC 3'); and the gene specific primers for *VLActin* as reported in Chapter 3. *VLActin* and *VL3GT* gene expression was repeated in at least duplicate, was quantified using Multigauge ver 3.0 (Fujifilm, Tokyo, Japan; www.fujifilm.ca), and the mean values were divided by the mean actin gene expression value to obtain a relative value for gene expression (% expression) in these tissues.

5.3.9 - Sequence alignment and Phylogenetic Analysis

The protein sequence of VL3GT (EF630356) was aligned with the full-length *Vitis vinifera* cv. Shiraz. flavonoid 3-*O*-glucosyltransferase sequence (AAB81683 - Ford et al., 1998) using ClustalW 2 (http://align.genome.jp/). Nucleotide sequence alignment and phylogenetic analysis of *VL3GT* with other glycosyoltransferase sequences was analyzed using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the DNA distance neighbour-joining phylogenetic analysis was visualized using Phylodraw (http://pearl.cs.pusan.ac.kr/phylodraw/).

5.4 - Results

5.4.1 - Molecular cloning and functional characterization of a UDP-glucose: flavonoid 3-O-glucosyltransferase from Concord grape

Oligonucleotide primers based on the previously characterized *Vitis vinifera* (cv. Shiraz) UDP-glucose: flavonoid 3-*O*-glucosyltransferase (*VV3GT*) (Ford et al., 1998) were used to amplify and clone the corresponding gene from *Vitis labrusca* (VL) week 12 exocarp-specific cDNA. The full-length *VL3GT* clone (1371 bp) encoded a putative protein of 456 amino acids, with an estimated pI of 6.29 and a calculated molecular weight of 50.1 kDa. The *VL3GT* was 98 % and 96 % identical in nucleotide and amino acid sequence, respectively, to the previously characterized *VV3GT* from *Vitis vinifera*. A search of the NCBI database identified 14 3GT-like sequences from *Vitis* spp., of which 8 were unique, but highly identical (96 - 99 % nucleotide sequence identity), including the Concord sequence obtained in this study (data not shown). Several amino acid residues which are essential for catalytic activity and important for donor and acceptor substrate binding in VV3GT (Offen et al., 2006) are also conserved in VL3GT (Figure 1). The VL3GT belongs to the family 1 glucosyltransferase group based on the presence of the Plant Secondary Product Glucosyltransferase (PSPG) consensus sequence at the C-terminus of the protein (Figure 1; Vogt and Jones, 2000).

After cloning *VL3GT* into an N-terminal GST-fusion vector, it was co-expressed with chaperone protein in *E. coli* cells for 20-24 h at 16-18°C. The recombinant VL3GT (rVL3GT) protein extracted from these cells was purified by affinity chromatography and

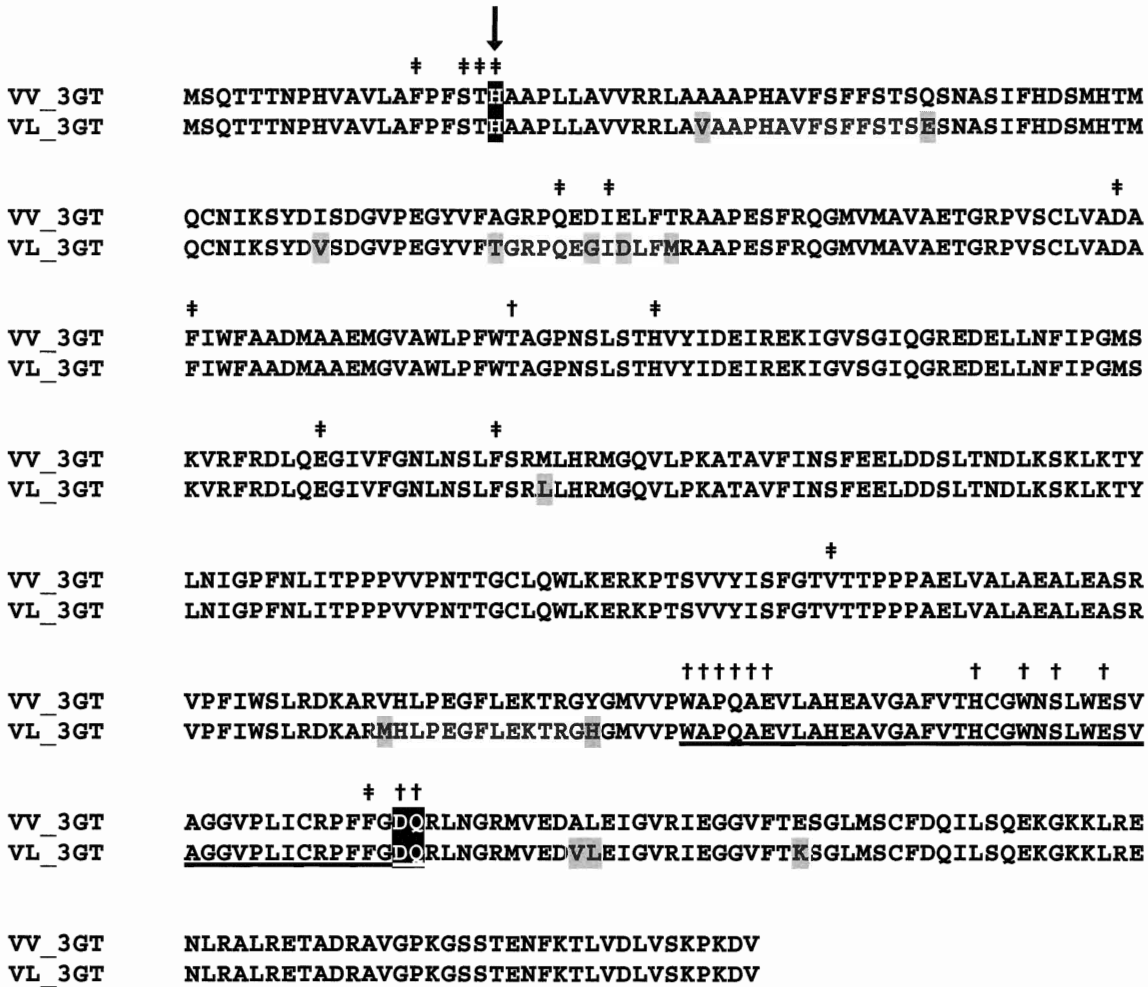

Figure 1. Protein sequence alignment of the functionally characterized flavonoid 3-*O*-glucosyltransferases (3GT) from *Vitis vinifera* cv. Shiraz and *Vitis labrusca* cv. Concord. The 14 unique amino acids between Concord and Shiraz are shaded in grey. Arrow indicates the catalytic base His20. Residues shaded in black are essential for enzyme activity and symbols indicate residues important for donor (†) and acceptor (‡) substrate binding (Offen et al., 2006). The plant secondary product glucosyltransferase (PSPG) consensus sequence is underlined. Abbreviations: VV_3GT - *Vitis vinifera* flavonoid 3-*O*-glucosyltransferase (AAB81683; Ford et al., 1998). VVL_3GT - *Vitis labrusca* flavonoid 3-*O*-glucosyltransferase (EF630356; this study).

assayed for glucosyltransferase activity with UDPG and either cyanidin or quercetin as substrates. Enzyme assays with cyanidin and UDPG remained linear for 3 min and produced a monoglucoside as shown by mass spectrometry (data not shown). The reaction product was confirmed to be cyanidin 3-*O*-monoglucoside (kuromanin) based on its identical UV spectrum and HPLC retention time (RT = 14.6 min) as the kuromanin standard. When rVL3GT was incubated with quercetin as the flavonoid substrate the reaction remained linear for 30 min to produce a single reaction product identified as quercetin 3-*O*-monoglucoside (RT = 19.3 min) as determined by HPLC, UV spectral analysis (hypsochromic shift to 356 nm) (Harborne, 1967; Vogt et al., 1997; Kramer et al., 2003) and mass spectrometry (data not shown). When the assay was carried out for longer than 15 min, two additional products (RT = 18.9 min and RT = 14.6 min) appeared that eluted earlier than quercetin 3-*O*-monoglucoside as determined by HPLC. Analysis by mass spectrometry (data not shown) revealed that a quercetin monoglucoside (RT = 18.9 min) and diglucoside (RT = 14.6 min) had been produced. These reaction products were identified as quercetin 7-*O*-monoglucoside and quercetin 3,7-*O*-diglucoside, respectively, as determined by UV spectral analysis (Harborne, 1967; Vogt et al., 1997; Kramer et al., 2003). Subsequent enzyme assays were carried out for only 5 min in order to eliminate the second glucosylation of quercetin at the 7-*O*-position. Assays with boiled rVL3GT protein, without UDPG or with purified protein extracts from bacteria expressing the empty vector (pGEX lacking *VL3GT*) produced no products with either cyanidin or quercetin (data not shown).

