INVESTIGATION INTO THE MOLECULAR BASIS FOR THE ABSENCE OF DIHYDROMYRICETIN-DERIVED FLAVONOIDS IN RASPBERRY

by

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ABSTRACT

Red raspberry (*Rubus idaeus*) is considered a health-promoting food with antioxidant properties. Flavonoid-3',5'-hydroxylase (F3'5'H), of the CYP75 family, is a member of the cytochrome P450 superfamily that catalyzes hydroxylation reactions to yield dihydromyricetin (DHM)-derived flavonoids. Raspberry and other Rosaceae family members such as apple (*Malus* spp.) and peach (*Prunus* spp.) preferentially accumulate dihydroquercetin (DHQ)-derived flavonoids, while lacking the DHM types. This peculiarity was investigated by comparative genomics and functional genomics of the F3’5’H gene(s).

To investigate the molecular basis for the lack of F3’5’H activity in raspberry that could be attributed to aberrations at the transcriptional level, I first validated DNA template quality and a PCR-based strategy for gene cloning in raspberry and found it to be feasible. Numerous PCR efforts failed to detect any F3’5’H transcripts in fruit cDNAs nor could F3’5’H gene(s) be detected in the raspberry genome. Apple and peach whole genome sequence data mining also did not identify F3’5’H. Taken together, I have shown that PCR, anthocyanin profiling, and comparative genomics data are corroborated and collectively make a strong case for the raspberry genome lacking an F3’5’H ortholog.

Genetic complementation of the absence of DHM-derived flavonoids in raspberry and apple callus lines was undertaken through inoculation of *Agrobacterium tumefaciens* harbouring a recombinant vector with grapevine F3’5’H gene driven by the 35S promoter. I established raspberry callus lines derived from leaves as well as apple callus lines derived from fruit. I then showed that both raspberry and apple callus lines are

amenable to *Agrobacterium*-mediated genetic transformation via GUS histochemical assay. I found that light together with nitrate and 2,4-D deficient culture medium rapidly and efficiently induced anthocyanin accumulation in apple callus. While anthocyanins and flavonols were readily detected in treated apple callus lines, only hydroxycinnamic acid derivatives and early pathway intermediates were detected in treated raspberry callus lines. PCR analyses were conducted on putative raspberry transgenic callus lines. It was found that F3'5'H gene expression was detected in actively proliferating raspberry transgenic callus lines, as well as F3'5'H gene insertion(s) in the raspberry transgenic callus genome in the same selected lines.

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LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
2iP	6-(γ,γ-dimethylallylamino) purine
2-ODD	2-oxoacid-dependent dioxygenase
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
ABA	abscisic acid
AIM	anthocyanin induction medium
BAP	benzylaminopurine
BC	British Columbia
BLAST	basic local alignment search tool
bp	base pairs
BSA	bovine serum albumin
$CaCl_2$	calcium chloride
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
CHS	chalcone synthase
CIM	callus induction medium
CODEHOP	consensus-degenerate hybrid oligonucleotide primer
CTAB	cetyl trimethylammonium bromide
cv(s)	cultivar(s)
DAD	diode array detector
DFR	dihydroflavonol 4-reductase
dH_2O	deionized water
DHK	dihydrokaempferol
DHM	dihydromyricetin
DHQ	dihydroquercetin
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionization
F3H	flavanone 3-hydroxylase
F3'H	flavonoid 3'-hydroxylase
F3'5'H	flavonoid 3',5'-hydroxylase
FLS	flavonol synthase
FS	fruit set
GBM	Gamborg B5 basal medium
GFP	green fluorescent protein
GH	green hard
GPJT	green pink just turning
GST	glutathione *S*-transferase
GUS	β-glucuronidase
HGHP	half green half pink
HPLC	high performance liquid chromatography
hr	hour
IBA	indole-3-butyric acid
IPGI	International Peach Genome Initiative
kDa	kiloDalton

kPa	kiloPascal
KOH	potassium hydroxide
L-Phe	L-phenylalanine
LB	lysogeny broth
LR	large reddish
m/z	mass-to-charge ratio
Md	*Malus* x *domestica*
$MgCl_2$	magnesium chloride
min	minute
MM	minimal medium
MR	medium reddish
MS	mass spectrometry
MS	Murashige and Skoog medium
MeJA	methyl jasmonate
n.d.	no date
NAA	naphthaleneacetic acid
NAPS	Nucleic Acid Protein Service
NCBI	National Center for Biotechnology Information
NDM	nitrogen deficient medium
NTC	no template control
NVC	no vector control
OD	optical density
ORF	open reading frame
P450	cytochrome P450
P	pink
PARC	Pacific Agri-Food Research Centre
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PMM	L-phenylalanine methyl jasmonate medium
PSM	L-phenylalanine sucrose medium
RACE	rapid amplification of cDNA ends
RE	restriction endonucleases
RH	red harvest
Ri	*Rubus idaeus*
RMS	red medium soft
RNA	ribonucleic acid
sec	second
UBC	The University of British Columbia
UFGT	UDP:glucosyltransferase
UTR	untranslated region
UV	ultraviolet
v/v	volume per volume
Vv	*Vitis vinifera*
w/v	weight per volume
wk	week
X-Gluc	5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid
YEP	yeast extract peptone

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1 LITERATURE REVIEW

1.1 Raspberries

1.1.1 Taxonomy

Red raspberry belongs to an economically-important family of fruit-producing crops and ornamental plants, *Rosaceae*. Some prominent Rosaceae genera include apple (*Malus*), cherry (*Prunus*), peach (*Prunus*), pear (*Pyrus*), raspberry (*Rubus*), rose (*Rosa*), and strawberry (*Fragaria*) (Shulaev *et al*., 2008). As Figure 1-1 indicates, roses, raspberries, and strawberries are included in the subfamily *Rosoideae*. The genus, *Rubus*, is subdivided into 12 subgenera with *Idaeobatus* and *Eubatus* being the largest (Alice and Campbell, 1999). Within the genus, red raspberry (*R. idaeus* L.), black raspberry (*R. occidentalis* L), and blackberry (*Rubus spp*.) are among the most recognizable species with the greatest commercial value.

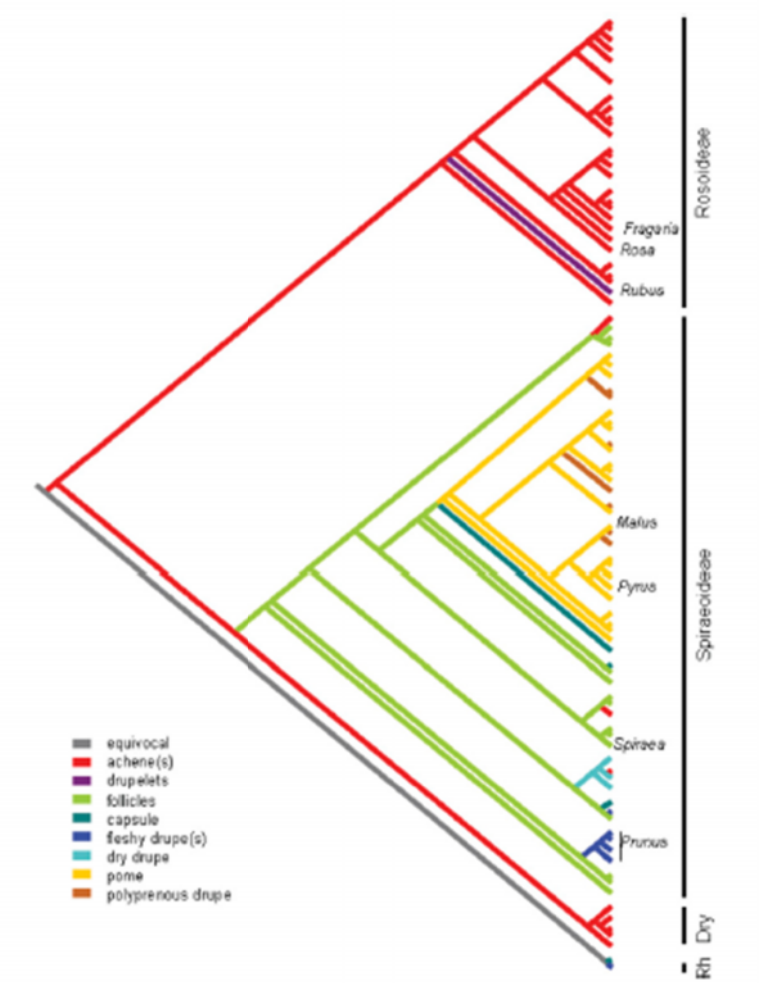


Figure 1-1. Phylogenetic tree of Rosaceae (Shulaev *et al*., 2008).

1.1.2 Distribution and Production

Wild raspberries are grown on every continent with the exception of Antarctica (Alice and Campbell, 1999). Domesticated raspberries are cultivated for commercial harvest primarily in Asia, Europe, and North America. The major production area in North America is the Pacific Northwest including British Columbia, Washington, and Oregon (Finn and Hancock, 2008). British Columbia is the largest raspberry producer in Canada, accounting for more than 80% of Canada's raspberries, with the raspberry crop

valued at 31.5 million dollars at the farm gate (Raspberry Industry Development Council, http://www.bcraspberries.com/).

1.2 Plant Pigments

1.2.1 ABC of Plant Pigments

Plant pigments are chemical compounds that absorb and reflect photons travelling at different wavelengths. With the exception of chlorophyll, the three main classes of plant pigments are anthocyanins, betalains, and carotenoids (Tanaka *et al.*, 2008; Grotewold, 2006; Delgado-Vargas *et al.*, 2000) (Figure 1-2). They are the natural colorants for leaves, flowers, fruits, and seeds. Plant pigments play important physiological and ecological roles in serving as attractants for pollinators and seed dispersal agents and acting as protectants from UV radiation (Stintzing and Carle, 2004).

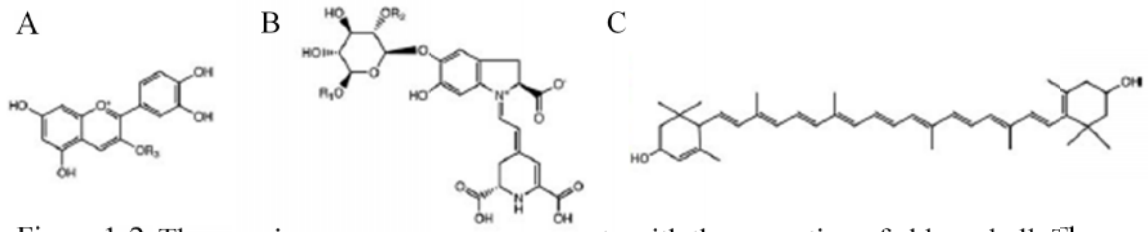


Figure 1-2. Three major classes of plant pigments with the exception of chlorophyll. They are (A) anthocyanins, (B) betalains, and (C) carotenoids (Grotewold, 2006).

Betalains are water-soluble, nitrogen-containing compounds derived from tyrosine. They confer yellow (betaxanthins) to red (betacyanins) colours to flowers and fruits. Interestingly, betalains are found exclusively in the order of Caryophyllalles (Tanaka *et al.*, 2008). Carotenoids are lipid-soluble and plastid-synthesized isoprenoid compounds. Unlike anthocyanins and betalains, carotenoids are essential in the plant life, playing roles in photosynthesis and phytohormone biosynthesis (Grotewold, 2006). Anthocyanins are a class of flavonoids with the basic structure of C6-C3-C6, of A ring-C

ring-B ring, respectively, consisting of two aromatic rings and one *O*-heterocycle (Forkmann, 1991) (Figure 1-3). They are water-soluble pigments responsible for a broad spectrum of colours from orange, red, blue, to purple. The key to such colour diversity, on a structural level, is dependent on the degree of hydroxylation of the B ring at the 3', 4', and/or 5' positions with an increased degree of oxygenation resulting in a "blueing" effect (Davies, 2004; Glover, 2007).

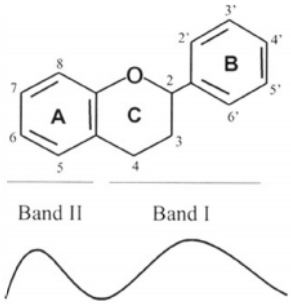


Figure 1-3. Basic structure of a flavonoid and its UV-visible absorption spectrum (http://www.globalspec.com/reference/70423/203279/chapter-5-on-line-identification-of-flavonoids-by-hplc-coupled-to-diode-array-detection).

1.2.2 Anthocyanins and Human Health

There is a growing body of evidence that has established a positive association between anthocyanins and human health. Anthocyanins are powerful antioxidants due to their particular chemical structures and are estimated to contribute to approximately 25% of the total antioxidant activity in ripe raspberries (Beekwilder *et al*., 2005a). Anthocyanins act as antioxidants through their ability to scavenge free radicals, chelate transition metals, and, to a lesser extent, suppress radical generating enzymes and protect antioxidant vitamins and detoxifying enzymes. It has been proposed through structure-activity studies that the presence of the ortho-dihydroxy structure on the B ring is a

chemical criterion for determining radical scavenging functions of anthocyanins (Bors *et al*., 1990; Rice-Evans *et al*., 1995).

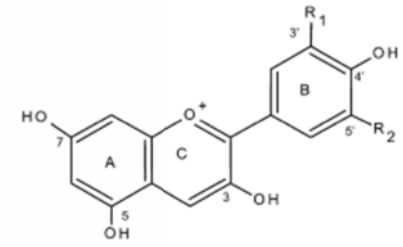
Oxidative stress leading to cell structural damage has been implicated as a predisposing factor to the initiation and the development of cancer. At high concentrations, reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated from endogenous and exogenous sources are the key factors responsible for oxidative damage in biological systems (Valko *et al.*, 2006). Because of the strong reducing nature of anthocyanins, they have been shown to exhibit various anti-carcinogenic properties in many *in vitro* and *in vivo* studies (Wang and Stoner, 2008; Seeram, 2008). Seeram *et al*. (2006) tested the abilities of anthocyanin-rich berry extracts from blackberry, black raspberry, raspberry, and strawberry to inhibit the proliferation of four human tumour cell lines, colon, prostate, breast, and oral. All berry extracts tested were shown to inhibit cell proliferation in a dose-dependent manner. They found that the anti-proliferative effect was correlated with the induction of apoptosis or cell cycle arrest. This corroborated with earlier findings by Chen *et al*. (2005) in which dietary polyphenols, cyanidin-3-glucoside and peonidin-glucoside, from *Oryza sativa* L. indica down-regulated cell cycle-related protein levels and induced caspase-3 activation and chromatin condensation. Furthermore, anti-angiogenic activity has been reported in edible berry extracts, which inhibits the induced expression of vascular endothelial growth factor (VEGF) (Roy *et al*., 2002; Atalay *et al*., 2003; Bagchi *et al*., 2004).

Anthocyanins are versatile biomedicinal compounds with a broad range of biological activities. In addition to their roles in cancer chemoprevention, anthocyanins have been linked to improved heart health, in particular, reducing the risk of coronary

heart disease (Mazza, 2007). The mechanisms involved are postulated to be through the reduction of platelet aggregation (Renaud *et al*., 1992) and the inhibition of lipid peroxidation (Afanas'ev *et al*., 1989). *In vivo* studies by Ahmet *et al*. (2009) and Ziberna *et al*. (2009) showed that anthocyanin-enriched diets provide protection against ischemia-induced reperfusion injury.

1.2.3 Anthocyanins in Raspberries

Anthocyanins are commonly found in fruits and vegetables as part of a balanced daily diet. In comparison, berries are especially rich in anthocyanins. There are considerable variations in the concentrations of anthocyanins and the types of aglycones present among foods. Taken together, the food intake data from The National Health and Nutrition Examination Survey (NHANES) from 2001-2002 (Wu *et al*. 2006) estimated the daily intake of anthocyanins to be 12.5 mg/day/person in the United States, whilst the total anthocyanin concentration in raspberries was measured to be 116 mg/serving, far in excess of the average. Of the six major anthocyanidins (Figure 1-4), only cyanidin and pelargonidin aglycones are found in raspberries (Wu *et al.*, 2006; Scalzo *et al.*, 2008). Cyanidin and its glycosylated and/or acylglycosylated derivatives are the predominant anthocyanins in nature. As a genus, *Rubus* species show a relatively limited range of individual anthocyanidin diversity. Grapevine (*Vitis vinifera* L.) berry contains five of the six anthocyanidins: cyanidins, peonidins, delphinidins, petunidins, and malvidins (Wu *et al.*, 2006).



Anthocyanidins	Substitutes		λ_{max} (nm) visible spectra	Molecular Weight
	R_1	R_2		
Pelargonidin	H	H	494 (orange)	271
Cyanidin	OH	H	506 (orange-red)	287
Delphinidin	OH	OH	508 (red)	303
Peonidin	OCH_3	H	506 (orange-red)	301
Petunidin	OCH_3	OH	508 (red)	317
Malvidin	OCH_3	OCH_3	510 (bluish-red)	331

Figure 1-4. Chemical structures of six common anthocyanidins (Jing *et al*., 2008).

1.2.4 Anthocyanin Structure and Chemistry

Naturally occurring anthocyanins exist as glycosides and acylglycosides of anthocyanidins. Glycosylation occurs, most frequently, at the C3 position (Figure 1-4), followed by the C5 position, and more rarely, at the C7 position (Andersen and Jordheim, 2006; Stevens and Verhe, 2004). In order of occurrence in nature, glucose, rhamnose, xylose, galactose, arabinose, and fructose are the most commonly substituted sugars replacing hydroxyl groups at C3 and C5 (Wang *et al*., 1997). These sugars then provide additional sites for esterification with aromatic (such as hydroxycinnamoyl groups) and aliphatic (generally malonyl group) acyl groups (Yonekura-Sakakibara *et al*., 2008).

In acidic (pH 1-6) aqueous solutions, anthocyanins exist in four forms in equilibrium: quinoidal base (A), flavylium cation (AH^+), carbinol pseudobase (B), and chalcone (C) (Figure 1-5) (Brouillard and Delaporte, 1977; Mazza and Brouillard, 1987). As reviewed by Castaneda-Ovando *et al*. (2009), at pH 1, anthocyanins exist predominantly as the flavylium cation conferring red colours. The quinoidal base is

considered the prevalent structural species present at pH values between 2 and 4 and contributes to blue colours. At pH 5 and 6, the colourless carbinol pseudobase and chalcone can be observed. Anthocyanins begin to degrade at pH values greater than 7; however, all four structural transformations co-exist at pH values between 4 and 6. In addition, the stability of anthocyanins, at neutral pH, can be influenced by the number and the position of hydroxyl and methoxyl groups substituted on the B ring, as well as C ring glycosylations.

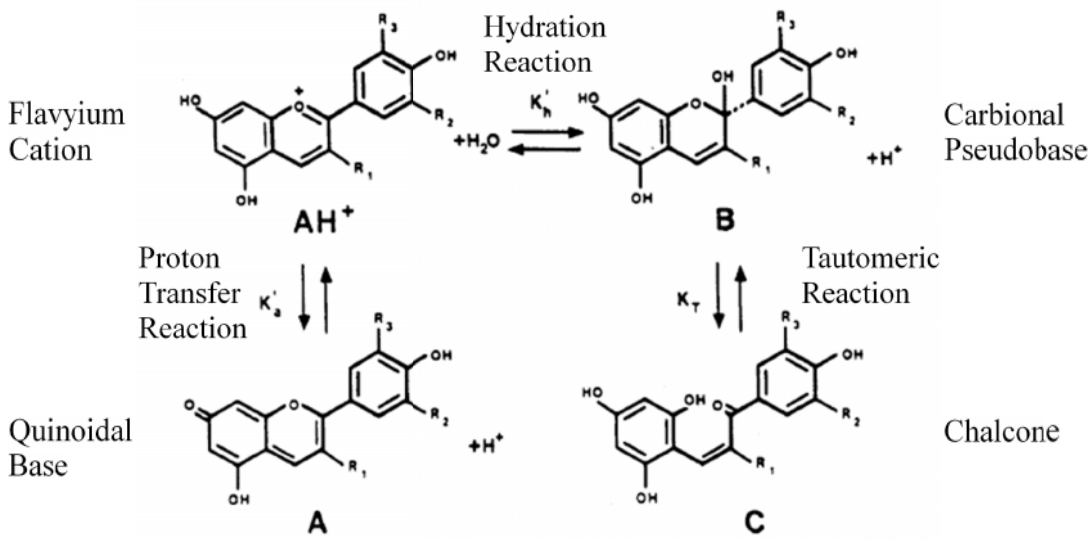


Figure 1-5. Four structural transformations of anthocyanins. Figure adapted from Mazza and Brouillard (1987).

1.3 Anthocyanin Biosynthetic Pathway

1.3.1 Structural Genes

Anthocyanins are synthesized from branches from the general flavonoid biosynthetic pathway, a well-conserved pathway in plant secondary metabolism (Figure 1-6). The entry point of the pathway is a stepwise condensation of one molecule of 4-coumaroyl-CoA and three molecules of malonyl-CoA by chalcone synthase (CHS), a

polyketide synthase, to mark the first committed step in the formation of tetrahydroxychalcone. The precursor 4-coumaroyl-CoA is a direct product of phenylpropanoid metabolism through the sequential activities of phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate:CoA ligase (4CL). The yellow-coloured tetrahydroxychalcone rapidly undergoes stereospecific isomerization to form colourless naringenin by chalcone isomerase (CHI). Naringenin, a flavanone, is hydroxylated to produce dihydrokaempferol (DHK), a dihydroflavonol, through the activity of flavanone 3-hydroxylase (F3H), which belongs to a large gene family of 2-oxoglutarate-dependent dioxygenases (2-ODDs). At this juncture, up to three pathways are possible giving rise to anthocyanin diversity, depending on the species.

Two separate hydroxylation events can occur at two variable positions of the B ring of DHK. Flavonoid 3'-hydroxyase (F3'H), a cytochrome P450 enzyme (P450), selectively hydroxylates at the 3' position to yield dihydroquercetin (DHQ). Flavonoid 3',5'-hydroxylase (F3'5'H), also a P450, hydroxylates at both the 3' and 5' positions to yield dihydromyricetin (DHM). Following the introduction of hydroxyl groups to DHK and DHQ, DHK, DHQ, and DHM are reduced by dihydroflavonol 4-reductase (DFR) to form leucopelargonidin, leucocyanidin, and leucodelphinidin, respectively, also collectively known as the leucoanthocyanidins. Anthocyanidin synthase (ANS), formerly named leucoanthocyanidin dioxygenase (LDOX), through a series of oxidation and dehydration reactions, converts leucoanthocyanidins to colourless aglycones, anthocyanidins. To achieve stability, anthocyanidins are modified at various positions by glycosylation, acylation, and/or methylation. Most commonly, anthocyanidins are glucosylated at the 3 position of the C ring by UDP-3-*O*-glucosyltransferases (UFGT).

Methylation is postulated to follow glucosylation and has been shown to increase thermal stability *in vitro* (Lucker *et al*., 2010).

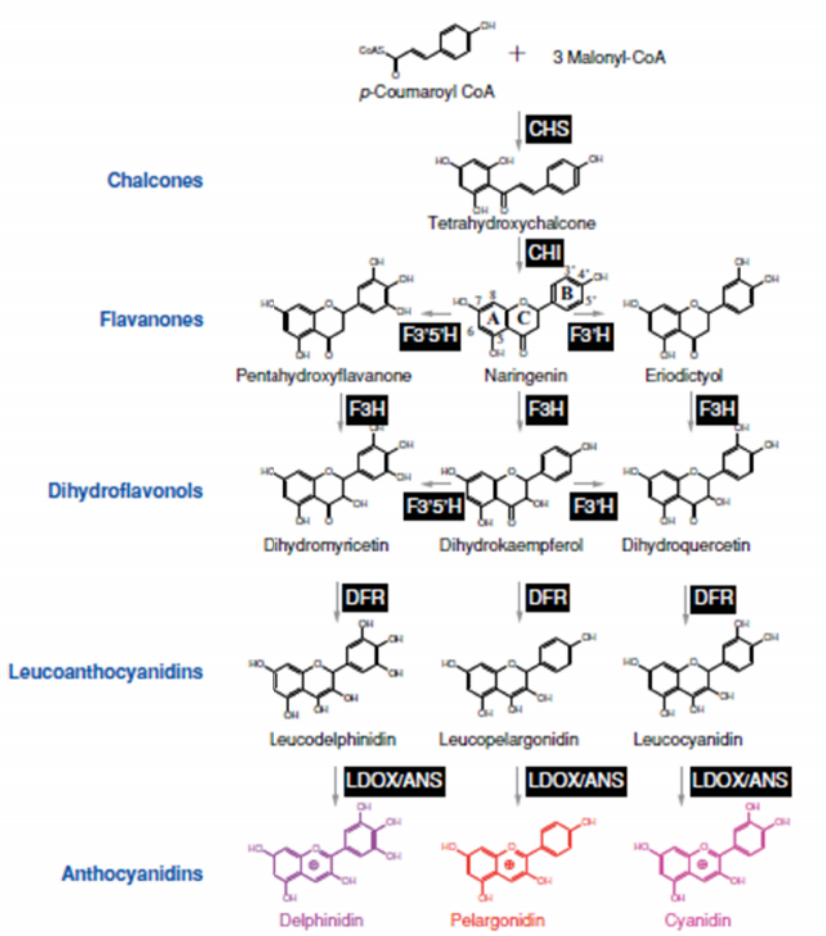


Figure 1-6. Simplified schematic of anthocyanin biosynthetic pathway (Grotewold, 2006).

Anthocyanins are synthesized in the cytosol. The most recent models postulate that metabolons, or multienzyme complexes, localized on the cytosolic side of the endoplasmic reticulum (ER) membrane are involved in flavonoid biosynthesis (Winkel-Shirley, 1999) based on precursor feeding (Margna and Vainjarv, 1981), membrane association (Hrazdina *et al*., 1987), coordinate enzyme and gene expression (Dooner, 1983), and protein interaction (Burbulis and Winkel-Shirley, 1999) experiments. Many genes of the anthocyanin biosynthetic pathway have been cloned and characterized in

petunia (CHS, Reif *et al*., 1985; CHI, van Tunen *et al*., 1988; F3H, Britsch *et al*., 1992; DFR, Beld *et al*., 1989; ANS, Weiss *et al*., 1993), maize (CHS, Dooner, 1983; CHI, Grotewold and Peterson, 1994; DFR, Reddy *et al*., 1987; ANS, Menssen *et al*., 1990), and snapdragon (CHS, Sommer and Saedler, 1986; CHI, Martin *et al*., 1991; F3H, Martin *et al*., 1991; DFR, Almeida *et al*., 1989; ANS, Martin *et al*., 1991).

Aside from anthocyanins, the formation of flavonols and proanthocyanidins (or condensed tannins) also funnels through the dihydroflavonols. Flavonol synthase (FLS), a 2-ODD enzyme that includes F3H, catalyzes the desaturation reactions from DHK, DHQ, and DHM to kaempferol, quercetin, and myricetin, respectively (Pelletier *et al*., 1997; Fujita et *al*., 2006). Copigmentation of anthocyanins with flavonols through intermolecular complex formation has been shown to result in bathochromatic (to longer wavelength) and hyperchromatic (colour intensity) shifts, thus conferring a blueing effect (Asen et al., 1972; Rein, 2005). While a single enzyme has been attributed to the synthesis of flavonols, two enzymes are responsible for catalyzing the reduction reactions of terminal units of proanthocyanidins: Leucoanthocyanidin reductase (LAR) and anthocyanidin reductase (ANR) (Bogs *et al*., 2005). LAR reduces leucocyanidins to catechins, while ANR reduces cyanidins to epicatechins. The extension units of proanthocyanidin polymers are provided by the precursor, leucocyanidins. Proanthocyanidins contribute to fruit flavour qualities due to their bitter and astringent properties.

1.3.2 Transport and Storage

Anthocyanins are transported from the cytosol to the weakly acidic vacuole for storage. Several transport mechanisms have been reported. Glutathione *S*- transferase (GST) was shown to act either as a carrier protein or a "tagging" protein that is required for anthocyanin transport in petunia (Mueller *et al*., 2000), carnation (Larsen *et al*., 2003), and grapevine (Conn *et al*., 2008). In maize (Goodman *et al*., 2004), the multidrug resistance-associated proteins (MRPs) of the ATP-binding cassette (ABC) protein family are active transporters playing a role in anthocyanin sequestration. Furthermore, multidrug and toxic extrusion (MATE) proteins are implicated as vacuolar H^+-dependent transporters for acylated anthocyanins in grapevine (Gomez et al., 2009). The involvement of ER-derived vesicle-like structures or anthocyaninic vacuolar inclusions (AVIs) in mediating transport was demonstrated in lisianthus (Markham *et al*., 2000; Zhang *et al*., 2006) and Arabidopsis seedlings (Poustka *et al*., 2007). Taken together, these findings provide evidence that transport mechanisms may be redundant and/or species-dependent.

1.3.3 Regulation

The regulation of anthocyanin biosynthesis is dependent on the spatial and temporal expression of its structural genes. The activity and interaction of R2R3 MYB, basic helix-loop-helix (bHLH), and WD40 transcription factors are essential to the activation of transcription of some or all structural genes within the anthocyanin biosynthetic pathway (Figure 1-7). This regulatory network has been reviewed most extensively in Arabidopsis, petunia, and maize (Koes *et al*., 2005).

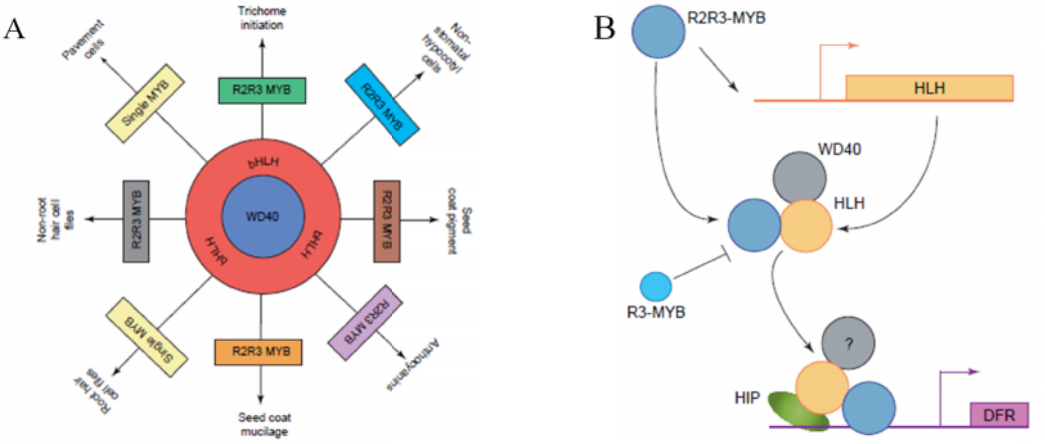


Figure 1-7. Model depicting the interactions between WD40, bHLH, and R2R3 MYB transcription factors resulting in (A) different epidermal cell types (Ramsay and Glover, 2005) and (B) transcriptional activation of anthocyanin biosynthetic gene (Koes *et al.*, 2005).

WD40 proteins are more or less ubiquitously expressed throughout the plant (Walker et al., 1999) and conserved across distant species (de Vetten *et al.*, 1997). WD40 proteins are not known to possess any catalytic activity (Neer *et al.*, 1994) but, rather, serve as a platform to facilitate protein-protein interactions (Ramsay and Glover, 2005).

Similar to WD40-repeat proteins, bHLH proteins are pleiotropic and regulate additional processes to anthocyanin biosynthesis (Tanaka *et al.*, 2008). Functional analysis revealed a direct interaction between bHLH and MYB proteins (Goff *et al.*, 1992). Although bHLH proteins do not bind to DNA, they have been shown to enhance MYB DNA-binding affinity and may recruit secondary factors to anthocyanin *cis*-regulatory elements in the promoters of some anthocyanin biosynthetic genes (Hernandez *et al.*, 2004).

R2R3 MYB proteins are DNA-binding proteins that specify epidermal cell fate. There is considerable sequence divergence between R2R3 MYB proteins, but they are functionally interchangeable (Ramsay and Glover, 2005). Several R2R3 MYB proteins involved in anthocyanin biosynthesis have been isolated from non-model species, such as

apple (Espley *et al*., 2007), mangosteen (Palapol *et al*., 2009), and pepper (Borovsky *et al*., 2004). Mutations in MYB or MYB-related genes can have paramount consequences leading to the loss of pigmentation. Kobayashi *et al*. (2004) found that white grapevine cultivars arose from a retrotransposon-induced mutation of *VvmybA1*. Lijavetzky *et al*. (2006) further determined that berry colour in more than 95% of the analyzed cultivars can be explained by sequence variations in VvmybA1, citing retrotransposon-related instability as the principal contributor.

1.4 Plant Cytochrome P450s

1.4.1 Activities

Plant cytochrome P450s (P450s) catalyze a vast array of biochemical reactions including hydroxylations, epoxidations, dealkylations, isomerizations, and oxygenations, to name a few. These reactions comprise a myriad of metabolic pathways, such as the biosynthesis of alkaloids, lipids, phenylpropanoids, and terpenoids and the breakdown of endogenous signalling molecules or exogenous compounds. Typically, P450s are membrane-bound heme-containing proteins that either utilize NADPH and/or NADH directly or require the association of a P450 reductase for electron transfer to cleave molecular oxygen (O_2) to produce a substrate and a molecule of water (Bolwell *et al*., 1994; Chapple, 1998; Schuler and Werck-Reichhart, 2003).