5.4.2 - Recombinant VL3GT glucosylates several anthocyanins and flavonols in vitro

Substrate specificity studies for rVL3GT were performed by testing several anthocyanidin- and flavonol-type substrates. The rVL3GT glucosylated several anthocyanidins (malvidin, peonidin, cyanidin and delphinidin) (Figure 2A) and flavonols (isorhamnetin, quercetin, dihydroquercetin and kaempferol) (Figure 2B) when assayed in the presence of UDP-glucose. The enzyme was not active if UDP-galactose was used as the donor substrate, or if kuromanin was used as the acceptor substrate (data not shown). The rVLGT glucosylated the anthocyanidin substrates peonidin and cyanidin at 93.5 - and 6.4 - fold higher rates than their corresponding flavonol substrates, isorhamnetin and quercetin (Figure 2). In addition the *O*-methylated substrates isorhamnetin, peonidin and malvidin were glucosylated at 3.6, 14.6 and 115.0 fold higher rates respectively than their non-*O*-methylated derivatives (quercetin, delphinidin and cyanidin) (Figure 2).

5.4.3 - Kinetic analysis of the recombinant VL3GT

The kinetic parameters of rVL3GT were determined with the acceptor substrates cyanidin and quercetin and the donor substrate UDPG (Table 1). The recombinant enzyme shows high affinity (K_m) for both quercetin (2.2 μM) and cyanidin (4.8 μM), but has a 6.2-fold higher turnover number (K_{cat}) and a 2.8-fold higher calculated catalytic efficiency ($K_{cat}\ K_m^{-1}$) for cyanidin (K_{cat} = 7.02 x 10^{-4} s^{-1}; $K_{cat}\ K_m^{-1}$ = 146 $M^{-1}s^{-1}$) than for quercetin (K_{cat} 1.13 x 10^{-4} s^{-1}; $K_{cat}\ K_m^{-1}$ = 52 $M^{-1}s^{-1}$); which suggests that rVL3GT preferentially glucosylates cyanidin compared to quercetin. Kinetic analysis of rVL3GT with 6 μM cyanidin and varying concentrations of UDPG calculated a K_m value of 914 μM, a K_{cat} value of 2.47 x 10^{-4} s^{-1}, and a calculated catalytic efficiency of 0.27 $M^{-1}s^{-1}$

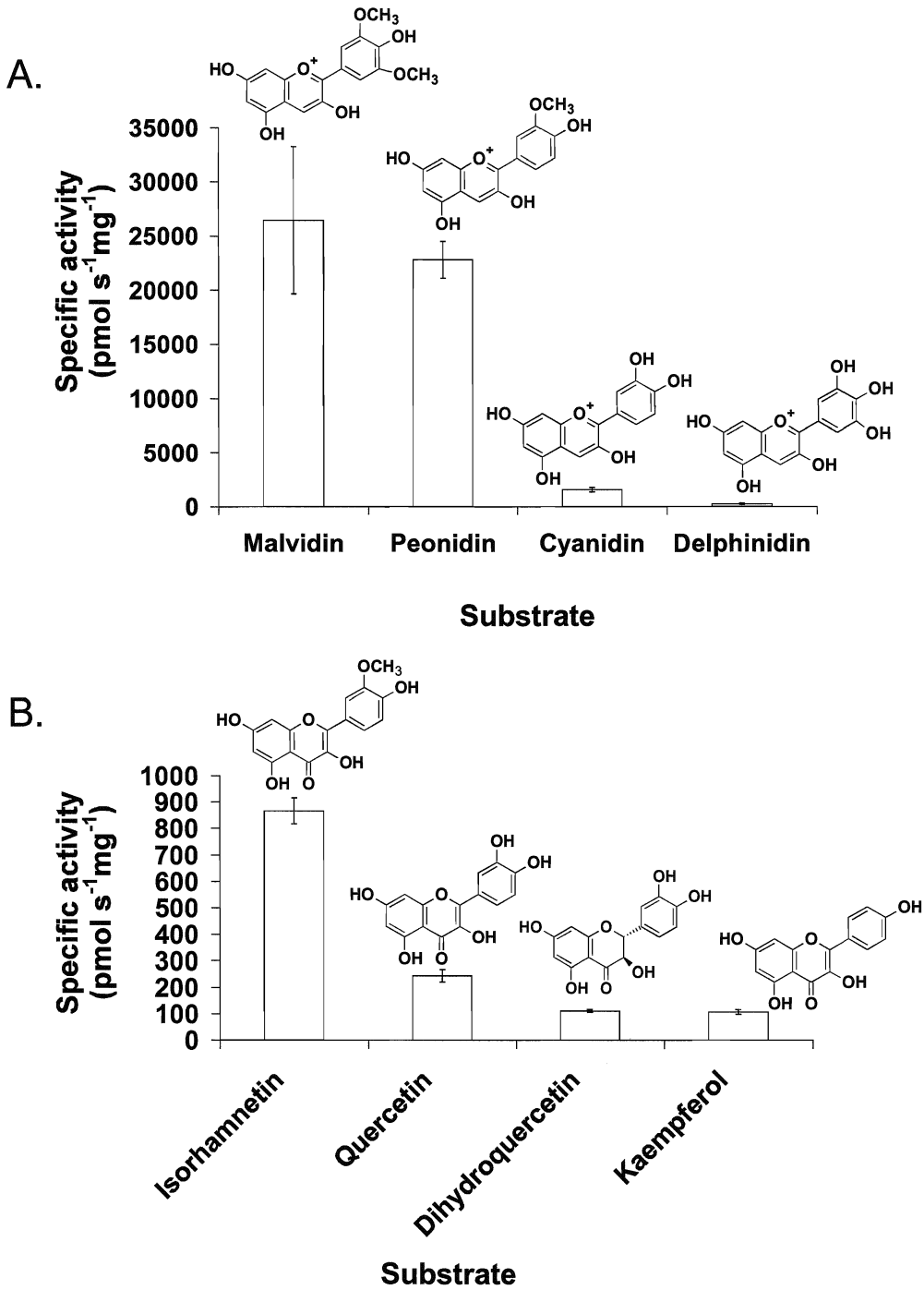

Figure 2. Substrate specificity of *Vitis labrusca* flavonoid 3-*O*-glucosyltransferase with anthocyanidin (A) and flavonol (B) substrates. Each bar respresents the mean of three independent trials ± standard deviation

Table 1. Kinetic analysis of VL3GT and VV3GT with cyanidin, quercetin and UDPG

All values represent the mean of at least three independent trials ± standard deviation.

Substrate	Km (μM)		Kcat ($x10^{-4}$ s^{-1})		Kcat/Km (M^{-1} s^{-1})	
	VL3GT[a]	VV3GT[b]	VL3GT[a]	VV3GT[b]	VL3GT[a]	VV3GT[b]
Cyanidin	4.79 ± 0.69	30	7.02	47.6	146	159
Quercetin	2.16 ± 0.28	15	1.13	0.99	52	6.6
UDPG (cyanidin)[a] (quercetin)[b]	914 ± 0.38	1880	2.47	0.99	0.27	0.014

[a] - present study

[b] as determined in Ford et al., 1998

(Table 1). The corresponding kinetic parameters of VV3GT were previously calculated and revealed K_m values of 30 μM and 15 μM , K_{cat} values of 47.6 x 10^{-4} s^{-1} and 0.99 x 10^{-4} s^{-1}, and K_{cat} K_m^{-1}values of 159 $M^{-1}s^{-1}$ and 6.6 $M^{-1}s^{-1}$ for cyanidin and quercetin respectively as acceptor substrates (Table 1; Ford et al., 1998).

5.4.4 - VL3GT gene expression and enzyme activity throughout grape berry development

Protein and total RNA was extracted from several developmental stages of *Vitis labrusca* berry exocarp and mesocarp tissue, as well as from leaves, flowers and flower buds. Similar extracts were also produced for comparative purposes from *Vitis vinifera* cv. Pinot Noir (PN) and cv. Pinot Noir Droit (PND) post-veraison (week 14 AF) berry exocarp and mesocarp tissue. Template generated from reverse transcription without reverse transcriptase was checked for genomic DNA contamination (data not shown). Primers designed to amplify *Vitis vinifera* actin were used with the cDNA templates to determine baseline expression between samples. Glucosyltransferase gene expression was quantified and divided by the mean *Actin* gene expression values to obtain the relative % expression of *VL3GT* compared to those of *Actin* throughout grape berry development in exocarp and mesocarp tissues (Figure 3). While there was little expression of *VL3GT* before veraison, these transcripts began to accumulate at week 8 and 10 AF to reach maximal levels at week 12 AF and decreases at week 14 and 16 AF (Figure 3A). Similarly, *VV3GT* expression was detected in exocarp extracts of week 14 AF *Vitis vinifera* cv. Pinot Noir (PN) and cv. Pinot Noir Droit (PND) (Figure 3B). *VL3GT* gene expression is detected at low levels in *Vitis labrusca* leaf, flower bud and

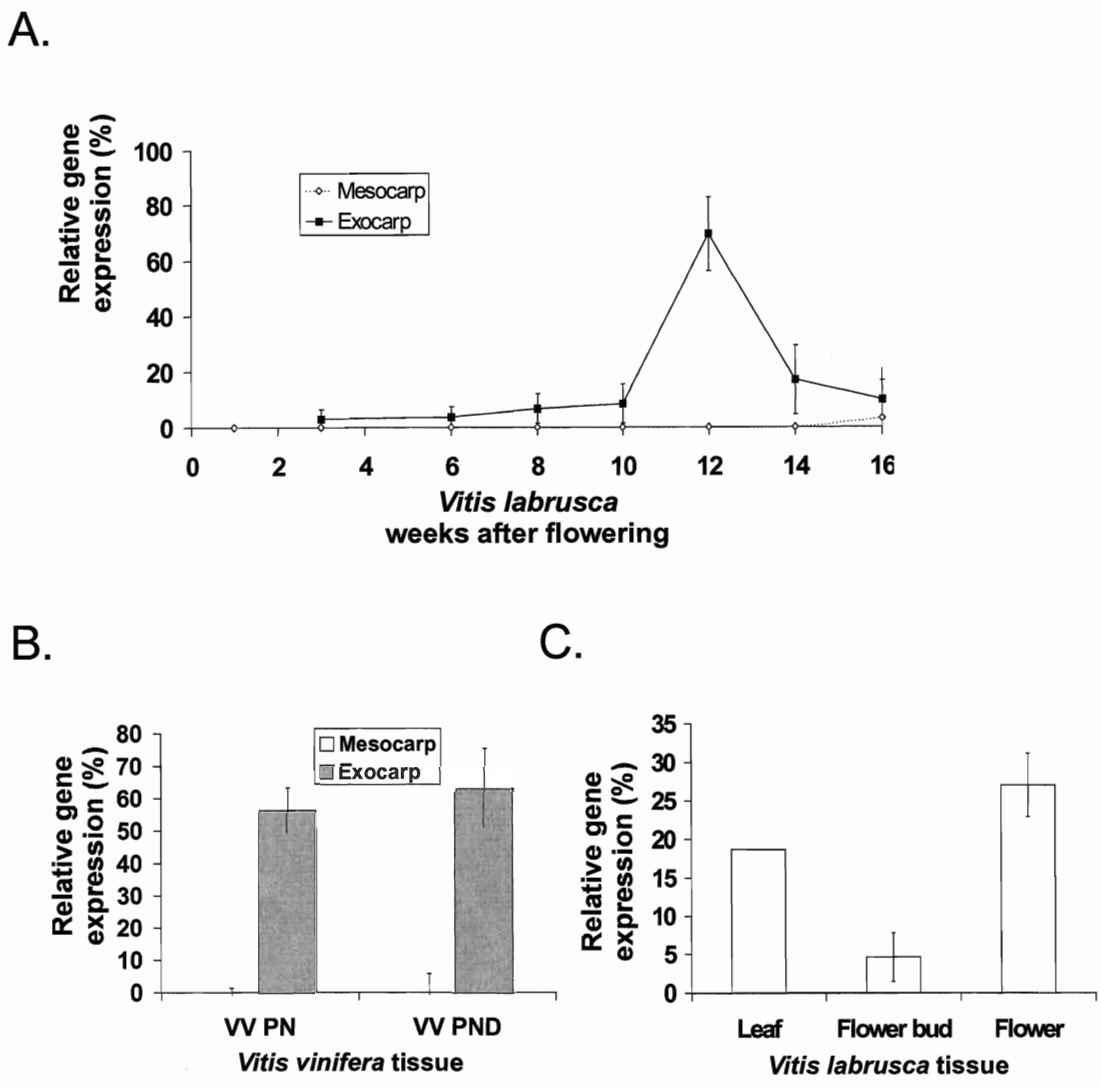

Figure 3. Relative gene expression of *VL3GT* compared to *VLActin* expression. Developmental gene expression profile of *VL3GT* in relation to *VLACTIN* gene expression in *Vitis labrusca* (*VL*) cv. Concord berry mesocarp and berry exocarp tissues (A) and in *Vitis vinifera* cvs. Pinot Noir (PN) and Pinot Noir Droit (PND) berry mesocarp and exocarp tissues (B). C. Relative gene expression of *VL3GT* in relation to *VLACTIN* gene expression in *Vitis labrusca* leaf, flower bud and flower tissue. All values represent the mean of at least two (mesocarp, leaf, flower, flower bud) or four (exocarp) trials ± standard deviation.

flower tissues (Figure 3C) but is not detected in the mesocarp tissues of either *Vitis labrusca* or *Vitis vinifera* berries (Figure 3A,B, Appendix I).

5.4.5 - Detection of VL3GT activity during grape berry ripening.

No glucosyltransferase activity with cyanidin as a substrate was detected in week 1 to week 6 AF berries in either *Vitis labrusca* mesocarp or exocarp tissue (Figure 4 A,B). However, cyanidin 3GT activity was first detected in *Vitis labrusca* grape berry exocarp, at 8 week AF, and rapidly increased to maximal levels between week 12 and 16 AF (Figure 4B). Similarly, high levels of cyanidin 3GT activity were also detected in week 14 AF *Vitis vinifera* Pinot Noir and Pinot Noir Droit exocarp tissue, respectively. Cyanidin 3GT activity was first detected at week 10 AF in *Vitis labrusca* berry mesocarp tissue and it increased to maximal levels at week 14 AF (Figure 4A); whereas low levels of cyanidin 3GT activity were detected in *Vitis vinifera* Pinot Noir Droit mesocarp tissue and no activity was detected in *Vitis vinifera* cv. Pinot Noir mesocarp tissue (Figure 4B,C). Cyanidin 3GT activity was also detected at low levels in grape flowers and flower buds but not in leaf tissue (Figure 4C), and in general, this activity was 10-fold lower in mesocarp than in exocarp tissue.