1.4.2 Characteristic Structural Features

While P450s are functionally diverse, considerable secondary and tertiary structural integrity is maintained. Generally, plant P450s are 50-60 kDa proteins with

three structural domains: membrane anchor, hinge, and globular domain (Figure 1-8). The hydrophobic N-terminus represents the most variable region, which carries a targeting sequence and anchors the protein to the membrane. Immediately following the hydrophobic helix, a series of basic residues precedes a proline-rich region ((P/I)PGPx(G/P)xP), which forms the hinge that optimally orients the protein on the cytosolic face of the membrane. Toward the C-terminus, residues obeying the consensus (A/G)Gx(D/E)T(T/s) form a threonine-containing binding pocket for molecular oxygen. The most conserved and characteristic P450 consensus sequence (FxxGxRxCxG) can be found in the heme-binding loop near the C-terminus. The cysteine residue, which serves as the fifth ligand to the heme iron, is essential to P450-mediated catalysis (Bolwell et al., 1994; Chapple, 1998; Werck-Reichhart and Feyereisen, 2000; Schuler and Werck-Reichhart, 2003).

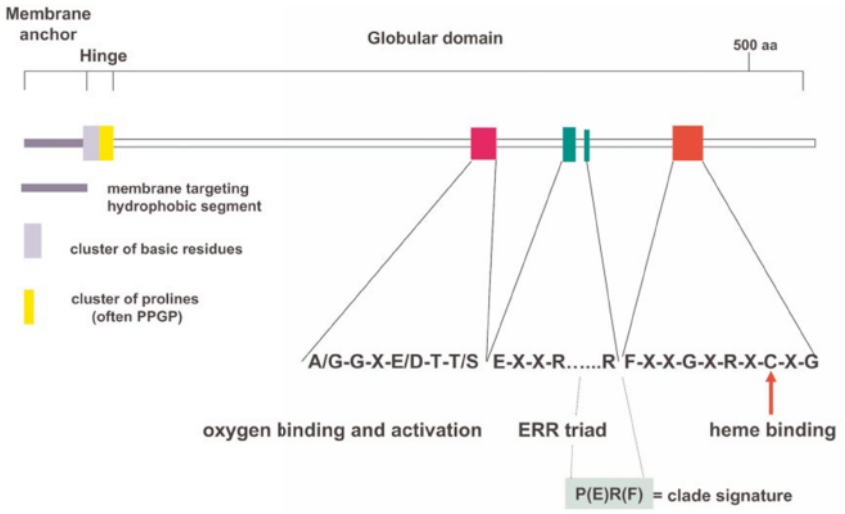


Figure 1-8. Conserved structural domains of plant P450s (Werck-Reichhart *et al.*, 2002).

The structural conservation between P450s allowed Gotoh (1992) to assign substrate recognition sites (SRSs) in eukaryotic CYP2 from bacterial P450s through comparative analyses of amino acid and coding nucleotide sequences. Based on

molecular modeling, Rupasinghe *et al*. (2003) compared four divergent P450s in the Arabidopsis phenylpropanoid pathway. They found that the substrate docking mechanism appears to be common, indicating consensus P450 SRSs.

1.4.3 Flavonoid 3'-Hydroxylase

Flavonoid 3'-hydroxylase (F3'H), belonging to the family CYP75B, is a key enzyme in directing the flux of anthocyanin intermediates toward the formation of red/magenta-coloured cyanidins and their derivatives. F3'H converts DHK to DHQ through the addition of a single hydroxyl group at the 3' position of the B ring.

The isolation of a F3'H clone was first reported in petunia by Brugliera *et al*. (1999). Since then, gene(s) encoding F3'H have been identified in many species, including Arabidopsis (Schoenbohm *et al*., 2000), oilseed rape (Xu *et al*., 2007), grapevine (Jeong *et al*., 2006), and apple (Han *et al*., 2009). The genomic structure of F3'H determined in grapevine and apple revealed a composition of three exons and two introns; however, an additional intron is known to exist in Arabidopsis and oilseed rape. In apple, a putative open reading frame (ORF) of 1536 bp encodes a protein of 511 amino acids.

Expression analyses of F3'H have been carried out primarily in grapevine due to its contribution to red wine colour and flavour (specifically, astringency due to tannins). F3'H mRNA was detected in various grape organs such as flower, root, stem, tendril, leaf, seed, and skin (Jeong *et al*., 2006). The expression of F3'H was monitored throughout grape berry development. In the skin, Bogs *et al*. (2006) found that F3'H transcript levels decreased prior to the onset of ripening, known as 'veraison' by

viticulturists, but increased steadily over the course of ripening until at least 8 weeks after veraison. This differential up-regulation was also observed at the protein level, quantitated through iTRAQ MS/MS implementation (Lucker *et al*., 2009).

1.4.4 Flavonoid 3',5'-Hydroxylase

Flavonoid 3',5'-hydroxylase (F3'5'H), a member of the CYP75A family, catalyzes sequential or simultaneous hydroxylation reactions at the 3' and 5' positions of the B ring of the flavylium cation. F3'5'H can utilize both DHK and DHQ as substrates to form DHM. The production of DHM leads to the accumulation of blue/purple-coloured delphinidin-based anthocyanins.

While cyanidin-derived anthocyanins are the most common anthocyanins in nature, some plant species such as Arabidopsis, rose (*Rosa* spp.), carnation (*Dianthus caryophyllus*), and chrysanthemum (*Chrysanthemum* spp.), lack delphinidins and their derivatives (Sheahan *et al*., 1998; de Vetten *et al*., 1999). It is presumed that these species lack F3'5'H activity.

Two F3'5'H cDNAs, Hf1 and Hf2, were first isolated from petunia by Holton *et al*. (1993). Petunia Hf1 and Hf2 share 75% and 86% identity with grapevine F3'5'H (VvF3'5'H). VvF3'5'H contains an ORF of 1527bp, which encodes a protein of 508 amino acid residues (Bogs *et al*., 2006). The genomic sequence of VvF3'5'H contains two exons and one 403bp intron (Jeong *et al*., 2006). Several copies of VvF3'5'H were found to tightly cluster in the grape genome (Castellarin *et al*., 2006).

In addition to the association of a P450 reductase, it has been reported *in vivo* that a cytochrome b_5, difF, may be required for the full activity of petunia F3'5'H enzyme by

acting as an alternative electron donor (de Vetten *et al*., 1999). Interestingly, F3'H activity was not substantially reduced in *difF* mutants. A functional interaction between cytochrome b_5 and F3'5'H has yet to be shown in other plant species.

F3'5'H transcripts are detected in any tissue in the grapevine that accumulates flavonoids, particularly, in the leaf and the skin (Jeong *et al*., 2006). In the grape berry, the expression of F3'5'H decreased until veraison was reached. After veraison, F3'5'H expression increased rapidly, concomitant with the biosynthesis of delphinidin-based anthocyanins (Bogs et al., 2006). Similarly, expression data at the protein level demonstrated up-regulation at veraison (Lucker *et al*., 2009).

1.5 Rosaceae Genome Sequencing

1.5.1 Preface

Historically, traditional breeding practices have dominated the process by which rosaceous crops are selected for desirable quality traits, such as high nutritional values, aesthetics, pest and disease resistance, and machine harvestable cultivars; however, recent advances in the development of molecular tools have made possible the various Rosaceae genomics initiatives. Although the raspberry genome is not yet sequenced, there is interest currently in building an international consortium to fund this. For now, several other Rosaceae species draft genomes are available.

1.5.2 Apple

Apple (*Malus x domestica* Borkh.) is a diploid (2*n*=34) pome fruit with a genome size of 750 Mb per haploid (Tatum *et al*., 2005). The genetic map of the apple genome

contains 17 linkage groups with over 800 molecular markers (Liebhard *et al*., 2003). Currently, 260,581 apple ESTs have been deposited in Genbank accounting for 23,284 contigs and 53,200 singlets (Genome Databse for Rosaceae, http://www.rosaceae.org/). A BAC-based physical map of the apple genome has been constructed, which represents the first draft genome sequence (Han *et al*., 2007). Recently, an international consortium led by Dr. Riccardo Velasco of the Instituto Agrario San Michele all'Adige Research and Innovation Centre in Trento, Italy, reported a high-quality draft genome sequence of the domesticated apple (*Malus* x *domestica*) (Velasco *et al*., 2010). They found that genome-wide duplication some 50 million years ago resulted in the expansion of haploid chromosome numbers to 17 (Velasco *et al*., 2010).

1.5.3 Peach

Peach (*Prunus persica*) is a model species for *Prunus* and is considered to be the best genetically characterized species in Rosaceae (Jung *et al*., 2008). The peach diploid genome (2*n*=16) (Jelenkovic and Harrington, 1972) is now believed to be ~220-230 Mb in size (Genome Database for Rosaceae). The peach genome sequencing project, initiated by Department of Energy's Joint Genome Institute, involved 8X whole genome shotgun Sanger sequencing of the double haploid cultivar 'Lovell' (Genome Database for Rosaceae). An initial draft of the assembled and annotated genome was made available on April 1, 2010 by the International Peach Genome Initiative (IPGI) (Genome Database for Rosaceae). A general *Prunus* physical and transcript map has been constructed from a peach cDNA library of developing fruit mesocarp (Horn *et al*., 2005). Currently, 89,166

peach ESTs are available from many sequencing projects around the world, which represent 24,307 putative unigenes (Genome Database for Rosaceae).

1.5.4 Strawberry

The octoploid cultivated strawberry has a complex phylogenetic ancestry; however, the diploid (2*n*=14) woodland strawberry (*Fragaria vesca*) has emerged as a probable ancestor (Folta and Davis, 2006). The small genome size of 206 Mb and a short generation time of 10-16 weeks have established *F. vesca* as a model plant for strawberry genome analysis (Shulaev et al., 2008). The Strawberry Genome Sequencing Consortium has generated an 8X full genome coverage by 454 sequencing technology. To date, 49,132 ESTs have been assembled in Genbank, 13,896 of which are putative unigenes, (Genome Database for Rosaceae) and approximately 1.75 Mb of genomic sequence derived from fosmid clones has been deposited (Davis *et al*., 2010).

1.6 Anthocyanin Biosynthesis in Plant Cell Cultures

1.6.1 Light

Photoinduction of anthocyanin biosynthesis has been demonstrated in many plant cell culture systems, including carrot (Takeda, 1990), grapevine (Zhang *et al*., 2002), strawberry (Sato *et al*., 1996), and sour cherry (Blando *et al*., 2005). This is further supported by the inhibition of anthocyanin biosynthesis in the dark (Takahashi *et al*., 1991; Dong *et al*., 1998). Of the total radiation spectrum, both the visible and ultraviolet (UV) regions are important stimuli to the induction mechanism (Chalker-Scott, 1999). The involvement of phytochrome was evident in the observed photoreversibility of

anthocyanin biosynthesis upon exposure to red and far-red light in apple fruit (Arakawa, 1988). While phytochrome may act as the primary photoreceptor for the photoinduction of anthocyanin biosynthesis, UVB photoreceptors will function in lieu in phytochrome-deficient mutants (Brandt *et al*., 1995) and following UVB irradiation (Ambasht and Agrawal, 1995). In addition, a UVA photoreceptor was found to mediate UVA-specific induction of anthocyanin accumulation (Zhou *et al*., 2007).

Expression analyses of anthocyanin biosynthetic genes in response to photoinduction revealed a coordinated up-regulation of PAL, CHS, CHI, F3H, DFR, ANS, and UFGT in parallel with anthocyanin accumulation (Hrazdina and Creasy, 1979; Alokam *et al*., 2002; Ahmed *et al*., 2009; Toguri *et al*., 1993; Lu and Yang, 2006; Hennayake *et al*., 2007). In Arabidopsis, anthocyanin regulatory genes of the MYB (*PAP1* and *PAP2*) and bHLH (*TT8*, *EGL3*, and *GL3*) families showed strong light induction, the expression of which preceded that of anthocyanin structural genes (Cominelli *et al*., 2008). In contrast, the expression of *TTG1* of the WD40 family was constitutive. Similar light-induced expression of MYB genes (*C1* and *pl*) was detected in maize (Procissi *et al*., 1997).

1.6.2 Abiotic Stress

While the role of anthocyanins in plant reproductive organs seems clear, their physiological significance in vegetative organs has begun to be elucidated through recent research. The ameliorating effects of anthocyanins in response to environmental stresses are critical to a plant's well-being and survival (Gould, 2004). Anthocyanins have

functional implications to stressors such as low temperature, high osmoticum, and nutrient deficiency.

Low temperature stress enhances the accumulation of anthocyanins in cabbage (Rabino and Mancinelli, 1986), maize (Christie *et al*., 1994), apple (Leng *et al*., 2000), and parsley (Hasegawa *et al*., 2001). Conversely, elevated temperatures reduce anthocyanin accumulation in grape (Mori *et al*., 2005). Transcriptional activation of anthocyanin biosynthetic genes is thought to mediate the increase in pigmentation in response to low temperature *in planta* (Christie *et al*., 1994; Hasegawa *et al*., 2001); similar findings were demonstrated in cell suspension cultures of *Perilla frutescens* (Zhong and Yoshida, 1993) and strawberry (Zhang *et al*., 1997).

Under natural conditions, high salinity, drought, and freezing are the major causes of osmotic stress to plants (Xiong and Zhu, 2002). Presumably, anthocyanins are produced to scavenge ROS brought about by oxidative stress (Khavari-Nejad *et al*., 2008) and to function as osmoregulators in preventing the loss of turgor (Chalker-Scott, 2002). Under cell culture conditions, osmotic stress can be induced by solutes, such as glucose (Tholakalabavi *et al*., 1994), sucrose (Solfanelli *et al*., 2006), mannitol (Do and Cormier, 1990), and salt (Khavari-Nejad *et al*., 2008). Increasing the osmotic potential of the medium, thereby placing the cells under water stress, resulted in the enhanced accumulation of anthocyanins (Deroles, 2008).

The macronutrients, nitrogen (N), phosphorus (P), and potassium (K), are essential to plant growth and development. Tuomi *et al*. (1984) argued that plant secondary metabolite production is inversely related to nutrient availability. As a result of nutrient stress, cell growth is arrested and the surplus of carbon is reallocated in part

towards anthocyanin biosynthesis (Deroles, 2008). Nitrogen deficiency has been shown to positively influence anthocyanin accumulation in carrot callus cultures (Rajendran et al., 1992) and grapevine cell suspension cultures (Do and Cormier, 1991). Similarly, phosphate starvation enhanced anthocyanin production in cell suspension cultures of strawberry (Sato *et al*., 1996) and grapevine (Dedaldechamp *et al*., 1995).

1.6.3 Phytohormones

The presence of exogenous phytohormones in cell cultures elicits variable responses that may be unique to individual plant species. The five major classes of such signalling molecules are abscisic acid (ABA), auxins, cytokinins, ethylene, and gibberellins. In cell cultures, phytohormone regimes determine the differentiation state and the aggregate size of the cell which, in turn, affect the induction of anthocyanin biosynthesis (Deroles, 2008). Narayan *et al*. (2005) found that the phase of the culture medium may influence the interplay between different growth regulators.

While auxins positively regulate cell enlargement and root initiation, low levels of auxins are known to stimulate anthocyanin production. The effect of 2,4-D (2,4-dichlorophenoxyacetic acid), a biologically active synthetic auxin, in cell cultures has been extensively examined. Pasqua *et al*. (2005) found that the maximal anthocyanin output was achieved when using low concentrations of 2,4-D. This is consistent with earlier studies in carrot (Ozeki and Komamine, 1986), *Catharanthus roseus* (Hall and Yeoman, 1986), and *Oxalis reclinata* (Makunga *et al*., 1997) cell cultures, in which the removal of 2,4-D restored anthocyanin biosynthesis. Other commonly used auxins in cell

culture experiments include NAA (naphthaleneacetic acid), IBA (indole-3-butyric acid), and IAA (indoleacetic acid).

Cytokinins are important in regulating cell division and shoot formation. In addition, cytokinins are capable of inducing anthocyanin accumulation in whole plant systems of Arabidopsis (Deikman and Hammer, 1995), maize (Piazza et al., 2002), and olive (Shulman and Lavee, 1973); however, low levels of cytokinin together with auxin were found to decrease cell aggregate size resulting in a parallel increase in anthocyanin yield in carrot cell cultures (Kinnersley and Dougall, 1980). Generally, kinetin, BAP (6-benzylaminopurine), and 2iP (6-(γ,γ-dimethylallylamino) purine) are considered highly active cytokinins in plant tissue culture practices.

Initially thought to specifically play a role in leaf abscission, ABA is now known to be a key regulator in the ripening of non-climacteric fruits (Zhang *et al*., 2009). Fruit ripening corresponds to a series of biochemical and physiological changes that are manifested through colour, texture, and flavour (Lelievre *et al*., 1997). ABA has been shown to promote the accumulation of anthocyanins in grapevine berry (Pirie and Mullins, 1976; Ban *et al*., 1998) and strawberry fruit (Jiang *et al*., 2003). However, this inductive effect of ABA was not limited to fruit organs. Similar enhancement of anthocyanin production was observed in maize (Hattori *et al*., 1992) and rice (Hung *et al*., 2008). Fambrini *et al*. (1993) further suggested that ABA deficiency in sunflower prevented anthocyanin biosynthesis; however, others have presented evidence for inhibitory effects of ABA on anthocyanin biosynthesis in radish (*Raphanus sativus*) seedlings and petunia flowers (Guruprasad and Laloraya, 1980; Weiss *et al*., 1995).

1.6.4 Elicitors

Formerly, elicitors were defined as substances which induce phytoalexin production (Darvill and Albersheim, 1984). Currently, elicitors are defined as signalling molecules of abiotic or biotic origin which induce a defence response that stimulates secondary metabolism. Elicitation has been widely used in plant cell cultures to enhance the production of anthocyanins (Rajendran *et al*., 1994; Mori and Sakurai, 1996; Ramachandra Rao *et al*., 1996; Curtin *et al*., 2003; Wang *et al*., 2004).

Some examples of elicitors include cell extracts of fungi, bacteria, or yeast, jasmonic acid (JA) or methyl jasmonate (MeJA), chitosan, and the inorganic ions, Ca, Mn, Zn, Co, Fe, and V (Zhang and Furusaki, 1999). Among the elicitors used, the effects of JA and MeJA have been well-documented. Gundlach *et al*. (1992) deduced the role of JA and its derivatives as signal transducers in the intracellular signal cascade that initiates *de novo* transcription of genes, such as PAL. The elicitation of anthocyanin accumulation with the exogenous application of JA or MeJA in plant cell cultures has been demonstrated in Ohelo (Fang *et al*., 1999), grapevine (Zhang *et al*., 2002), and sour cherry (Blando *et al*., 2005).

1.7 Metabolic Engineering of Anthocyanin Biosynthesis in Plants

1.7.1 Flowers

In nature, flower colour serves as an attractant for pollinators and agents for seed dispersal. In society, flower colour is paramount to the floriculture industry in providing a genetic playground for novelty, given the relatively limited spectrum of anthocyanins accumulated in each plant species. For example, roses, carnations, and chrysanthemums

lack violet to blue flower varieties because they do not accumulate delphinidin-based anthocyanins. In contrast, petunias and cymbidiums lack orange to brick-red flower varieties due to the absence of pelargonidin-based anthocyanins.

Recent advances in plant biotechnology have enabled the manipulation of structural genes within the anthocyanin biosynthetic pathway for the purpose of engineering novel flower colours. In rose, Katsumoto *et al*. (2007) successfully generated blue-hued flowers accumulating delphinidins by introducing the viola F3'5'H gene and replacing the endogenous DFR gene with the iris DFR gene. In carnation, Brugliera *et al*. (2000) co-expressed a petunia cytochrome b_5 gene with a petunia F3'5'H gene to enhance the activity of F3'5'H, which resulted in the efficient production of delphinidin-based anthocyanins. In chrysanthemum, sense and antisense CHS constructs were introduced to produce white-flowering transgenic plants. Courtney-Gutterson *et al*. (1994) attributed the white-flowering trait to an accumulation of CHS precursors and a reduction in CHS expression.

1.7.2 Fruits

While metabolic engineering of anthocyanin biosynthesis has been carried out predominantly in flowers for the development of new commercial varieties, a growing interest exists in the enrichment of fruits and vegetables with health-promoting phytonutrients.

In tomato, the expression of two transcription factors, *Del* (bHLH) and *Ros1* (MYB), from snapdragon up-regulated the genes required for anthocyanin biosynthesis (Butelli *et al*., 2008). The transgenic tomato line accumulated substantial levels of

anthocyanins equivalent to those found in blackberries. The total antioxidant capacity was measured to be threefold higher in transgenic tomato fruit than that in the wildtype. This translated into a prolonged lifespan in cancer-susceptible mice when fed with a diet supplemented with freeze-dried transgenic tomatoes.

Szankowski *et al*. (2007) introduced the maize *Lc* (bHLH) transcription factor gene into apple, which led to increased expression of most anthocyanin structural genes, especially ANS, and subsequent phenotypic colour change. In strawberry (*Fragaria* x *ananassa*), an antisense CHS gene construct down-regulated CHS mRNA accumulation (Lunkenbein *et al*., 2006). As a result, precursors of the flavonoid biosynthetic pathway were shunted toward the formation of cinnamoyl glucose, caffeoyl glucose, and feruloyl glucose.

1.7.3 Genetic Transformation in Raspberry

Genetic transformation of plants began in the early 1980s (Vasil, 2008). Considerable progress has been made in the development of related methods. While there has been a lack of emphasis on fruit modification, several genetic transformation experiments have been reported in raspberry. Graham *et al*. (1990) used GUS as a selectable marker for *Agrobacterium*-mediated transformation of raspberry leaf discs and intermodal stem segments. Mathews *et al*. (1995) successfully introduced the gene encoding *S*-adenosylmethionine hydrolase into three raspberry cultivars, Meeker, Chilliwack, and Canby, via *Agrobacterium*-mediated gene transfer. To engineer resistance to *Raspberry Bushy Dwarf Virus* (RBDV), a pollen-transmitted virus that causes crumbly fruit, Martin and Mathews (2001) introduced mutant and wildtype coat

protein and movement protein genes into a RBDV susceptible cultivar. They found that approximately one third of transformants when grafted with RBDV infected material demonstrated resistance.

2 COMPARATIVE GENOMICS: THE WHY

2.1 Introduction

The structural diversity of anthocyanidins is primarily determined by the individual activity of flavonoid 3'-hydroxylase (F3'H) and flavonoid 3',5'-hydroxylase (F3'5'H) through single or dual hydroxylation reactions, respectively, of the B ring of the flavylium ion. It has been said that progressive hydroxylation in the B ring has a blueing effect on colour manifested by the anthocyanins. Interestingly, raspberry and other Rosaceae family members such as apple (*Malus* spp.) and rose (*Rosa* spp.) preferentially accumulate 3'-hydroxylated dihydroquercetin-derived (DHQ) anthocyanins, such as cyanidins and peonidins, while lacking the 3',5'-dihydroxylated dihydromyricetin (DHM) types, such as delphinidins, petunidins, and malvidins. Unlike raspberry, grapevine berries (*Vitis vinifera* L.) predominantly accumulate DHM-derived anthocyanins. To date, the majority of studies undertaken in raspberry involves characterizing the bioavailability and antioxidant activities of anthocyanin-rich extracts in animal models. However, the molecular basis for the absence of DHM-derived anthocyanins in raspberry remains largely unknown.

Structural enzymes of the anthocyanin biosynthetic pathway are divided into two groups designated as "early" and "late" (Pelletier *et al*., 1997; Ahmed *et al*., 2009). These enzymes are shared components within the pathway that funnel a range of flavonoid substrates towards anthocyanin formation. F3'H and F3'5'H function at the branch point that, in most cases, separate the early and late structural genes and give rise to anthocyanin diversity. Here, this peculiarity is investigated by comparative genomics of

the F3’5’H gene given that the activity of F3’5’H is required for the formation of all 3’,5’-hydroxylated flavonoids and, more specifically, anthocyanins. I hypothesize that a functional F3’5’H enzyme is lacking in raspberry as a result of aberrations in one or more of the following: gene presence in genome, genomic sequence, and/or gene expression. The experimental approaches taken here include validating DNA template quality and PCR-based strategy for gene cloning in raspberry, testing for the presence of F3’5’H gene(s) and transcript(s) in raspberry, and mining other Rosaceae genomes for the presence for F3’5’H gene(s).

2.2 Materials and Methods

2.2.1 Plant Material

Seedlings from *Rubus idaeus* cv. Chemainus were generously donated by Chaim Kempler, lead scientist in small fruit breeding at the Pacific Agri-Food Research Centre (PARC), Agassiz, BC. Potted seedlings were grown in the greenhouse at UBC. Young leaves were sampled and used immediately for genomic DNA isolation.

A developmental series of fruits from each of *Rubus idaeus* cvs. Meeker and Chemainus was collected from a PARC research field in Abbotsford, BC, in summer 2008. Tissues were snap-frozen and transported in an ethanol-dry ice bath to UBC and stored at -80°C until use. The berry stages were named based on pigmentation and size phenotypes as follows: fruit set (FS), green hard (GH), green pink just turning (GPJT), medium reddish (MR), large reddish (LR), half green half pink (HGHP), pink (P), red medium soft (RMS), and red harvest (RH) (Figure 2-1).

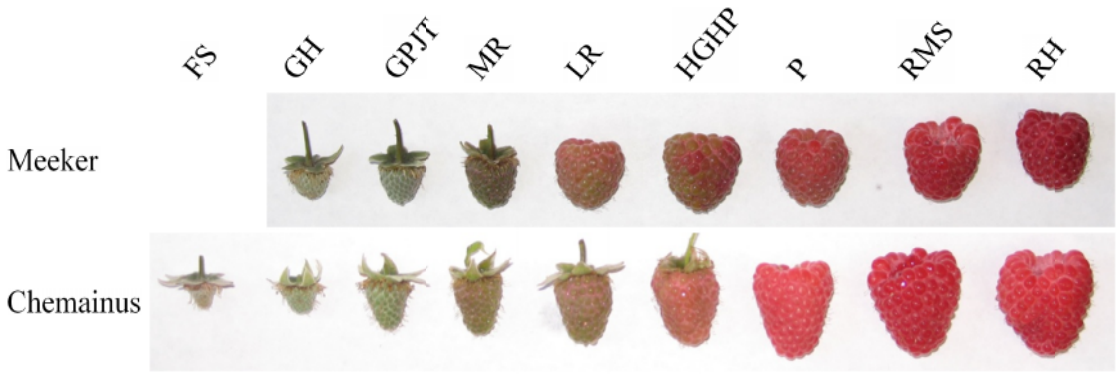


Figure 2-1. Developmental series of *R. idaeus* cvs. Meeker and Chemainus. Berry stages are abbreviated as follows: FS, fruit set; GH, green hard; GPJT, green pink just turning; MR, medium red; HGHP, half green half pink; P, pink; RMS, red medium soft; RH, red harvest.

Grapevine canes (dormant shoots) were collected in winter 2008 from a commercial (Vincor International) vineyard at Osoyoos, BC. Young leaves and mature berries from *Vitis vinifera* L. cv. Pinot Noir were harvested from potted canes grown in the greenhouse at UBC. Leaves were used immediately upon sampling, while berries were promptly stored at -80°C until use.

2.2.2 Total Anthocyanin Extraction

Our in-house protocol for total anthocyanin extraction from raspberries was developed by Tatiana Pirogovskaia which she adapted from Mullen *et al*. (2002). RH berries from *R. idaeus* cv. Chemainus and *V. vinifera* L. cv. Pinot Noir were collected for crude anthocyanin compositional analyses. Approximately 4 mL of extraction buffer (methanol plus 0.1% (v/v) hydrochloric acid) was added to 1 g of whole berries. Samples were homogenized in extraction buffer using a handheld homogenizer and centrifuged at 3500 x *g* for 15 min. A 1 mL aliquot of the supernatant was transferred to an amber vial for HPLC analysis.

2.2.3 HPLC-MS Apparatus and Conditions

HPLC-MS analyses were performed on an Agilent 1100 HPLC system equipped with a diode array detector (DAD) and a HP ChemStation software and coupled with an Agilent 1100 Series LC/MSD Trap XCT Plus mass spectrometer. HPLC separation was carried out at a column temperature of 60°C. The mobile phases were acetonitrile with 2% (v/v) formic acid (solvent B) and water with 2% (v/v) formic acid (solvent A) at a flow rate of 1.2 mL/min according to the gradient program listed in Table 2-1. Detection was carried out between 190-600 nm. Anthocyanins were detected at 520 nm. Electrospray ionization (ESI) was performed in positive ion mode and data were collected over a mass range of *m/z* 100-800.

Table 2-1. HPLC gradient program.

Time (min)	Solvent B (%)	Flow Rate (mL/min)	Pressure (bar)
0.70	5.0	1.200	400
6.00	16.0	1.200	400
6.10	90.0	1.200	400
7.00	90.0	1.200	400
8.00	5.0	1.200	400
9.00	5.0	1.200	400

2.2.4 Total RNA Extraction

Total RNA was isolated from a developmental series of fruits from *R. idaeus* cv. Meeker according to a quick and simple method developed by Jones *et al*. (1997). Berry exocarp excised longitudinally with a razor blade on dry ice was used for all stages to avoid seed contamination.

RNA samples were treated with recombinant DNase I (Roche Applied Science, Laval, Quebec) according to the manufacturer's instructions, they were then purified

using the RNeasy Mini Kit (Qiagen, Mississauga, Ontario) according to the manufacturers' instructions and eluted in RNase-free water. Final RNA quality and quantity were determined by gel electrophoresis (Figure 2-2) and spectrophotometry. RNA samples were stored at -80°C.

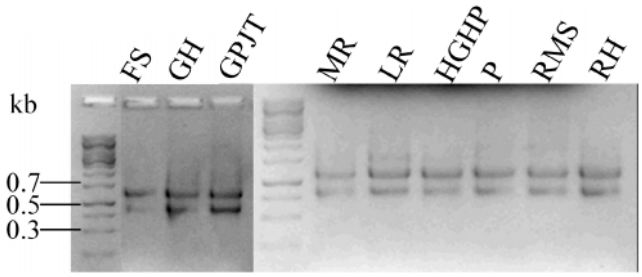


Figure 2-2. Agarose gel electrophoresis of total RNA isolated from a developmental series of raspberry cv. Meeker. Berry stages are abbreviated as follows: FS, fruit set; GH, green hard; GPJT, green pinkish just turning; MR, medium reddish; LR, large reddish; HGHP, half green half pink; P, pink; RMS, red medium soft; RH, red harvest.

2.2.5 First-Strand cDNA Synthesis

Total RNA was converted to first-strand cDNA using the SuperScript VILO cDNA Synthesis Kit (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions.

2.2.6 Genomic DNA Extraction

The following protocol for genomic DNA isolation was modified by Dr. Joost Lücker from an in-house CTAB protocol. Mortar and pestle were washed with 100% ethanol and pre-chilled at -80°C. Young leaves from *R. idaeus* cv. Chemainus and *V. vinifera* L. cv. Pinot Noir were sampled for extraction. 1 g of leaves was ground to a fine powder in liquid nitrogen with a mortar and pestle. 20 mL of extraction buffer (2% CTAB (w/v), 100 mM Tris-HCl pH 8, 20 mM EDTA, 1.4 mM NaCl; 4 µL of 2-

mercaptoethanol was added to each 1 mL of extraction buffer) was added to the homogenized sample, collected in a sterile 50 mL Falcon tube, and mixed by inverting for several minutes. The extraction mixture was incubated in a 65°C water bath for 1 hr.

An equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added and mixed well by inverting. Sample was centrifuged at 3500 rpm for 20 min. The upper aqueous phase was collected and transferred to a new sterile 50 mL falcon tube. This extraction was repeated until the dissolution of phase boundary between aqueous and organic phases has been reached.

To precipitate DNA, 0.625 volume of isopropanol was added to the aqueous phase, incubated at -80°C for 15 min, and centrifuged at 3500 rpm for 30 min. Subsequently, the supernatant was discarded and the pellet was washed with 70% ethanol, centrifuged briefly, and air dried in a 37°C incubator. Extracted DNA was resuspended in 10 mM Tris-HCl (pH 8.5) and assessed for quality and quantity via gel electrophoresis and spectrophotometry, respectively.

Resuspended DNA was treated with 1 µL RNase A per 100 µL DNA and incubated at 37°C for 1 hr, then 2 µL proteinase K was added and incubated at 37°C for 30 min. Treated DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v), inverted to mix, and centrifuged at 15000 rpm for 10 min. The upper aqueous phase was collected and extracted repeatedly with an equal volume of chloroform:isoamyl alcohol (24:1, v/v) by inverting to mix and centrifuging at 15000 rpm for 5 min until the interphase disappeared.

Lastly, DNA was precipitated with 2.5 volume of 100% ethanol and 0.1 volume of 3 M sodium acetate, incubated at -80°C for 1 hr, and centrifuged at 15000 rpm for 20

min. DNA was then washed with 70% ethanol, centrifuged briefly, and air dried in 37°C incubator. Isolated genomic DNA was resuspended in 10 mM Tris-HCl (pH 8.5) and assessed for quality and quantity via gel electrophoresis (Figure 2-3) and spectrophotometry.