In contrast to cyanidin 3GT activity, quercetin 3GT activity was detected before veraison in *Vitis labrusca* exocarp and mesocarp tissues (Figure 4A,B). While quercetin 3GT activity was detected in week 1 and 3 AF grape mesocarp, it decreased and was absent in week 6 and 8 AF before rapidly increasing to maximal levels at week 10 AF and subsequently decreasing to a constant level to week 12 to 16 AF (Figure 4A). Quercetin 3GT activity was detected at low levels in pre-veraison *Vitis labrusca* exocarp

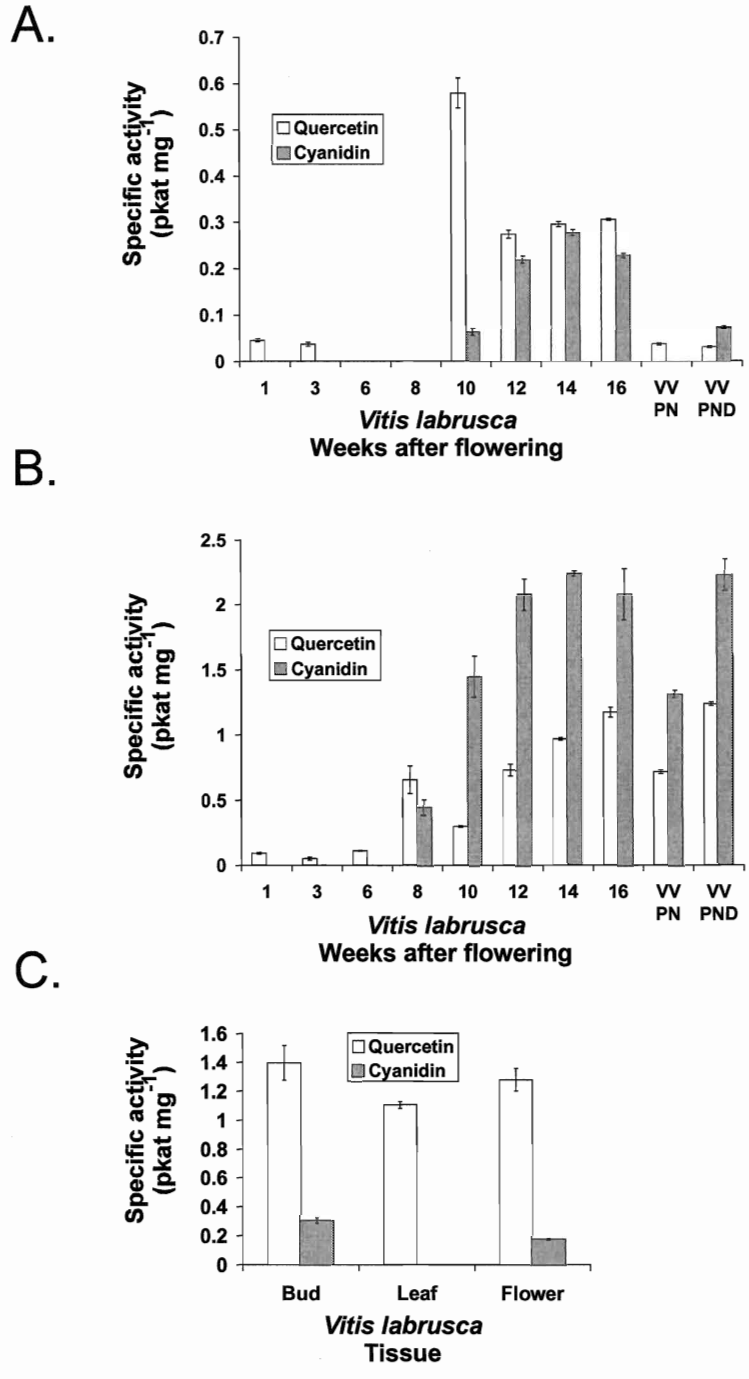

Figure 4. Developmental profile of flavonoid 3-*O*-glucosyltransferase activity with quercetin and cyanidin as substrates in *Vitis labrusca* and *Vitis vinifera* tissues. Flavonoid 3-*O*-glucosyltransferase activity in *Vitis labrusca* and *Vitis vinifera* mesocarp (A) and exocarp (B) tissues with quercetin and cyanidin as substrates. C. Flavonoid 3-*O*-glucosyltransferase activity in *Vitis labrusca* flower bud, leaf and flower tissue. Abbreviations: VV PN - Week 14 after flowering *Vitis vinifera* cv. Pinot Noir tissue; VV PND - Week 14 after flowering *Vitis vinifera* cv. Pinot Noir droit tissue. Bars indicated the mean of three independent trials ± standard deviation.

tissue and it increased throughout ripening to reach maximal levels at week 16 AF (Figure 4B). Similarly, quercetin 3GT activity was detected in post-veraison week 14 AF *Vitis vinifera* Pinot Noir and Pinot Noir Droit exocarp and mesocarp tissues (Figure 4A,B); and is highly active in *Vitis labrusca* leaf, flower and flower bud tissues (Figure 4C). In grape berry post-veraison exocarp tissue, cyanidin 3GT activity was 1.8 to 4.8 fold higher than quercetin 3GT activity, in contrast to pre-veraison exocarp tissue, pre- and post-veraison mesocarp tissue and leaf, flower bud and flower tissues where quercetin glucosyltransferase activity was favoured (Figure 4).

A neighbour-joining phylogenetic tree that included several enzymes that add sugars to various small molecules, places the *VL3GT* in a clade with several other GTs that add glucose, rhamnose or galactose to the 3-*O*-position of anthocyanidin and flavonol substrates (Figure 5).

5.5 - Discussion

The current report describes the molecular cloning, heterologous expression and biochemical characterization of a UDP-glucose: flavonoid 3-*O*-glucosyltransferase (VL3GT) from *Vitis labrusca*. A search of the NCBI database identifies several 3GT-like sequences from *Vitis* spp. including 8 unique but highly identical full-length genes. The ORF of the *Vitis labrusca* sequence has 96 % amino acid sequence identity to the corresponding enzyme from *Vitis vinifera* cv. Shiraz (Figure 1), that has been cloned, functionally characterized (Ford et al., 1998) and crystallized (Offen et al., 2006). Substrate specificity studies indicate that VL3GT glucosylates the 3-*O*-position of anthocyanidin and flavonol substrates (Figure 2), while kinetic analyses suggests that this

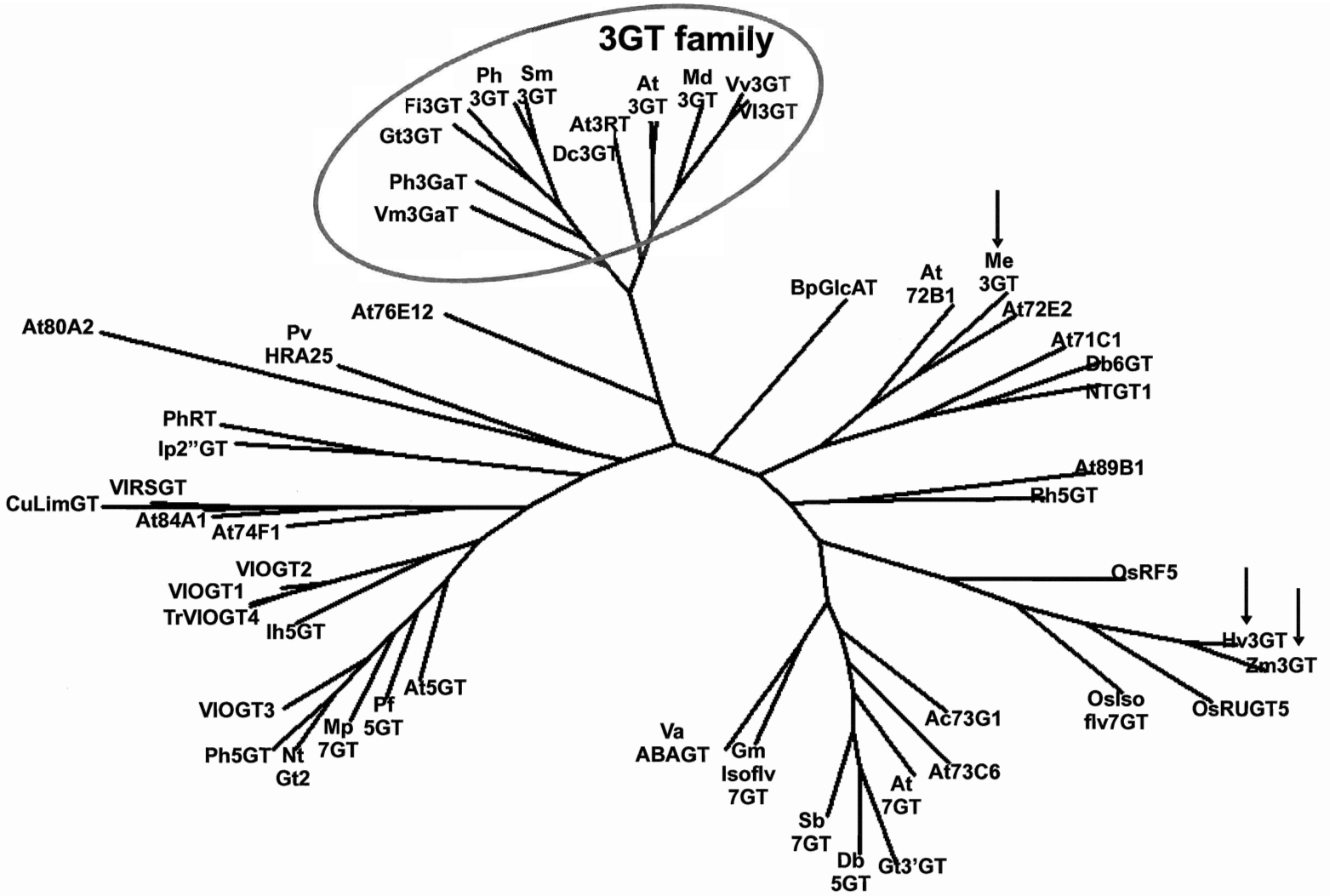

Figure 5. Neighbour joining phylogenetic tree of several plant glycosyltransferases including flavonoid 3-*O*-glycosyltransferases. Arrows indicate genes which glucosylate the 3-*O*-position of flavonols and anthocyanidins which are not members of the 3GT gene family. Abbreviations on following page.

Figure 5 Abbreviations:
Vm 3GaT - *Vigna mungo* flavonoid 3-*O*-galactosyltransferase (AB009370); Ph 3GaT - *Petunia hybrida* flavonoid 3-*O*-galactosyltransferase (AF165148);Gt 3GT - *Gentiana triflora* flavonoid 3-*O*-glucosyltransferase (D85186); Fi 3GT - *Forsythia intermedia* flavonoid 3-*O*-glucosyltrnasferase (AF127218); Ph 3GT - *Petunia hybrida* flavonoid 3-*O*-glucosyltransferase (AB027454); Sm 3GT - *Solanum melongena* flavonoid 3-*O*-glucosyltransferase (X77369); ; At 3GT - *Arabidopsis thaliana* UGT78D2 UDP-glucose flavonoid 3-*O*-glucosyltransferase; Dc 3GT - *Dianthus caryophyllus* flavonoid 3-*O*-glucosyltransferase (AB191247); At 3RT - *Arabidopsis thaliana* UGT 78D1 UDP-rhamnose flavonol 3-*O*-glucosyltransferase; At 3GT - *Arabidopsis thaliana* UGT 78D2 UDP-glucose flavonol 3-*O*-glucosyltransferase; Md 3GT- *Malus domesticus* flavonoid 3-*O*-glucosyltransferase (AY663784); Vv 3GT - *Vitis vinifera* flavonoid 3-*O*-glucosyltransferase (AF000372); VL 3GT - *Vitis labrusca* flavonoid 3-*O*-glucosyltransferase (EF630356); BpGlcAT - *Bellis perennis* UDP-glucuronic acid: anthocyanin glucuronosyltransferase (AB190262); At72B1 - *Arabidopsis thaliana* UGT72B1 hydroxybenzoic acid *O*-glucosyltransferase; Me 3GT - *Manihot esculenta* flavonoid 3-*O*-glucosyltransferase (CAA54612); At72E2 - *Arabidopsis thaliana* UGT72E2 TCP glucosyltransferase (NM126067); At71C1 - *Arabidopsis thaliana* UGT71C1 phenolic alcohol glucosyltransferase (NM128529); DbBet6gt - *Dorotheanthus bellidiformis* betanidin 6-*O* glucosyltransferase (AF374004); NtGt1 - *Nicotiana tabacum* glucosyltransferase 1 (AB052557); At89B1 - *Arabidopsis thaliana* UGT89B1 hydroxybenzoic acid glucosyltransferase (NM106048); Rh5GT - *Rosa hybrida* anthocyanidin 5-*O* glucosyltransferase (AB201050); Os Rf5 - *Oryza sativa* flavonoid *O*-glucosyltransferase Rf5 (NM_001050705); Hv 3GT - *Hordeum vulgare* flavonoid 3-*O*-glucosyltransferase (X15694); Zm 3GT - *Zea mays* flavonoid 3-*O*-glucosyltransferase (X13501); Os RUGT - *Oryza sativa* flavonoid *O*-glucosyltransferase RUGT (XM_463383); OsIsoFlv7GT - *Oryza sativa* isoflavonoid 7-*O*-glucosyltransferase (BAC80066); Ac73G1 - *Allium cepa* 7GT UGT73G1 (AY262062); At73C6 - *Arabidopsis thaliana* UGT73C6 flavonoid 7-*O*-glucosyltransferase; At7gt73B1 - *Arabidopsis thaliana* UGT73B1 flavonol 7-*O*-glucosyltransferase; Gt3'GT - *Gentiana triflora* anthocyanin 3'-*O*-glucosyltransferase (AB076697); DbBet5gt - *Dorotheanthus bellidiformis* betanidin 5-*O* glucosyltransferase (AF374004), Sb7gt - *Scutellaria baicalensis* flavonoid 7-*O* glucosyltransferase (AB031274); GmIsoflv7gt - *Glycine max* isoflavonoid glucosyltransferase (DQ278439); VaABAgt - *Vigna angularis* abscisic acid glucosyltransferase (AB065190); At5GT - *Arabidopsis thaliana* UGT75C1 anthocyanin 5-*O*-glucosyltransferase; Pf5GT - *Perilla frutescens* anthocyanin 5-*O*-glucosyltransferase (AB013596); Mp7gt - *Maclura pomifera* flavonoid 7GT (DQ985179); Vh5GT - *Verbena* x *hybrida* anthocyanin 5-*O*-glucosyltransferase (Ab013598). NtGt2 - *Nicotiana tabacum* flavonol 7-*O* glucosyltransferase (AB072919); Ph5gt - *Petunia* x *hybrida* anthocyanin 5-*O* glucosyltransferase (AB027455); VLOGT3 - *Vitis labrusca O*-glucosyltransferase 3 (EF533706); Ih5gt - *Iris hollandica* anthocyanin 5-*O*-glucosyltransferase (AB113664); trVLOGT4 - truncated *Vitis labrusca O*-glucosyltransferase 4 (EF533707); VLOGT1 - *Vitis labrusca O*-glucosyltransferase 1 (EF533704); VLOGT2 - *Vitis labrusca O*-glucosyltransferase 2 (EF533705); At84A1 - *Arabidopsis thaliana* UGT74F1 salicylic acid glucosyltransferase (BT015796); At84A1 - *Arabidopsis thaliana* UGT84A1 hydroxycinnamic acid glucosyltransferase;

Figure 5 Abbreviations continued.
CuLimgt - *Citrus unshiu* liminoid glucosyltransferase (AB033758); VLRSGT - *Vitis labrusca* resveratrol/hydroxycinnamic acid *O*-glucosyltransferase (DQ832169); Ip2''GT - *Ipomoea purpurea* anthocyanidin 3-*O*-glucoside-2''-*O*-glucosyltransferase (Ab192315); PhRT - Petunia x *hybrida* anthocyanidin 3-O-glucoside-6''-*O*-rhamnosyltransferase (CAA50377); At80A2 - *Arabidopsis thaliana* UGT80A2 sterol glucosyltransferase; PvHRA25 - *Phaseolus vulgaris* glucosyltransferase HRA25 (AF303396); At76E12 - *Arabidopsis thaliana* UGT76E12 flavonol *O*-glucosyltransferase.

enzyme preferentially glucosylates anthocyanidins (Table 1). *VL3GT* is expressed in post-veraison exocarp tissue (Figure 3), consistent with anthocyanin accumulation and high levels of cyanidin glucosylation in these tissues (Figure 4).

5.5.1 - The anthocyanin accumulation profile of Vitis labrusca does not reflect the in vitro substrate specificity of VL3GT

The high level of protein sequence identity which exists between VL3GT and VV3GT suggests a common function in the glucosylation of anthocyanidins and flavonols at the 3-*O*-position, which was confirmed *in vitro*. Despite having nearly identical protein sequences, *in vitro* biochemical characterization of VL3GT reveals several functional differences between these two proteins. Whereas rVV3GT can use several donor substrates including UDP-Gal (Offen et al., 2006), rVL3GT is specific for UDPG as a substrate. Interestingly, rVV3GT glucosylates the 7-*O*-position of flavonoids at low levels *in vitro* (Offen et al., 2006), and incubation of rVL3GT with quercetin for 30 min identified low levels of a homologous activity (producing quercetin 7-*O*-glucoside and quercetin 3,7-*O*-diglucoside).