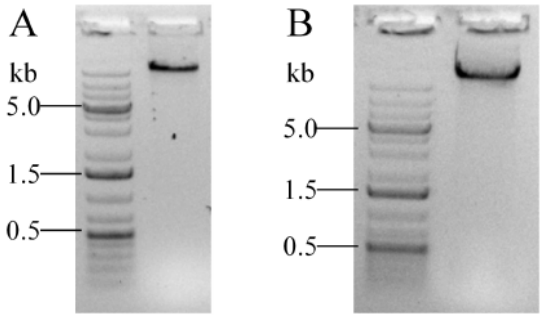


Figure 2-3. Agarose gel electrophoresis of genomic DNA isolated from (A) raspberry cv. Chemainus and (B) grapevine cv. Pinot Noir.

2.2.7 Degenerate PCR

2.2.7.1 Oligonucleotides

Oligonucleotide primers (Table 2-2) were designed to sense and antisense strands of F3H, F3'H, and F3'5'H DNA sequences and were commercially synthesized by Integrated DNA Technologies (Toronto, Ontario) or Eurofins MWG Operon (Huntsville, Alabama).

Table 2-2. Oligonucleotides used in degenerate PCR amplifications.

Gene ID	Primer ID	Primer Sequence 5' to 3'
RiF3H	DegRiF3H_Outer_158F	TGGCCTACAACGAGTTCTCCRAYGANRTNCC
	DegRiF3H_Outer_158R	GGCATCTTCCAGGTGGTGRAYCAYGGNGT
	DegRiF3H_Nested_159F	GTTGGAGTTCACCACGGCYTGRTGRTC
	DegRiF3H_Nested_159R	CTGGCCAGCTCCAGGTCYTTNSHCAT
RiF3'H	F3'H_Degenerate_ACCTGC_72F	ACCTGCGGATGGGCTTYGTNGAYG T
	F3'H_Degenerate_GGTCAG_72R	GGTCAGGCCGTAGGCCTCNTCCATRTT
	F3'H_Degenerate_CCAAGC_73F	CCAAGCACATGGCCTACAAYTAYCARGA
	F3'H_Degenerate_GCAGTC_73R	GCAGTCCATCAGCTCCCARTYRAA
RiF3'5'H	RiF35H_Degenerate_Outer_110F	TGCTGGGCAACATGCCNCAYGT
	RiF35H_Degenerate_Outer_110R	GCGAGGCCGAAGGTCTCNTCCATRTTNA
	RiF35H_Degenerate_Inner_111F	CCTGAAGACCCAGGACATGAAYTTYWSNAA
	RiF35H_Degenerate_Inner_111R	GAAGTGGTTGCCCCGGKGNTCDATYTT
	RiF35H_DegenerateBlkE_120F	GCTGACCTACGCCATGGCNAAYATGHT
	RiF35H_DegenerateBlkJ_120R	GGGTCCCGGCCGATNGCCCADAT
	DegRiF3'5'H_SRS1_167F	TGGAAGTTRCTDAGRAAAYTAAGCAAC
	DegRiF3'5'H_SRS5_167R	GGAAGRTTBAGTGGHGTDGAWGGGT

2.2.7.2 PCR Conditions

Degenerate primers were designed using the Consensus-Degenerate Hybrid Oligonucleotide Primer (CODEHOP) program from conserved blocks of amino acids within multiply-aligned protein sequences (Rose *et al*., 2003). Degenerate PCRs were carried out with Phusion High-Fidelity DNA Polymerase (Finnzymes, Woburn, Massachusetts). The reaction was set up according to the manufacturer's instructions. PCR conditions were as follows: 1 cycle of 98°C for 1 min, followed by 35 cycles of 98°C for 10 sec, T_A (Table 2-3) for 30 sec, and 72°C for 15-30 sec/kb, and a final extension cycle of 72°C for 5 min.

Table 2-3. Annealing temperatures used in degenerate PCR amplifications.

Gene ID	Primer ID	T_A (°C)
RiF3H	DegRiF3H_Outer_158F	60-55
	DegRiF3H_Outer_158R	60-55
	DegRiF3H_Nested_159F	60-55
	DegRiF3H_Nested_159R	60-55
RiF3'H	F3'H_Degenerate_ACCTGC_72F	3x 35, 3x 45, 25x 55
	F3'H_Degenerate_GGTCAG_72R	3x 35, 3x 45, 25x 55
	F3'H_Degenerate_CCAAGC_73F	3x 35, 3x 45, 25x 55
	F3'H_Degenerate_GCAGTC_73R	3x 35, 3x 45, 25x 55
RiF3'5'H	RiF35H_Degenerate_Outer_110F	60-55, 58-53
	RiF35H_Degenerate_Outer_110R	60-55
	RiF35H_Degenerate_Inner_111F	60-55
	RiF35H_Degenerate_Inner_111R	60-55
	RiF35H_DegenerateBlkE_120F	60-55
	RiF35H_DegenerateBlkJ_120R	60-55, 58-53
	DegRiF3'5'H_SRS1_167F	55-50
	DegRiF3'5'H_SRS5_167R	55-50

2.2.8 Rapid Amplification of cDNA Ends (RACE)

2.2.8.1 Oligonucleotides

Oligonucleotide primers (Table 2-4) were designed to sense and antisense strands of F3H and F3'H DNA sequences and were commercially synthesized by Integrated DNA Technologies or Eurofins MWG Operon.

Table 2-4. Oligonucleotides used in RACE PCR amplifications.

Gene ID	Primer ID	Primer Sequence 5' to 3'
RiF3H	RiF3H_5'RACEOuter_160R	GACGCCATGATCAACAATCT
	RiF3H_5'RACEInner_161R	TCACAGGCCTCGACAATCTT
	RiF3H_3'RACEOuter_162F	TAGAGATGGTGGAAAGACGTG
	RiF3H_3'RACEInner_163F	ATTTTTTGAGCAACGGGAGA
RiF3'H	RiF3'H_5'RACE_356-378_146R	CTCTCCCACTACTCATCTTCTTC
	RiF3'H_5'RACE_206-225_147R	CCAACACCATCATCTCCACC
	RiF3'H_3'RACEOuter_80F	GTGCTGGGCGAAGAATATGT

2.2.8.2 PCR Conditions

RACE PCR was carried out using the FirstChoice RLM-RACE Kit (Applied Biosystems, Carlsbad, California) according to the manufacturer's instructions. PCR conditions were as follows: 1 cycle of 98°C for 1 min, followed by 35 cycles of 98°C for 10 sec, 60-55°C for 30 sec, and 72°C for 15-30 sec/kb, and a final extension cycle of 72°C for 5 min.

2.2.9 Isolation of PCR Products

If a single PCR product was amplified from a given reaction, DNA was purified using the GeneJET PCR Purification Kit (Fermentas, Burlington, Ontario) according to the manufacturer's instructions. If multiple PCR products were amplified from a given reaction, PCR products were run on a 1% TopVision Low Melting Point Agarose (Fermentas) and the desired band was excised. DNA was recovered by using the GeneJET Gel Extraction Kit (Fermentas) according to the manufacturer's instructions.

2.2.10 Cloning of PCR Products

2.2.10.1 Blunt-End Cloning

Blunt-end PCR products generated by proofreading DNA polymerases, such as Phusion High-Fidelity DNA Polymerase, were cloned into the pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (Fermentas) according to the manufacturer's instructions.

Cloned DNA was transformed into α-Select Chemically Competent Cells Gold Efficiency (Bioline, Taunton, Massachusetts) according to the manufacturer's suggested transformation procedure.

To quickly screen for the presence of DNA insert directly from *E. coli* colonies, the method of colony PCR was used. Individual colonies were picked with a pipette tip and resuspended in sterile Milli-Q water. A PCR master mix was prepared with *Taq* DNA Polymerase (Bioline) according to the manufacturer's instructions. 5 µL of bacterial water suspension was added to each 20 µL reaction. PCR conditions were as follows: 1 cycle of 96°C for 7 min, followed by 35 cycles of 96°C for 25 sec, 45°C for 30 sec, and 72°C for 15-30 sec/kb, and a final extension cycle of 72°C for 5 min. The presence of a PCR product was then analyzed on a 1% agarose gel.

2.2.10.2 Plasmid DNA Isolation

Select positive clones for DNA insert were individually inoculated in 4 mL LB containing 30 µg/ml kanamycin. Inoculated cultures were incubated at 37°C on a rotating shaker at 250 rpm overnight. Plasmid DNA was isolated from recombinant *E. coli* cultures using the GeneJET Plasmid Miniprep Kit (Fermentas) according to the manufacturer's instructions and stored at -20°C.

2.2.11 DNA Sequencing

Prior to submission to the sequencing facility, samples were prepared with 3 µL BigDye v3.1, 225 ng double-stranded plasmid DNA, 5 pmol primer, and sterile deionized water to a volume of 10 µL. Post-reaction clean-up, electrophoresis, and DNA

sequencing were carried out by the Nucleic Acid Protein Service (NAPS) unit at UBC using Applied Biosystems chemistries and equipment.

2.2.12 Sequencing Data Assembly and Alignment

Sequence data were processed and assembled into contigs using the DNASTAR Lasergene software suite (http://www.dnastar.com/default.aspx) and the CLC Main Workbench software (http://www.clcbio.com/). BLAST searches with raspberry genomic sequences were carried out against the NCBI database. By default, the threshold for the E-value on the BLAST web page is 10. Characteristic structural features of P450s were manually identified. Multiple sequence alignments were performed using GeneDoc (http://www.nrbsc.org/gfx/genedoc/) and the CLC Main Workbench software with default parameters.

2.2.13 Apple Genome Sequence Data Mining

A TBLASTN search of translated apple whole genome sequence data with grapevine F3’5’H (VvF3’5’H, ABH06585; E-value set to 1E-9) and petunia F3’5’H amino acid sequences (PhF3’5’H, P48418; E-value set to 1E-10) was kindly carried out by Dr. Paolo Fontana, Department of Genetics and Molecular Biology, Fondazione Edmund Mach-Istituto Agrario San Michele all’Adige (FEM-IASMA), Michele all’Adige, Italy.

2.2.14 Peach Genome Sequence Data Mining

A TBLASTN search of the initial draft of the peach genome (peach v1.0) made available by the International Peach Genome Initiative (IPGI) was carried out using grapevine and petunia F3'5'H amino acid sequences as queries (VvF3'5'H, ABH06585; PhF3'5'H, P48418; E-value set to 0.1).

2.3 Results

2.3.1 Rapid Characterization of Anthocyanin Composition in Raspberry and Grapevine Crude Extracts

In order to corroborate previous findings that raspberries do not accumulate dihydromyricetin-derived anthocyanins, compositional analyses were performed on total anthocyanin extracts from RH berries from raspberry cv. Chemainus and grapevine cv. Pinot Noir. These cultivars were selected based on usage for subsequent genomic analyses. Figures 3-5 and 3-6 show the chromatograms of the crude extracts for raspberry and grapevine, respectively. Tentative identification of peaks was determined based on their UV-visible absorption spectra and their mass spectra.

Four anthocyanin peaks were identified in raspberry fruit crude extracts: cyanidin-3-sophoroside (Cy-3-S), cyanidin-3-glucoside (Cy-3-G), cyanidin-3-rutinoside (Cy-3-R), and pelargonidin-3-glucoside (Pel-3-G) (Fig 2-4). Based on the relative peak areas, Cy-3-S was the most abundant anthocyanin compound in Chemainus berries. Although fewer anthocyanins were identified than reported in previous studies (Mullen *et al*., 2002; Beekwilder *et al*., 2005b; Borges *et al*., 2007; Kassim *et al*., 2009), these data strengthen the conclusion that raspberry fruits lack any 3',5'-dihydroxylated anthocyanins.

Five anthocyanin peaks were identified in grapevine berry crude extracts. They were delphinidin-3-glucoside (Del-3-G), cyanidin-3-glucoside (Cy-3-G), petunidin-3-glucoside (Pet-3-G), peonidin-3-glucoside (Peo-3-G), and malvidin-3-glucoside (Mal-3-G) (Fig 2-5). In accordance with previous reports (Mattivi *et al*., 2006), pelargonidins were not detected in grapevine berries. Additionaly, Mattivi *et al*. (2006) calculated the ratio of 3',5'-hydroxy to 3'-hydroxy anthocyanin derivatives for 64 red-skinned grapevine varieties and found that red-skinned grapevine varieties are particularly rich in 3',5'-dihydroxylated anthocyanins (delphinidin, petunidin, and malvidin).This is consistent with the findings presented here for Pinot Noir. Altogether, these compositional analyses re-affirm the differential anthocyanin accumulation between raspberry and grapevine berries pertaining to the pattern of B ring hydroxylations.

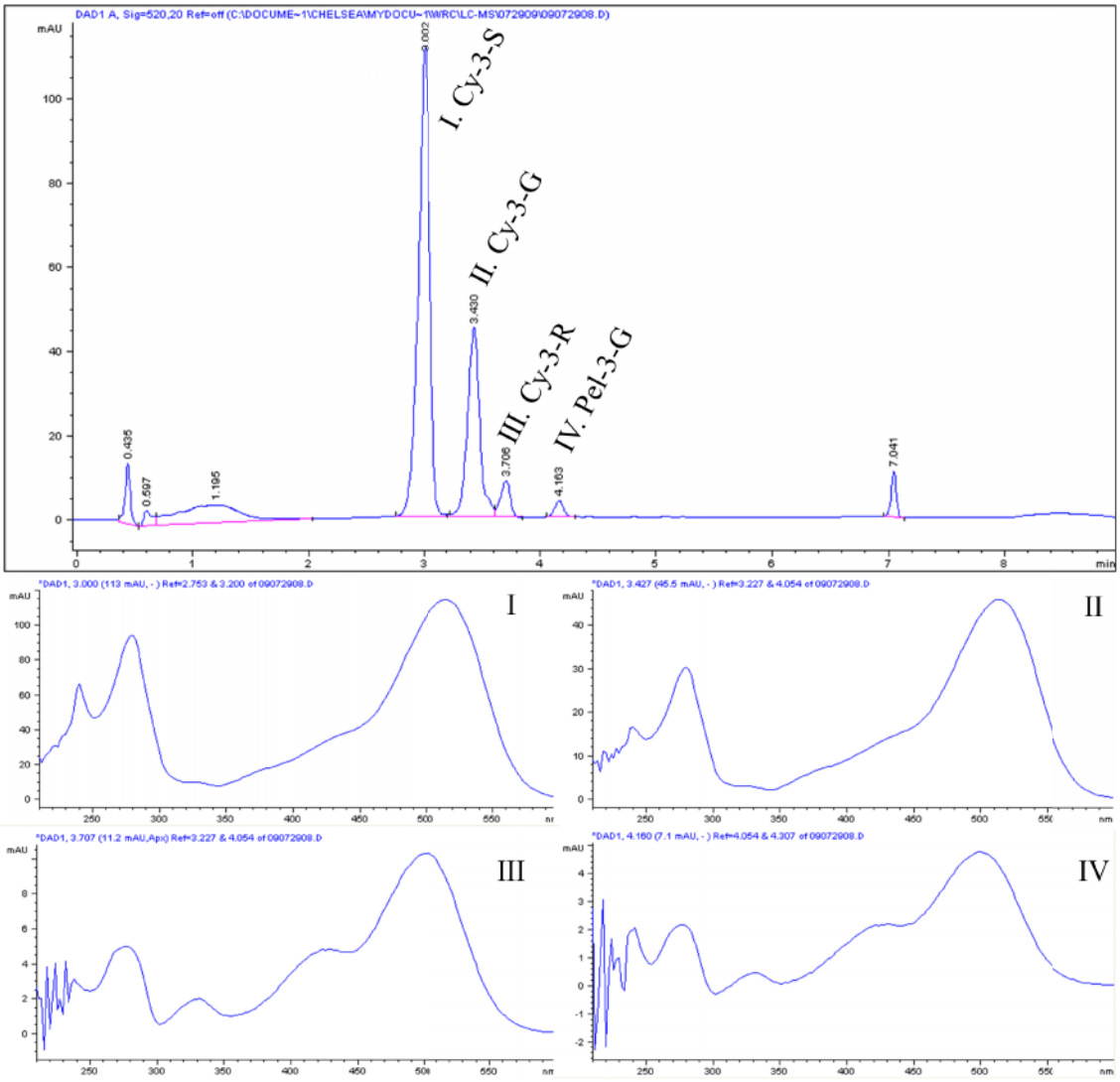


Figure 2-4. HPLC chromatogram at 520 nm of acidified methanol extract of red raspberry fruit, *Rubus idaeus* cv. Chemainus, and UV spectrum of each peak. Peak annotations are as follows: Cy-3-S, cyanidin-3-sophoroside; Cy-3-G, cyanidin-3-glucoside; Cy-3-R, cyanidin-3-rutinoside; Pel-3-G, pelargonidin-3-glucoside.