Substrate specificity studies indicate that rVV3GT preferentially glucosylates hydroxylated acceptor substrates (cyanidin, delphinidin, quercetin) (Ford et al., 1998); whereas rVL3GT preferentially glucosylates their *O*-methoxylated derivatives (peonidin, malvidin, isorhamnetin) (Figure 2). Surprisingly this difference is not reflected by the anthocyanin profiles of the grape berries; as *Vitis vinifera* cv. Shiraz accumulates high levels of malvidin-3-*O*-glucosides and *Vitis labrusca* cv. Concord accumulates high levels of cyanidin- and delphinidin-3-*O*-glucosides in the berry exocarp (Mazza and

Miniati, 1993; Boss et al., 1996a, b; Wu and Prior, 2005; Wu et al., 2006). These results suggest that the anthocyanin profiles of grapes are not dependent on the substrate specificity of the 3GT, instead these profiles reflect the broad substrate specificity of 3GTs which allows this enzyme to gluocosylate the substrate with which it is presented *in vivo*. Methylation generally precedes glucosylation in flavonoid biosynthesis and it is likely that the anthocyanin profiles of grape reflect the substrate specificity of the *O*-methyltransferase(s) that *O*-methylate the B-ring hydroxyl groups of anthocyanidins and flavonols.

5.5.2 - Calculated kinetic values indicate that VL3GT preferentially glucosylates anthocyanidins

The kinetic parameters of rVL3GT were determined for the donor substrate UDP-glucose and the acceptor substrates quercetin and cyanidin in order to compare these to those obtained for rVV3GT (Ford et al., 1998). Both rVV3GT and rVL3GT have two-fold lower affinity for cyanidin ($K_{m\ VL}$= 4.8 μM; $K_{m\ VV}$ = 30 μM) than for quercetin ($K_{m\ VL}$= 2.2 μM; $K_{m\ VV}$ = 15 μM) (Table 1); and both rVL3GT and rVV3GT have higher turnover numbers with cyanidin ($K_{cat\ VL}$= 7.02 x $10^{-4}s^{-1}$; $K_{cat\ VV}$ = 47.6 x $10^{-4}s^{-1}$) than with quercetin ($K_{cat\ VL}$= 1.13 x $10^{-4}s^{-1}$; $K_{cat\ VV}$ = 0.99 x $10^{-4}s^{-1}$), although the rVV3GT is able to glucosylate cyanidin at a faster rate than is the rVL3GT. The calculated catalytic efficiencies for the rVV3GT ($K_{cat}\ K_m^{-1}$ = 159 $M^{-1}s^{-1}$) and the rVL3GT ($K_{cat}\ K_m^{-1}$ = 146 $M^{-1}s^{-1}$) with cyanidin as a substrate are almost identical, whereas with quercetin, the $K_{cat}\ K_m^{-1}$ values indicated that quercetin is a better substrate for rVL3GT ($K_{cat}\ K_m^{-1}$ = 52 $M^{-1}s^{-1}$) than for rVV3GT ($K_{cat}\ K_m^{-1}$ = 6.6 $M^{-1}s^{-1}$) (Table 1). In general, calculation of the kinetic

parameters indicates that both enzymes preferentially glucosylate cyanidin as compared with quercetin.

5.5.3 - VL3GT Gene expression profile is consistent with a role in anthocyanin modification.

VL3GT gene expression was monitored in Concord berry exocarp and mesocarp tissues throughout grape berry development (Figure 3). *VL3GT* transcript was detected post-veraison in *Vitis labrusca* and *Vitis vinifera* exocarp tissues; at low levels in *Vitis labrusca* leaf, flower bud and flower tissue; but was not detected in *Vitis labrusca* mesocarp tissue or in the exocarp of pre-veraison berries (Figure 3). These results are consistent with the well-documented expression of *3GT* in post-veraison *Vitis vinifera* berry exocarp tissues from red (but not white) cultivars, concurrent with the biosynthesis and accumulation of anthocyanins in these tissues (Boss et al., 1996a,b; Kobayashi et al., 2001).

5.5.4 - Crude protein extracts from grape berry exocarp and mesocarp differentially glucosylate cyanidin and quercetin throughout development.

Protein was extracted from post-veraison *Vitis vinifera* and several stages of pre- and post- veraison *Vitis labrusca* mesocarp and exocarp tissue. The extracts were desalted and assayed for 3-*O*-glucosylation of cyanidin and quercetin. As was previously reported for *Vitis vinifera*, enzyme assays of crude desalted extracts were complicated by the presence of several endogenous enzymes capable of glucosylating flavonols *in vitro* (Ford et al., 1998). In *Vitis labrusca,* glucosylation of quercetin at the 3-*O*-position was

detected in berry mesocarp, exocarp, leaf, flower bud and flower tissues, while anthocyanidin 3GT activity was restricted to grape exocarp. These results: a) suggest that the quercetin 3GT activity in the berry mesocarp, flower bud, leaf and flower is catalyzed by at least one separate enzyme that preferentially glucosylates flavonols; and b) are consistent with the lack of anthocyanins and the accumulation of several 3-*O*-glucosylated flavonols in these tissues (Moore and Giannasi, 1994; Park and Cha, 2003). Cyanidin 3-*O*-glucosylation was detected at low levels in post-veraison mesocarp tissue, and at 10-fold higher levels in post-veraison exocarp tissue, consistent with the gene expression profile of *VL3GT* (Figure 3) and the accumulation of anthocyanins in berry exocarp but not mesocarp tissue (Boss et al., 1996 a, b; Kobayashi et al., 2001).

5.5.5 - The position of VL3GT in relation to other members of the 3GT phylogenetic clade.

Phylogenetic analysis places *VL3GT* in a clade with other enzymes which transfer sugars from an activated donor to the 3-*O*-position of flavonoids (Figure 5). The recent crystal structure of rVV3GT has allowed the identification of several residues which are important for catalytic activity and both acceptor and donor substrate binding (Figure 1; Offen et al., 2006). Protein sequence alignment of rVL3GT with rVV3GT reveals the conservation of these essential residues, in both sequences (Figure 1), which is consistent with their homologous function glucosylating the 3-*O*-position of flavonoids.

In addition to glucosyltransferases, this clade also contains the *Arabidopsis thaliana* flavonoid 3-*O*-rhamnosyltransferase (Jones et al., 2003) and the *Vigna mungo* and *Petunia* x *hybrida* flavonoid 3-*O*-galactosyltransferases (Mato et al., 1998; Miller et

al., 1999) (Figure 5). Recently, it was demonstrated that a single point mutation (G375H) at the C-terminus of the PSPG consensus sequence changed the substrate specificity of a flavonoid 3-*O*-galactosyltransferase, allowing it to also glucosylate the 3-*O*-position of flavonoids (Kubo et al., 2004) and site-directed mutagenesis of an isoflavonoid 7-O-glucosyltransferase changed the activity and kinetic parameters of this protein towards its endogenous substrate (Noguchi et al., 2007). Similarly, domain swapping of the PSPG box from the curcumin *O*-glucosyltransferase from *Catharanthus roseus* with the corresponding sequence from the *Nicotiana tabacum* NTGT1b eliminated curcumin glucosyltransferase activity, which was restored by site-directed mutagenesis of a single amino acid (R377C) (Masada et al., 2007b); suggesting that non-conserved amino acid residues within the PSPG box are important for acceptor substrate specificity, in addition to the well-documented role of conserved residues in this domain for donor substrate specificity. The necessity of single amino acid residues for protein function and the importance of these residues for donor and acceptor substrate specificity are consistent with the distinct functional properties of rVL3GT and rVV3GT revealed in this study, despite having 96 % amino acid sequence identity.

5.6 - Conclusion

Glucosylation of the 3-*O*-position of anthocyanidins is essential for their storage and the stability of their spectral characteristics (Prior and Wu, 2006). The molecular cloning and *in vitro* biochemical characterization of this enzyme from Concord grape berry shows its strict regiospecificity for the 3-*O*-position of anthocyanidins and flavonols and its use of UDP-glucose, but not UDP-galactose as a donor substrate. Kinetic analysis reveals that rVL3GT preferentially glucosylates anthocyanidins *in vitro*;

and RT-PCR analysis shows that *VL3GT* expression coincides with cyanidin 3GT enzyme activity (but not quercetin 3GT activity) and the accumulation of anthocyanins in post-veraison Concord grape exocarp tissues. A comparison of the *in vitro* biochemical parameters obtained with rVL3GT reveals distinct biochemical properties as compared to the highly identical and previously characterized rVV3GT.