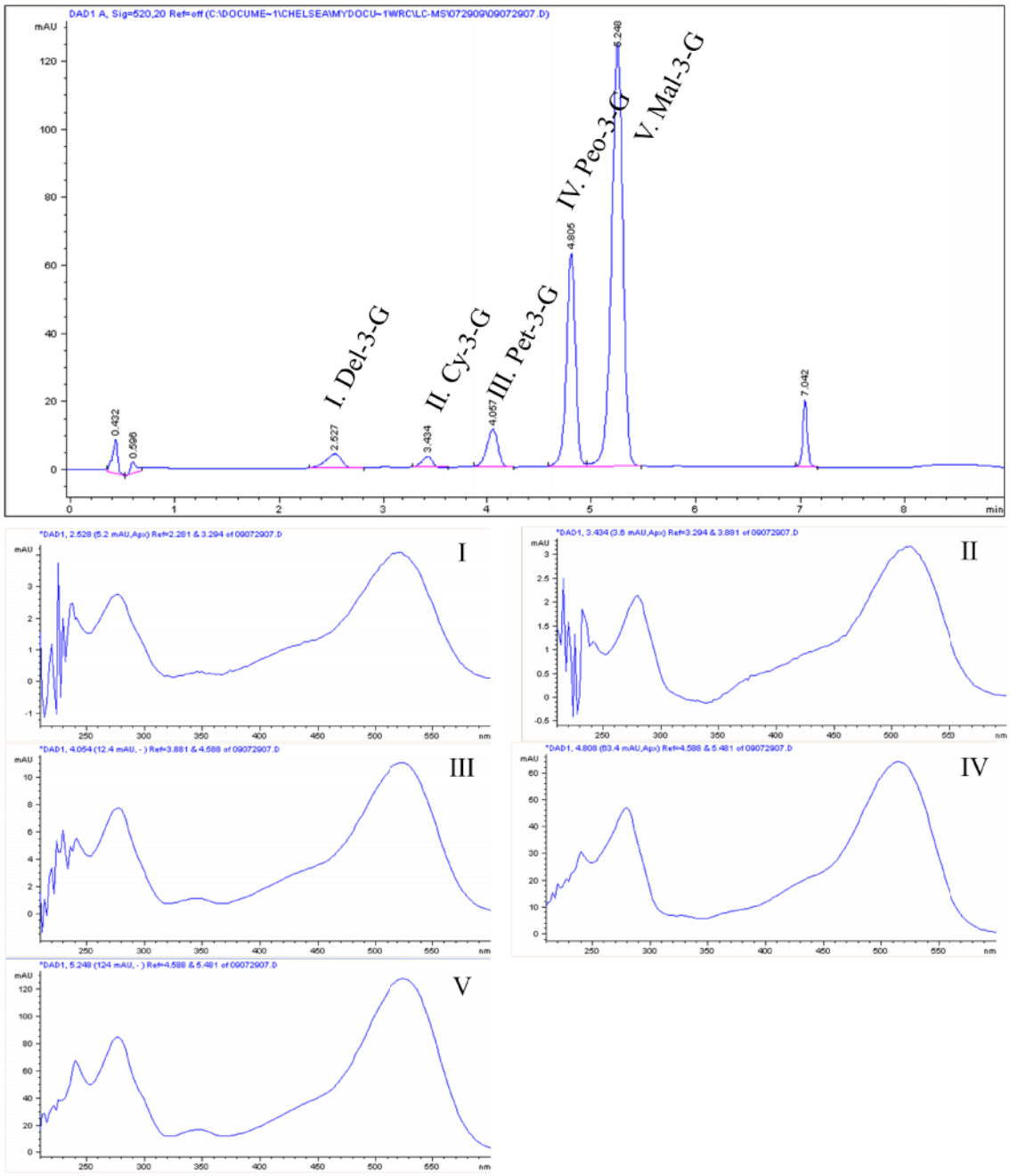


Figure 2-5. HPLC chromatogram at 520 nm of acidified methanol extract of grapevine berry, *Vitis vinifera* L. cv. Pinot Noir, and UV spectrum of each peak. Peak annotations are as follows: Del-3-G, delphinidin-3-glucoside; Cy-3-G, cyanidin-3-glucoside; Pet-3-G, petunidin-3-glucoside; Peo-3-G, peonidin-3-glucoside; Mal-3-G, malvidin-3-glucoside.

2.3.2 Quality Control for DNA Template

Following berry metabolite analyses, it was important to establish good quality and reproducibility of DNA templates via quality assurance measures. Although there was a very limited number of raspberry DNA sequences deposited in NCBI, Kumar and Ellis (2001) had reported that, in raspberry, phenylalanine ammonia lyase (PAL), a key enzyme in phenolic compound metabolism, is encoded by a family of two genes, RiPAL1 (AF237954) and RiPAL2 (AF237955). CLC Main Workbench software was used to obtain optimal primer pairs designed under standard PCR amplification conditions, which were used to monitor the quality of the DNA templates. It is worth noting that these primers do not span any introns. These exon primers were predicted to generate PCR products that are 522 bp and 550 bp for RiPAL1 and RiPAL2, respectively. PCR data (Fig. 2-6) showed that DNA fragments of the appropriate sizes were amplified from raspberry cDNA and genomic DNA. DNA fragments for the RiPAL2 gene were sequence verified. The additional, larger DNA fragment amplified from genomic DNA using the RiPAL1 primer pair was not further investigated, but could be the result of non-specific amplification, most likely of a pseudogene. These PCR results indicate good quality of DNA templates that can be used for downstream genomics analyses.

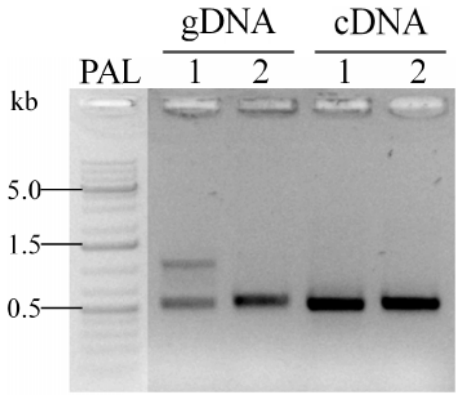


Figure 2-6. PCR analysis of two raspberry PAL genes for quality control of DNA template.

2.3.3 Quality Control for PCR-Based Cloning Strategy

To demonstrate that the cloning strategy employed here is effective for isolating partial or full-length gene fragments in raspberry, other structural genes of the anthocyanin biosynthetic pathway, including another P450, were isolated. As a proof of concept, a putative gene encoding flavonoid 3'-hydroxylase (F3'H) in raspberry (*Rubus idaeus* cv. Meeker) was isolated. Degenerate PCR primers were designed based on a multiple alignment of F3'H amino acid sequences obtained from NCBI. RACE was used to recover 5'- and 3'-end sequence data which were assembled using CLC Main Workbench software to obtain a full-length cDNA sequence. Putative RiF3'H cDNA contains an open reading frame of 1551 bp encoding a polypeptide of 516 amino acids. In addition, 26 bp of 5'-UTR and 139 bp of 3'-UTR were detected (Figure 2-7).

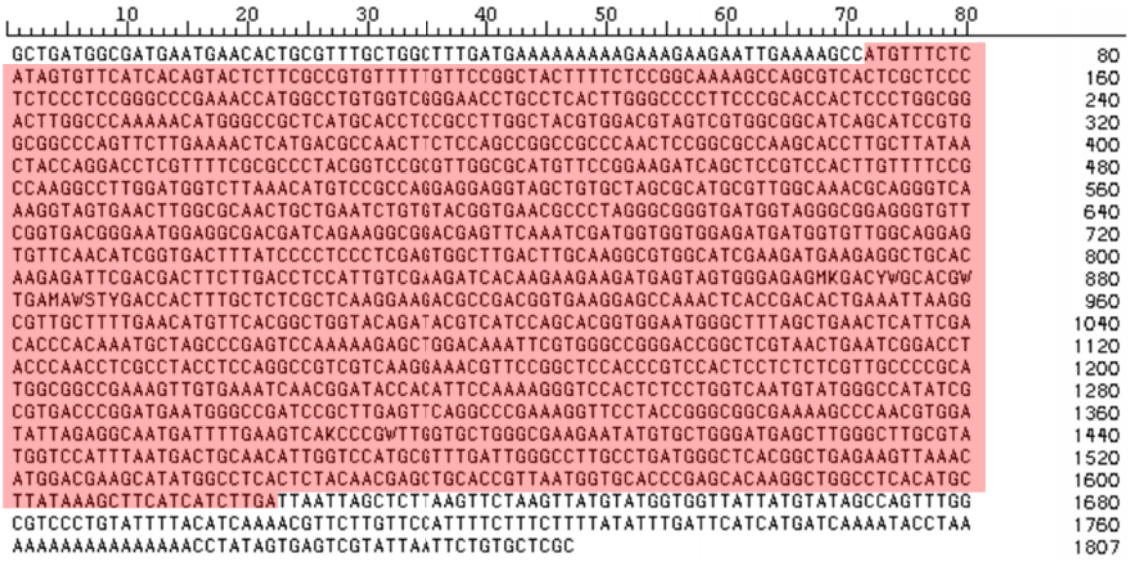


Figure 2-7. Nucleotide sequence of full-length putative RiF3'H including partial 5'- and 3'-UTR. ORF is highlighted in red.

Conserved domains characteristic of P450s were found in the deduced amino acid sequence of putative RiF3'H. Peptide signatures, GGEK and VVVAAS, that are thought to distinguish F3'H from F3'5'H (Brugliera *et al*., 1999; Boddu *et al*., 2004) were

identified in the predicted translation product of the cloned putative RiF3'H (Figure 2-8). A BLASTP search of the predicted RiF3'H amino acid sequence revealed 83% identity and 90% positives with MdF3'H I (*Malus* x *domestica*, ACR14867) and 75% identity and 86% positives with VvF3'H (*Vitis vinifera* L., CAI54278).

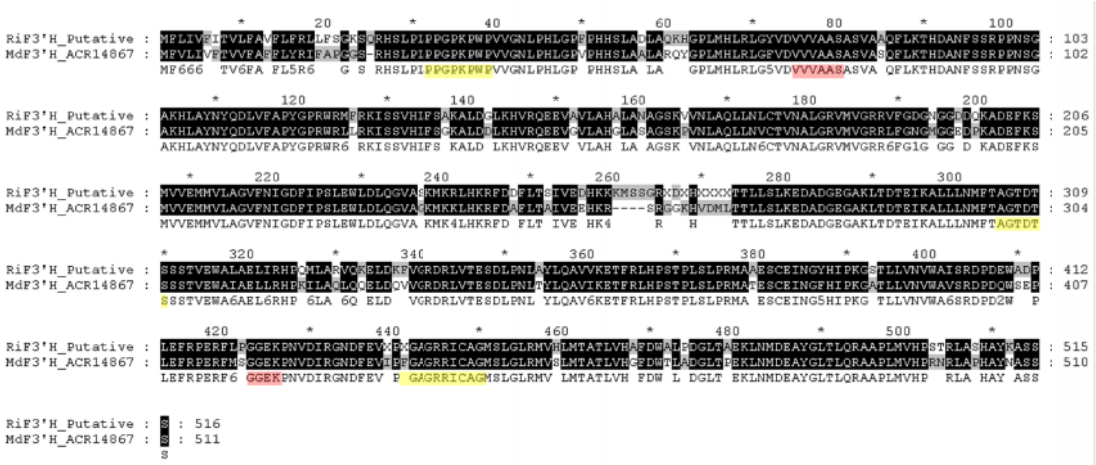


Figure 2-8. Alignment of putative RiF3'H and MdF3'HI (ACR14867) amino acid sequences. F3'H-specific peptide signatures (VVVAAS and GGEK) are highlighted in red. P450 conserved domains (PPGPxPxP, AGxDTS, and FxxGxRxCxG) are highlighted in yellow.

The nomenclature system for P450s is determined based on amino acid sequence identity (Chapple, 1998). As a rule-of-thumb, an arbitrary 40% amino acid sequence identity grants membership to a family and 55% dictates membership in a subfamily. If amino acid sequences are 97% identical or greater, they are assumed to be allelic variants (Chapple, 1998). Accordingly, the high amino acid sequence identity between putative RiF3'H and MdF3'H I (83% identity), putative RiF3'H and VvF3'H (75% identity), and MdF3'H I and VvF3'H (77% identity) indicates that they likely belong to the same P450 subfamily and are orthologous (i.e. functionally conserved). Moreover, putative RiF3'H and VvF3'5'H (ABH06585) share only 49% identity over 500 amino acid residues denoting affiliation with the same family but different subfamily.

Additionally, I isolated another putative gene in the anthocyanin biosynthetic pathway encoding flavanone 3-hydroxylase (F3H) in raspberry. As shown in Figures 2-9 and 3-10, I isolated a near full-length transcript with an open reading frame of 1007 bp, which encodes a predicted protein of 334 amino acids. A small fragment of the N-terminus was not recovered, as 5'RACE is generally a more difficult procedure than 3'RACE.

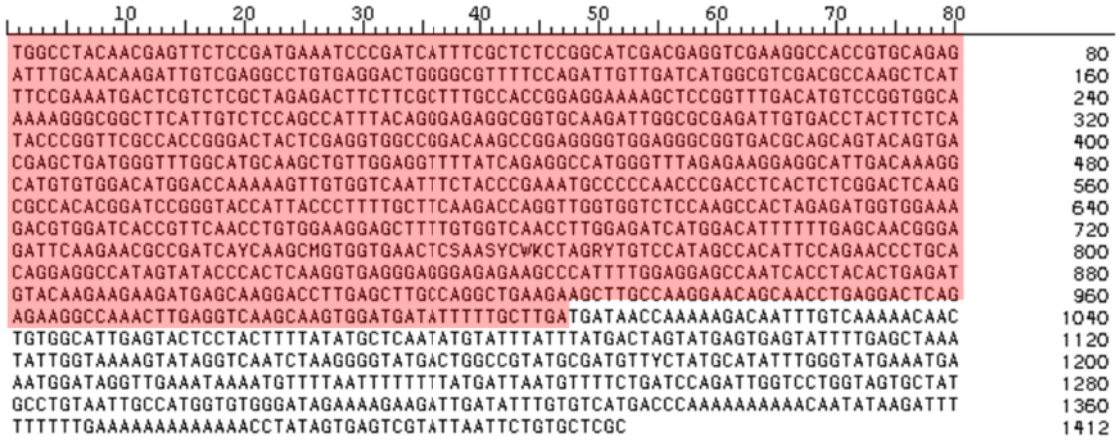


Figure 2-9. Nucleotide sequence of partial putative RiF3H and its 3'-UTR. Protein coding sequence (CDS) is highlighted in red.

An amino acid sequence alignment of the putative RiF3H with a published F3H sequence from Korean black raspberry (Baek *et al*., 2008) revealed a single amino acid substitution from arginine (R) to histidine (H) at position 54 based on three clones sequenced; however, I cannot be certain that this is not the result of a sequencing error, as this amino acid change was not observed in F3H sequences from *Fragaria* x *ananassa* (BAE17126), *Malus* x *domestica* (AAD26206), and *Pyrus communis* (AAX89400). The three unspecified/unknown amino acid residues (XXX) at positions 284-286 contains multiple ambiguous nucleotides that are likely due to poor sequence data with high background noise. F3H falls into the group of 2-oxoacid-dependent dioxygenases (2-ODD). 2-ODDs are soluble enzymes that require iron as a cofactor (Prescott and John,

1996). Conserved amino acid residues in plant 2-ODDs are found in RiF3H as marked in Figure 2-10. The successful isolation of F3'H and F3H transcripts in raspberry supported the use of similar cloning strategy towards F3'5'H gene detection.

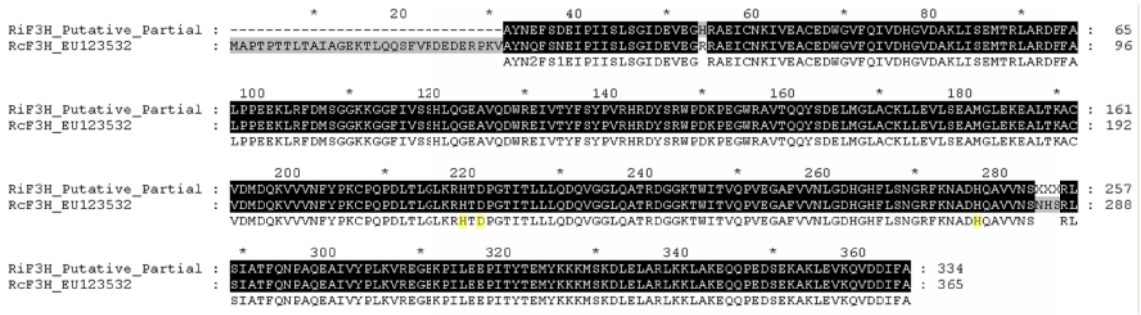


Figure 2-10. Alignment of putative RiF3H and RcF3H (EU123532) amino acid sequences. Conserved amino acid residues (H, 219; D, 221; H, 277) found in the 2OD-FeII-Oxy domain are highlighted.

2.3.4 Detecting F3'5'H Expression and Gene Presence in Raspberry

Here, substantiated by biochemical data and equipped with genomic resources, I aimed to detect F3'5'H expression and gene presence using previously validated PCR-based cloning strategy. A multiple alignment of seven F3'5'H amino acid sequences retrieved from GenBank (*Delphinium grandiflorum*, AAX51796; *Vitis vinifera* L., ABH06585; *Campanula medium*, O04773; *Eustoma grandiflorum*, O04790; *Solanum melongena*, P37120; *Petunia* x *hybrida*, P48419; *Gentiana triflora*, Q96581) was used to design three pairs of degenerate PCR primers (Figure 2-11). These primers were carefully chosen to hybridize to regions outside conserved P450 sequence motifs.

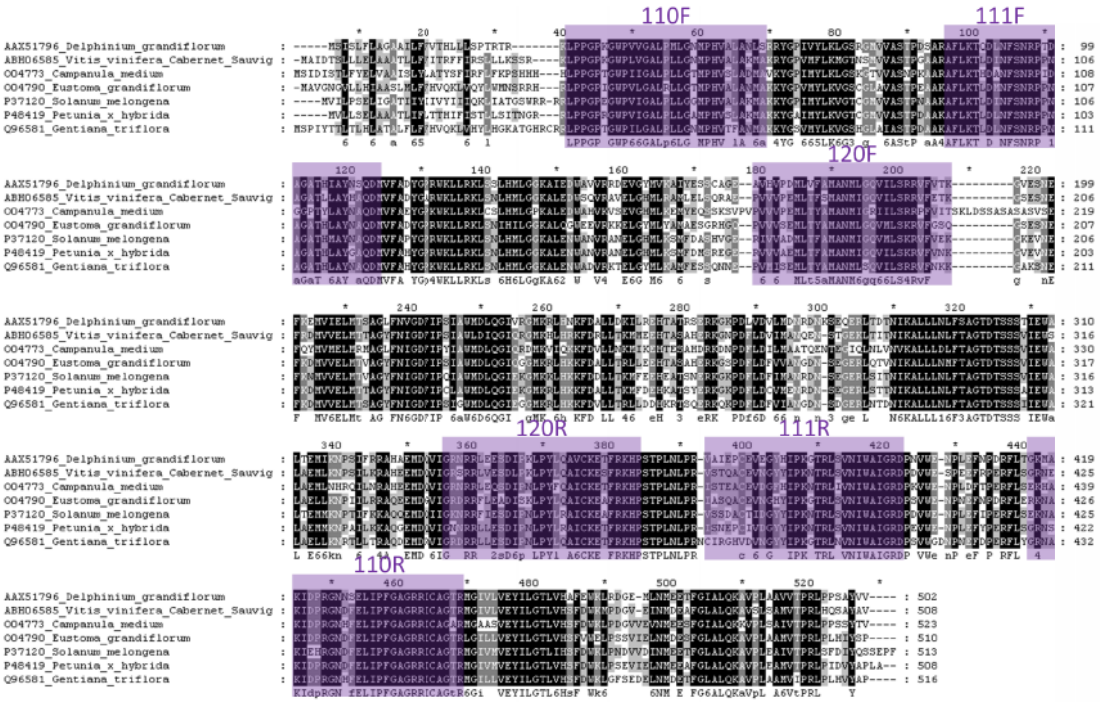


Figure 2-11. Multiple alignment of seven F3'5H amino acid sequences for designing degenerate primers. Sites of primer hybridization are highlighted in blocks.

I tested several different combinations of primer pairs using both genomic DNA and cDNA as templates. Touchdown PCR was routinely used for any given primer pair. Nested PCR was also carried out for additional specificity. Some PCRs were optimized with DMSO and $MgCl_2$. Consistently, PCR amplifications of cDNA template failed to yield any PCR products (Figure 2-12). PCR amplification of genomic DNA template typically produced multiple DNA fragments (Figure 2-12). Often, BLASTN, BLASTX, and TBLASTX searches (E-value set to 10) of these PCR products resulted in no significant similarity found. Some matches that arose include pyruvate kinase and phosphatase-associated protein. The closest match to F3'5'H was ferulate 5-hydroxylase/coniferaldehyde 5-hydroxylase, a P450, from poplar (*Populus trichocarpa*) that functions in lignin biosynthesis.

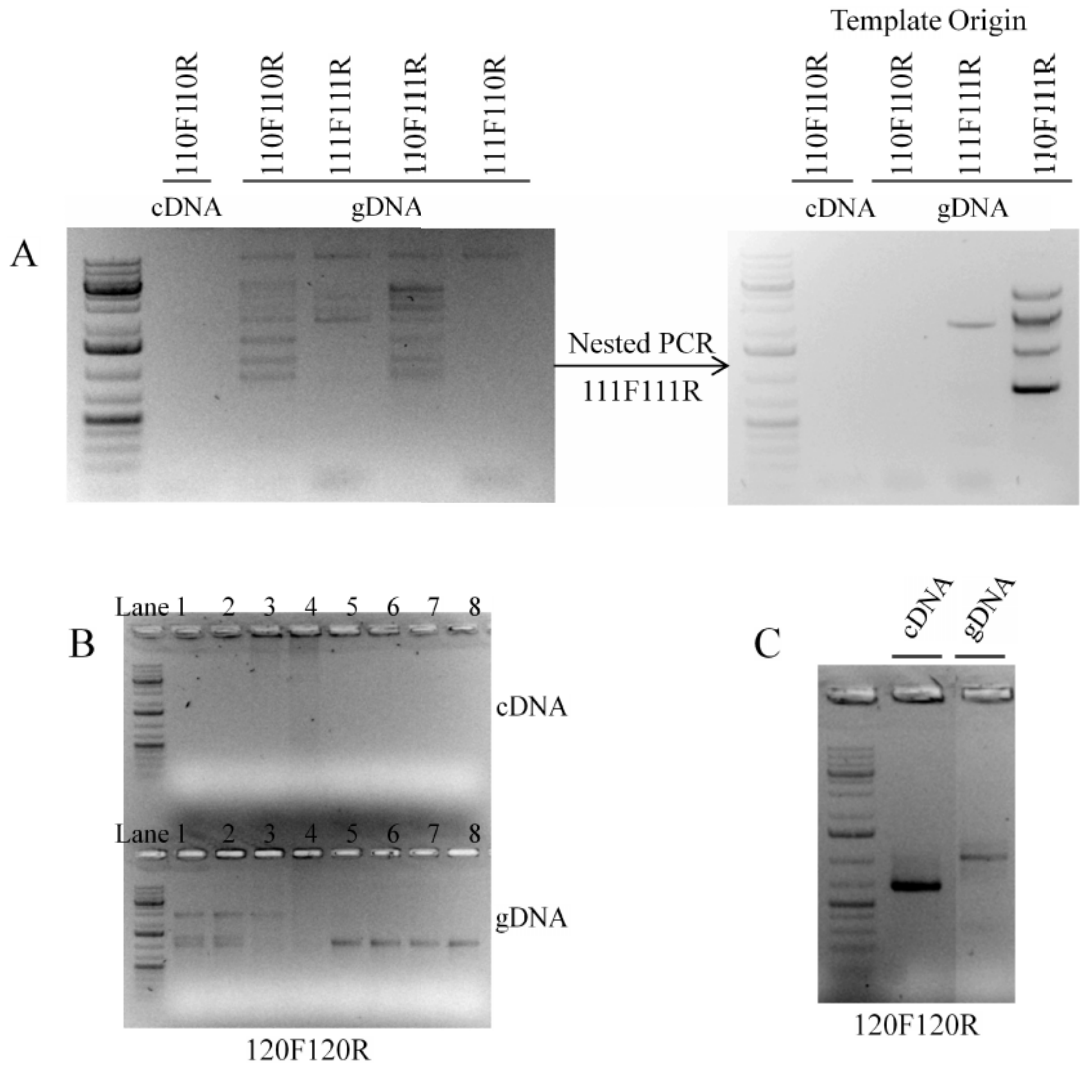


Figure 2-12. Agarose gel eletrophoresis of PCR-based strategy for detecting F3'5'H gene presence with various degenerate primer pair combinations designed by CODEHOP in (A) and (B) raspberry, and (C) grapevine. PCR optimizations are labelled as follows: Lane 1, 3% DMSO; Lane 2, 5% DMSO; Lane 3, 7.5% DMSOS; Lane 4, 12.5% DMSO; Lane 5, 2.0 mM $MgCl_2$; Lane 6, 2.5 mM $MgCl_2$; Lane 7, 3.0 mM $MgCl_2$; Lane 8, No additives.

Despite the extensive functional variations amongst P450 enzymes, there is a high degree of spatial structural conservation that is likely necessary to facilitate binding to heme iron and molecular oxygen (Johnson and Stout, 2005). The first x-ray crystallography data for a substrate-bound bacterial P450 from *Pseudomonas putida*, $P450_{cam}$, was presented by Poulos *et al*. in 1987. Through a multiple sequence alignment

of CYP2 family proteins and those of bacterial P450s, including $P450_{cam}$, Gotoh (1992) identified six putative substrate recognition sites (SRSs) that are involved in recognizing or binding of substrates that determine substrate specificity. Accordingly, I located conserved SRSs in CYP75 proteins by alignment of F3'H and F3'5'H sequences from multiple species (Figure 2-13). I then designed degenerate PCR primers located near SRS 1 and in SRS 5. Given the high degree of similarity between F3'H and F3'5'H, the difference in as little as one or two amino acid residues was used to distinguish F3'5'H from F3'H. These primers were selected to border the intron.

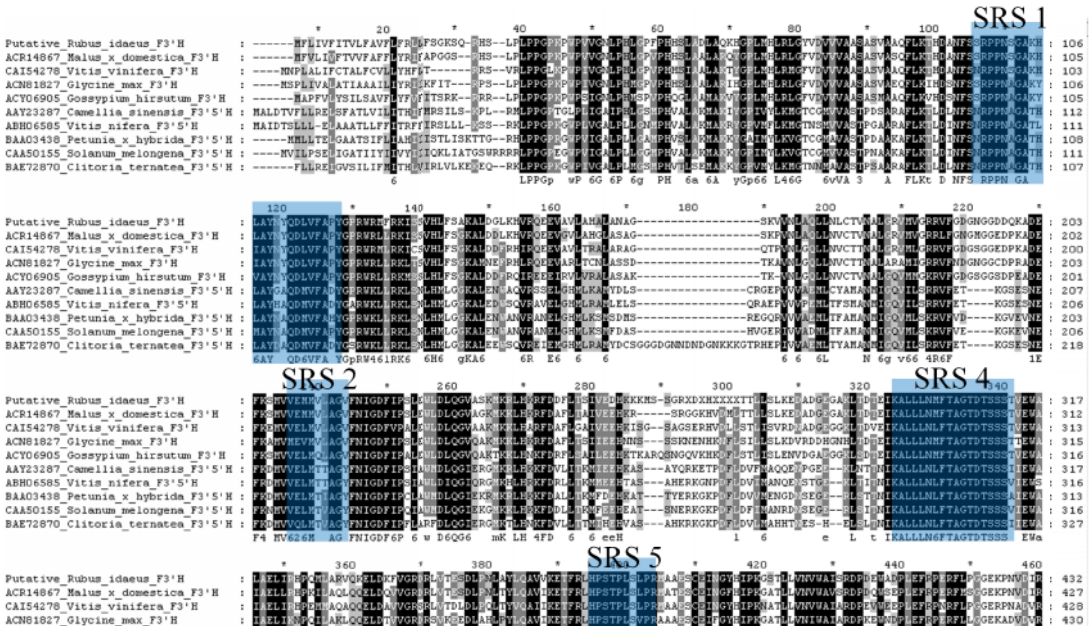


Figure 3-13. Multiple alignment of five F3'H and five F3'5'H amino acid sequences for designing SRS-specific degenerate primers. SRS locations are highlighted in blocks.

PCR optimizations were conducted involving varying concentrations of DMSO and $MgCl_2$. DMSO has been shown to be an effective enhancer in genomic DNA-based

PCRs by facilitating strand separation through the disruption of base pairings. The specificity of these SRS-based degenerate primers was demonstrated through the amplification of F3'5'H gene fragments in both grapevine genomic DNA and cDNA as shown in Figure 2-14b and via subsequent sequence confirmation (not shown). Here, PCR results support previous findings in which PCR with raspberry fruit-derived cDNA as template did not yield any products. In contrast, PCRs with raspberry genomic DNA produced significantly larger DNA fragments than would be expected. Cloning and sequencing of these DNA fragments produced similar results in that only non-specific sequences were amplified and F3'5'H was not identified.

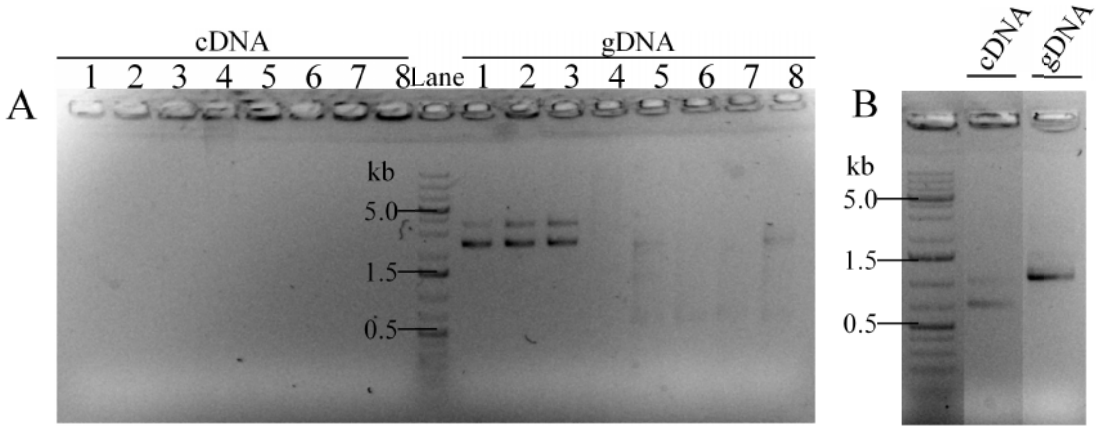


Figure 2-14. Agarose gel eletrophoresis of PCR-based strategy for detecting F3'5'H gene presence with P450 SRS-based degenerate primers in (A) raspberry and (B) grapevine. PCR optimizations for raspberry are labelled as follows: Lane 1, 3% DMSO; Lane 2, 5% DMSO; Lane 3, 7.5% DMSO; Lane 4, 12.5% DMSO; Lane 5, 2.0 mM $MgCl_2$; Lane 6, 2.5 mM $MgCl_2$; Lane 7, 3.0 mM $MgCl_2$; Lane 8, No additives.

2.3.5 Mining Apple Whole Genome Sequence Data for F3'5'H Gene

Comparative genomics takes advantage of the availability of Rosaceae genomes while exercising a computational approach. As such, the apple genome (Velasco *et al*., 2010) was mined for the F3'5'H gene that had not been identified in raspberry.

TBLASTN searches of apple whole genome sequence data with F3'5'H amino acid sequences from *Vitis vinifera* (ABH06585) and *Petunia* x *hybrida* (P48418) generated a large number of hits. Of the hits generated, only those top five hits mutually found in both TBLASTN searches were analyzed further. Sequence IDs are written in ranking order: MDP0000190489, MDP0000286933, MDP0000499282, MDP0000193880, and MDP0000209633.

Open reading frames were translated *in silico*. All deduced apple amino acid sequences were shown to contain characteristic P450 conserved motifs. These sequences were individually aligned with VvF3'5'H and PhF3'5'H amino acid sequences to determine percent identities, as summarized in Table 2-5. As alluded to previously, regarding P450 nomenclature, these percent identities under the 40% rule, can adequately allocate a new sequence to an existing P450 family. It was found that MDP0000190489 amino acid sequence shared 50% identity and 70% positives with VvF3'5'H, while MDP0000286933 amino acid sequence showed 49% identity and 68% positives with VvF3'5'H. This suggested that they likely belong to the same P450 family as VvF3'5'H, but not to the same subfamily. Protein alignment between VvF3'H (CAI54278) and VvF3'5'H (CAI54277) showed 50% identity and 69% positives. A BLASTP search (E-value set to 10) of the MDP0000190489 sequence revealed 99% identity to F3'H IIa from apple (MdF3'H2-1, ACR14868) indicating that they are likely allelic variants of the same gene in apple. Similarly, MDP0000286933 showed 93% identity to F3'H I from apple (MdF3'H1, ACR14867). Collectively, MDP0000499282, MDP0000193880, and MDP0000209633 amino acid sequences shared an average of 37% identities and an average of 62% positives with VvF3'5'H. Although MDP0000499282,

MDP0000193880, and MDP0000209633 share an average of 41% identities with PhF3'5'H, I cannot conclude with confidence that they are CYP75 family proteins, since the 40% amino acid sequence identity rule is more or less a rule of thumb. Similarly, Nelson (2004) argued that the P450 nomenclature relies more so on the evolutionary relationships as depicted through clustering in phylogenetic trees than the absolute percentage of identity.