Chapter 6 - General conclusion

This thesis has combined several biochemistry and molecular biology-based tools to identify, clone and biochemically characterize *O*-glucosyltransferases from *Vitis labrusca*. In addition to more than tripling the number of functionally characterized *Vitis* glucosyltransferases from two to seven, the identification and characterization of a novel dual function enzyme (VLRSGT) that glucosylates the neutraceutical resveratrol is an important finding which is relevant to both human epidemiological studies and to the field of basic plant research.

Despite the frequent consumption of high levels of resveratrol glucoside (piceid) in food and in wine, epidemiological studies concentrating on the uptake and effects of piceid in humans have been hindered by a lack of large quantities of pure glucoside. Expression of recombinant VLRSGT and enzymes assays with resveratrol as a substrate will produce high levels of piceid, which can be isolated and used in epidemiological studies. Based on the outcome of these epidemiological studies, plants which accumulate varied amounts of resveratrol and piceid becomes an attractive target for the genetic engineering of crop plants. By introducing VLRSGT to tissues that accumulate only resveratrol aglycone, plants can be generated and selected for high levels of piceid accumulation. Conversely, plant transformation with the VLRSGT antisense transcript would decrease the amount of piceid in a plant, with the expected result of increasing free resveratrol levels in these tissues. Overall, the biochemical characterization of VLRSGT enables the intensive study of the human effects of resveratrol and piceid and can be used to engineer crops with varied neutraceutical value.

Further biochemical characterization of VLRSGT revealed the unique ability of this enzyme to form glucosides with several flavonoids and stilbenes at pH 9.0, and to form glucose esters of hydroxycinnamic and hydroxybenzoic acids at pH 6.0. Although several previously characterized enzymes display broad substrate specificity, typical pH analyses concentrate on one substrate at several pHs or several substrates at one pH, but do not investigate the effect of pH on different classes of substrates. Glucose ester forming enzymes with high levels of sequence identity to VLRSGT have been biochemically characterized from several plant species at pH 6.0, however none of these studies assayed several substrates at several pHs. It is expected that additional characterization of these enzymes throughout a range of pHs with a broad range of substrates would reveal novel specificities, as found for VLRSGT. The pH-dependent functional group specificity of VLRSGT is novel for this group of enzymes and reinforces the importance of thorough biochemical characterization of proteins expressed *in vitro*.

Several unsuccessful attempts to clone the anthocyanin 5-*O*-glucosyltransferase (5GT) gene from *Vitis labrusca* highlighted the hazards involved in homology-based approaches for gene identification, and emphasized the importance of biochemical characterization before assigning a gene function. This result is of particular relevance in reference to the large scale plant genome sequencing efforts which are nearing completion and will be publicly available within the next few years. Currently, annotation of genes relies heavily on assigning function based on sequence identity to previously characterized proteins. The time and effort required for gene cloning and functional characterization often deters the complete biochemical characterization of a

given gene product. With the huge amount of sequence information generated from the genome sequencing initiatives, this study (in addition to several others) exemplifies the need to move away from homology-based annotation of gene function and towards the development of high-throughput technologies for biochemical characterization of gene function.

Vitis vinifera cv. Pinot Noir is anticipated to be the next plant genome to be completely sequenced and publicly available. Although the TIGR grape gene index has more than 90 EST sequences with sequence identity to glucosyltransferases, the additional sequence information obtained from the genome will undoubtedly contain novel glucosyltransferase sequence, including the identity of the 5GT. Further studies will include the cloning and biochemical characterization of this gene and attempt to address the question of why *Vitis* spp. expresses so many 5GT-like genes. The *Vitis vinifera* genome sequence can also be exploited to predict glucosyltransferase function by analyzing which genes cluster together on chromosomes, which genes are fused together and whether or not these genes share regulatory sites. This approach can also be used to confirm the participation of VLRSGT and the 5GT-like genes in the metabolic pathways suggested in this thesis.

Biochemical characterization of recombinant enzymes is a tool which is used to correlate information generated *in vitro* to a putative role within the plant. By correlating this biochemical information with gene expression and metabolite profiles of the plant, the *in planta* function of a protein can be suggested. Although these methods often assign the correct protein function, a more reliable assessment of the *in planta* role of a protein is to overexpress or knock down the gene within the plant and look for

phenotypic differences. Although both transient and stable grape transformation systems have been developed for almost five years, the use of these systems is limited by the long regeneration time required for grapevine tissue. An alternative and time-efficient approach would be to use the well-established *Arabidopsis* transformation systems to generate model plants which may display a phenotype, and correlate the observed phenotype with the expected results in grape tissues.

In conclusion, the knowledge generated in this thesis has multi-disciplinary applications and contributes to the general understanding of plant enzymology. By successfully combining several techniques, five full-length *O*-glucosyltransferases from Concord grape were identified, cloned and characterized. The hazards of homology-based approaches to gene identification, and the importance of biochemical characterization for assigning gene function were emphasized and future work using the grape genome sequence and *in planta* gene expression is necessary to confirm the putative roles of these genes within *Vitis* spp.

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Appendix I

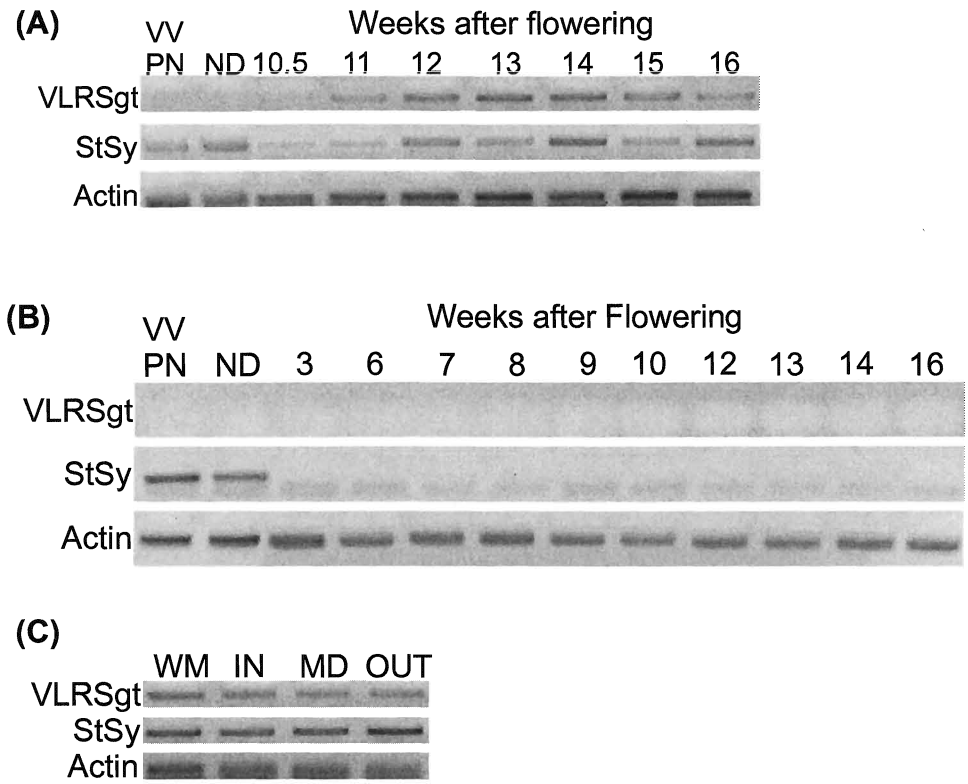

Figure 1. RT-PCR analysis of VLRSgt and Stilbene synthase (StSy) transcripts throughout development of *Vitis labrusca* cv. Concord. (A). Developmental gene expression in Concord grape (wk 10.5 to 16) and post-veraison (wk 14) *Vitis Vinifera* cv. Pinot Noir (VV PN) and Noir Droit (ND) berry mesocarp. (B). Developmental gene expression in Concord grape berry exocarp (wk 3-16) and post-veraison (wk 14) *V. vinifera* cv. Pinot Noir (VV PN) and Noir Droit (ND) berry exocarp. (C) Transcript expression in whole mesocarp (WM), inner (IN), middle (MID), and outer (OUT) wk 12 Concord grape mesocarp. Actin was used as a loading control.

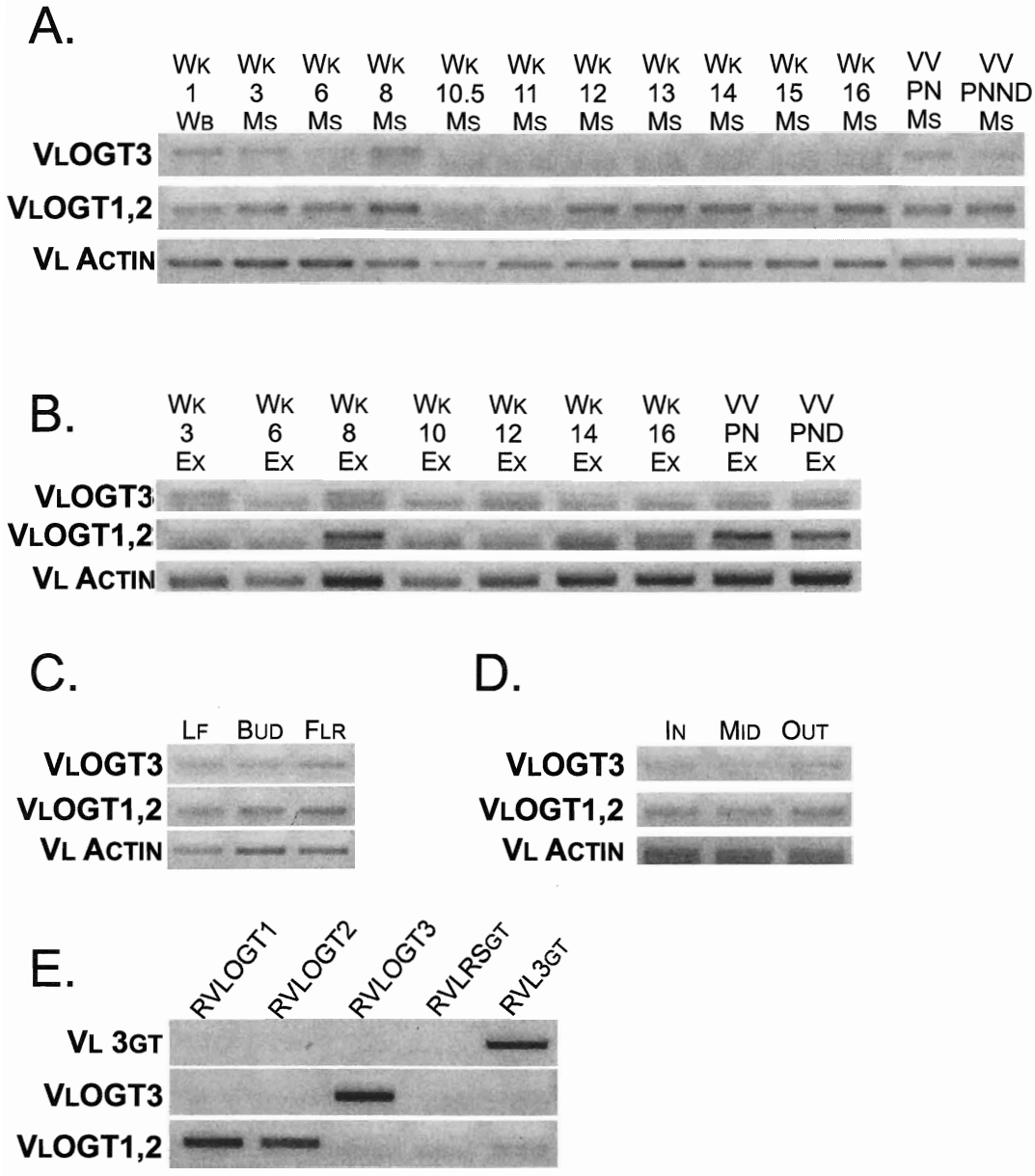

Figure 2. RT-PCR analysis of *VLOGT1,2*, AND *VLOGT3*. A. Developmental gene expression profile of *VLOGT1,2, VLOGT3,* and *VLACTIN* in *Vitis labrusca* cv. Concord *and Vitis vinifera* cvs. Pinot Noir (VVPN) and Pinot Noir Droit (VV PND) berry mesocarp (MS) tissue. B. Developmental gene expression profile of *VLOGT1,2, VLOGT3* and *VLACTIN* in *Vitis labrusca* cv. Concord *and Vitis vinifera* cvs. Pinot Noir (VVPN) and Pinot Noir Droit (VV PND) berry exocarp (Ex) tissue. C. Gene expression profile of *VLOGT1,2, VLOGT3,* and *VLACTIN* in *Vitis labrusca* leaf, flower bud and flower tissue. D. Gene expression profile of *VLOGT1,2,* and *VLOGT3* in *Vitis labrusca* inner (IN), middle (MID) and outer (OUT) wk 12 mesocarp tissue. E. PCR specificity of VL3GT, VLOGT1,2 and VLOGT3 primers to expression plasmids containing the full-length rVLOGT1, rVLOGT2, rVLOGT3, rVLRSGT and rVL3GT.

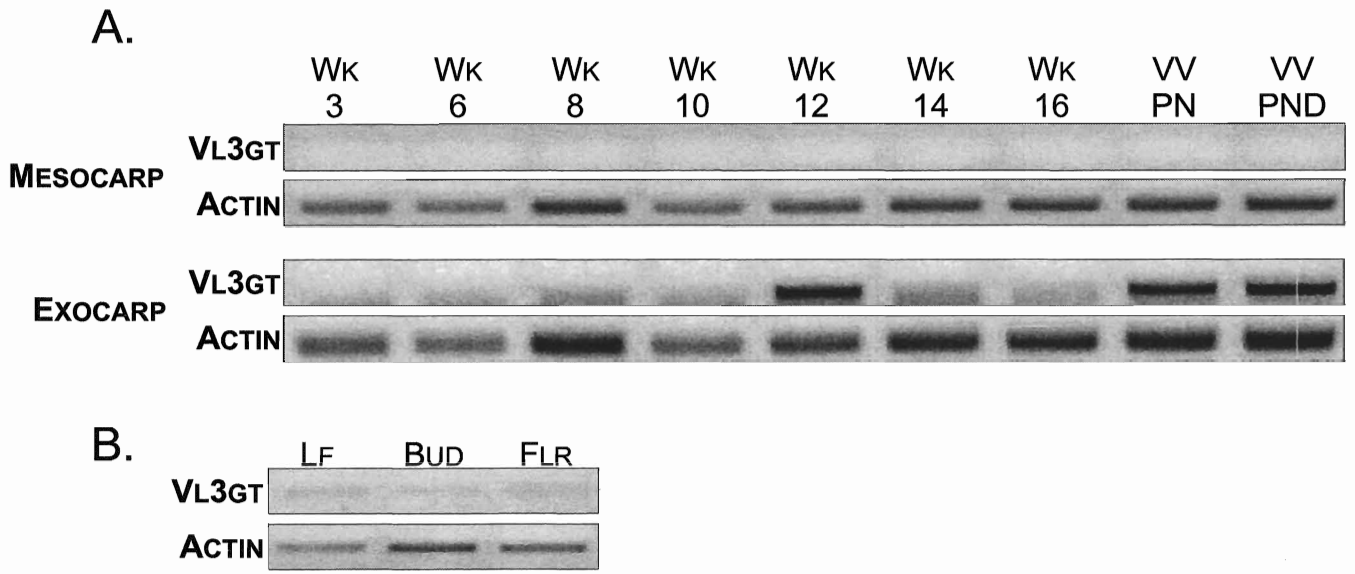

Figure 3. RT-PCR analysis of *VL3GT.* A. Developmental gene expression profile of VL3GT, and VLACTIN in *Vitis labrusca* cv. Concord and *Vitis vinifera* cvs. Pinot Noir and Pinot Noir Droit berry mesocarp and exocarp tissue. B. Gene expression profile of VL3GT, and VLACTIN in *Vitis labrusca* leaf, flower bud and flower tissue.