Table 2-5. Alignment analysis of five best apple hits mutually found in both grapevine and petunia F3'5'H TBLASTN searches with grapevine and petunia F3'5'H amino acid sequences.

		Alignment with VvF3'5'H			**Alignment with PhF3'5'H**		
Rank	ID	Identities	Positives	Gaps	Identities	Positives	Gaps
1	MDP0000190489	50%	70%	2%	52%	71%	4%
2	MDP0000286933	49%	68%	6%	50%	69%	6%
3	MDP0000499282	37%	62%	3%	41%	61%	3%
4	MDP0000193880	37%	63%	1%	41%	63%	2%
5	MDP0000209633	37%	61%	3%	41%	60%	4%

BLASTP searches (E-value set to 10) of MDP0000499282, MDP0000193880, and MDP0000209633 resulted in ambiguous matches of hypothetical proteins. Search results summarized in Table 2-6 suggested they may belong to a subset of P450 families, CYP92 and CYP736, which coincidently both belong to the CYP71 clan (Nelson *et al*., 2008). MDP0000499282, MDP0000193880, and MDP0000209633 amino acid sequences were aligned with a characterized CYP92A2 from tobacco (*Nicotiana tabacum*, X95342). While MDP0000499282 and MDP0000209633 showed 74% identity and 86% positives over a 93% query coverage and 71% identity and 84% positives over a 100% query coverage, respectively, MDP0000193880 shared only 39% identity and 59% positives over a 93% query coverage with tobacco CYP92A2. Expectedly, MDP0000499282 is highly similar to MDP0000209633 with 91% identity and 95% positives. Thus, MDP0000499282 and MDP0000209633 likely group within the CYP92A subfamily. An

amino acid sequence alignment of MDP0000193880 with a P450 monooxygenase CYP736B gene from grapevine (*V. arizonica* x *V. rupestris*, ACM89788) showed 61% identity and 79% positives. This result suggests that MDP0000193880 is a member of the CYP736B subfamily. Taken together, these comparative sequence analyses corroborate earlier findings which suggest a lack of F3'5'H orthologs in each of raspberry and apple.

Table 2-6. Summary of BLASTP analysis of five best apple hits obtained from TBLASTN searches.

Rank	ID	Length (a.a)	Score (bits)	E value
1	MDP0000190489	515	991	0.0
2	MDP0000286933	718	913	0.0
3	MDP0000499282	509	795	0.0
4	MDP0000193880	498	686	0.0
5	MDP0000209633	511	792	0.0

Rank	Sequences Producing Significant Alignments	P450	Identity
1	ACR14868_flavonoid 3'-hydroxylase IIa	CYP75B	99%
2	ACR14867_flavonoid 3'-hydroxylase I	CYP75B	93%
3	ABC59081_cytochrome P450 monooxygenase CYP92A29	CYP92	77%
4	XP_002310007_cytochrome P450	CYP736	65%
5	ABC59081_cytochrome P450 monooxygenase CYP92A29	CYP92	77%

2.3.6 Mining Peach Whole Genome Sequence Data for F3'5'H Gene

To strengthen the argument that the F3'5'H gene may be absent from the raspberry genome, a second Rosaceae genome was considered. Similar TBLASTN searches (E-value set to 0.1) of the peach (*Prunus persica*) genome with grapevine and petunia F3'5'H amino acid sequences retrieved a long list of P450-like sequences. The best five hits generated from each search are listed in Table 2-7. As shown in Table 3-7, three of the five best hits are mutually found in both searches. BLASTP searches (E-value set to 10) of these amino acid sequences did not identify F3'5'H among these hits.

Rather, likely members of the P450 families, CYP71, CYP76, CYP81, CYP84, CYP92, and CYP98, were identified. In accordance with what was found earlier with apple whole genome sequence data mining, the resulting TBLASTN hits likely represent P450 sequences of the CYP71 clan that are closely related to the CYP75 family.

As a diagnostic tool to assess the robustness of the TBLASTN search and its strength in identifying P450s within a plant genome, a TBLASTN search (E-value set to 0.1) of the peach genome with apple F3'H (MdF3'H2-1, ACR14868) produced ppa004433m as the hit with the highest score (420.2) and the lowest E value (1.1e-116). Alignment of the top peach sequence with MdF3'H2-1 showed 90% identity and 93% positives, suggesting that ppa004433m is an MdF3'H1 ortholog in peach. Not surprisingly, no F3'5'H ortholog was found in the peach genome. Coincidentally, apple and peach, like raspberry, do not accumulate dihydromyricetin-derived anthocyanins.

Table 2-7. Summary of TBLASTN searches of peach genome with (A) VvF3'5'H (GenBank accessions: ABH06585), (B) PhF3'5'H (GenBank accessions: P48418), and (C) MdF3'H (GenBank accessions: ACR14867) amino acid sequences and summary of BLASTP searches of peach hits agaisnt NCBI database.

A VvF3'5'H Query (508 residues)

ID	Scaffold	Score	E	Length	Coverage	CYP Family
ppa019048m	7	319.7	1.90E-86	509	43-506	CYP92
ppa014596m	7	319.7	1.90E-86	506	43-506	CYP92
ppa017339m	5	187.2	5.90E-85	519	44-499	CYP71
ppa016144m	5	187.2	5.90E-85	517	43-506	CYP98
ppa017329m	1	305.1	5.20E-82	489	45-496	CYP76

B PhF3'5'H Query (506 residues)

ID	Scaffold	Score	E	Length	Coverage	CYP Family
ppa019048m	7	354.0	9.30E-97	509	10-504	CYP92
ppa014596m	7	354.0	9.30E-97	506	40-504	CYP92
ppa022041m	6	341.3	5.60E-93	498	7-500	CYP84
ppa025296m	6	341.3	5.60E-93	496	44-498	CYP81
ppa016144m	5	184.1	1.10E-84	517	33-504	CYP98

C MdF3'H Query (511 residues)

ID	Scaffold	Score	E	Length	Coverage	CYP Family
ppa004433m	5	420.2	1.10E-116	510		CYP75B

2.4 Discussion

2.4.1 Rapid Characterization of Anthocyanins in Raspberry and Grapevine Crude Extracts

Here, I have shown that PCR, RT-PCR, anthocyanin profiling, and comparative genomics data are corroborated and collectively make a strong case for the raspberry genome lacking an F3'5'H ortholog. It has been established through compositional analyses from this investigation and previous works that the differential anthocyanin accumulation between raspberry and grapevine berry lies in the exclusive accumulation of 3'-hydroxylated, or DHQ-derived anthocyanins, and the absence of 3',5'-hydroxylated

anthocyanins, or DHM-derived anthocyanins, in raspberry; the cause of which is likely due to a lack of F3'5'H activity. This raises an interesting question: Does raspberry lack subsequent downstream products using DHM as a substrate? In other words, does raspberry lack other 3',5'-hydroxylated flavonoids, such as flavonols and proanthocyanidins? Like anthocyanins, flavonol and proanthocyanidin diversity relies on the activities of F3'H and F3'5'H. The synthesis of flavonols occurs through the conversion of dihydroflavonol precursors, such as DHK, DHQ, and DHM, to kaempferol, quercetin, and myricetin, respectively (Pang *et al*., 2007). Proanthocyanidin polymers are derived from the addition of multiple extension units of flavan-3,4-diol (such as leucoanthocyanidin) origin to a flavan-3-ol terminal unit (such as catechin and epicatechin) (Bogs *et al*., 2005). It is, thus, of special interest to examine the flavonol and proanthocyanidin profile of raspberry and grapevine and infer whether it can be attributed to the presence or the absence of F3'5'H activity.

The composition of flavonols in raspberry fruits has been largely determined via HPLC. Peak characterization revealed that quercetin is the predominant flavonol aglycone detected (Hakkinen *et al*., 1999; Maatta-Riihinen *et al*., 2004; Jakobek *et al*., 2007), while trace amounts of kaempferol were found (Rommel and Wrolstad, 1993). Myricetin was not detected in any capacity. These results mirror the anthocyanin profile observed in raspberry, where delphinidin-based anthocyanins are not produced. This is demonstrative of F3'H function, but not F3'5'H. In contrast, the flavonol profile of 64 red-skinned grape varieties showed a broad range of structural diversity of flavonol aglycones including kaempferol, quercetin, myricetin, and some *O*-methylated flavonols (Mattivi *et al*., 2006).

Proanthocyanidins have been detected in many fruits and berries. Data from several independent studies concur that raspberry contains both propelargonidin and procyanidin constituent units, while grapevine accumulates both the procyanidin- and prodelphinidin-type (Gu *et al*., 2003; Prior and Gu, 2005; Hellstrom et al., 2009). Again, since the synthesis of flavonols, proanthocyanidins, and anthocyanins share common substrates and enzymes in the flavonoid biosynthetic pathway, the pattern of flavonoid accumulation in raspberry is entirely consistent with a lack of F3'5'H activity.

2.4.2 PCR-Based Strategy for Detecting F3'5'H Gene Presence in Raspberry

The molecular basis underlying the lack of F3'5'H activity in raspberry has not been previously determined. It was found in this thesis that F3'5'H transcripts and gene presence could not be detected in raspberry. Various measures were taken to assay for quality assurance. Degenerate primer specificity was authenticated via PCR amplification in grapevine cDNA and genomic DNA and the yield of single PCR product. Although multiple PCR products were produced from raspberry genomic DNA, sequence verification revealed amplification of non-specific products.

There is a number of molecular level explanations that could account for this finding. I hypothesized that a functional F3'5'H enzyme is lacking in raspberry as a result of aberrations in one or more of the following: gene presence, genomic sequence, and/or gene expression. On the transcript level, the lack of amplification from raspberry cDNA suggests some type of post-transcriptional regulation involving alternative splicing, transcript accumulation, and/or transcript degradation. Alternately, a lack of transcriptional activation would produce the equivalent outcome.

On the genomic level, non-specific amplification suggests differences in F3'5'H gene structure in raspberry from previously characterized F3'5'H genes in other species. Two genomic structures of F3'5'H are known, consisting of two exons, as in grapevine (*V. vinifera* L.; Jeong *et al*., 2006) and gentian (*Gentiana scabra*; Nakatsuka *et al*., 2006) or three exons, as in soybean (*Glycine max*; Zabala and Vodkin, 2007), potato (Solanum tuberosum; Jung *et al.*, 2005) and tomato (Solanum lycopersicum; Olsen *et al.*, 2010). It is possible that raspberry has a different exon-intron arrangement and that the intervening intron(s) were simply too long to be amplified given the extension time. Secondly, single or multiple transposable element insertion(s) in anthocyanin structural genes, such as F3H, F3'H, and F3'5'H, have been discovered in morning glory (Hoshino *et al*., 2003), petunia (Matsubara *et al*., 2005), gentian (Nakatsuka *et al*., 2006), and soybean (Zabala and Vodkin, 2005). In pink-flowered gentians, the independent insertion of one of two different transposable elements, *dTgs1* or *GsTRIM1*, into the first exon of F3'5'H gene created two mechanisms of gene inactivation, which involves the introduction of nonsense stop codon or the induction of alternative splicing for the phenotypic expression of predominantly cyanidin-based anthocyanins. Yet, the possibility of mispriming cannot be ignored. Ultimately, it is not beyond the realm of possibility that F3'5'H ortholog is absent in the raspberry genome; however, the evolutionary relevance of F3'5'H gene loss is not known.

2.4.3 Mining Rosaceae Whole Genome Sequence Data

The advent of high-throughput DNA sequencing at the turn of the 21st century prompted a wave of whole genome sequencing projects. In 2000, the model system

Arabidopsis thaliana became the first plant genome to be sequenced (The Arabidopsis Genome Initiative, 2000). Since then, rice (*Oryza sativa*; Yu *et al*., 2002 and Goff *et al*., 2002), poplar (*Populus trichocarpa*; Tuskan *et al*., 2006), and grapevine (*V. vinifera*; Velasco *et al*., 2007 and Jaillon *et al*., 2007)) genome sequencing initiatives have followed. Increasing public demand for the production of sustainable and health-promoting Rosaceous crops has facilitated the developments in Rosaceae genomics and genetics research. In British Columbia, the tree fruit industry is valued at $58,757,000 per year with an average production of 312,200,000 lbs per year (Ministry of Agriculture and Lands, http://www.gov.bc.ca/al/). Because of the heterogeneity found within the Rosaceae family, which encompasses over 100 genera and 3000 species, the need for subfamily-specific model systems has shifted the focus to peach, apple, and strawberry given their strengths in structural genomics, functional genomics, and reverse genetics, respectively (Shulaev *et al*., 2008). This resulted in genome sequencing initiatives for these species. In April 2010, the International Peach Genome Initiative (IPGI) released an initial draft of the assembled and annotated peach genome prior to peer-reviewed publication of the data. Ensuingly, in October 2010, a high-quality draft genome sequence of the domesticated apple was reported (Velasco *et al*., 2010). In contrast, the number of ESTs available for raspberry is quite small, 323 (Genome Database for Rosaceae, http://www.rosaceae.org/). Although the lack of raspberry genomic resources presented a challenge for F3’5’H gene detection, the utility of comparative genomics, which takes advantage of sequenced genomes from phylogenetically closely related species, apple and peach, adequately allowed similarity determinations between genomes of different species.

Extensive TBLASTN searches of both apple and peach genomes with two functionally characterized F3'5'H amino acid sequences that are 76% identical yielded no putative matches to F3'5'H. This search method appears to be robust, since the best hit from a TBLASTN search of the peach genome with RiF3'H shares 90% identities with MdF3'H2-1 and is likely an F3'H ortholog in peach. Furthermore, P450 nomenclature affirms amino acid sequence similarities between members of the same family as in the case of F3'H (CYP75B) and F3'5'H (CYP75A). It is, thus, not surprising to find the manifestation of F3'H sequences from the apple genome when searched with an F3'5'H amino acid sequence. Plant P450 families are divided into four main clans (Nelson, 1999). CYP75 is a member of the CYP71 clan that is by far the largest and catalyzes many reactions in the phenylpropanoid pathway (Nelson, 2006). These P450 families fall within the same clan, as they likely represent genes that diverged from a single common ancestor. The phylogenetic relationship between P450 families of the CYP71 clan shows that CYP71, CYP76, CYP81, CYP84, CYP92, and CYP98 cluster together attesting to a heightened degree of sequence similarity and conservation. These circumstantial data suggest that an F3'5'H ortholog may be lacking in both the apple and peach genomes. It is unlikely that the lack of dihydromyricetin-derived anthocyanins in raspberry is due to a "glitch" in transport, storage, and/or regulation, since this would abolish the accumulation of all types of anthocyanins. While both metabolite and genomics analyses strongly suggest a lack of F3'5'H ortholog in raspberry genome, whole genome sequencing will be required to verify this.

Currently, the Lund lab has generated a *Rubus idaeus* cv. Chemainus 10X BAC library for public dissemination in collaboration with Dr. Christopher Saski, Clemson

University Genomics Institute. While it is beyond the scope of this thesis, it will be of interest to screen this BAC library for the F3’5’H gene, although cross-hybridization to RiF3’H sequences would be a concern with this approach.

3 FUNCTIONAL GENOMICS: THE HOW

3.1 Introduction

While most current genetic transformations of fruit crops involve the enhancement of horticultural traits for disease resistance, pest resistance, herbicide tolerance, and cold tolerance, there is a growing interest towards the cultivation of functional foods by altering their inherent metabolite profiles. Currently, one of the most sought-after targets in the development of functional foods is to enrich foods with physiologically active compounds, such as polyphenolic antioxidants. A range of different polyphenolic compounds is found in soft fruits. In addition to vitamin C, anthocyanins contribute to 25% of total antioxidant capacity (Beekwilder *et al*., 2005a; Beekwilder *et al*., 2005b). Functional foods can be achieved through, traditionally, plant breeding, and now, genetic engineering.

Like rose, carnation, and chrysanthemum, raspberry lack blue-coloured varieties due to the absence of dihydromyricetin-derived anthocyanins. The key enzyme in the biosynthesis of dihydromyricetin-derived anthocyanins is flavonoid 3',5'-hydroxylase (F3'5'H). F3'5'H belongs to a large and diverse group of enzymes, the cytochrome P450 superfamily. The activity of F3'5'H requires NADPH as a cofactor and may require a cytochrome b5 for full activity as shown in petunia (de Vetten *et al*., 1999). Natural substrates utilized by F3'5'H are flavanones (naringenin, eriodictyol) and dihydroflavonols (dihydrokaempferol, dihydroquercetin). F3'5'H is known to catalyze 3',5'-hydroxylations, 3',4'-hydroxylations, and 3',4',5'-hydroxylations of the B ring *in vitro* (Seitz *et al*., 2006). Ectopic expression of F3'5'H has been very important in the

floricultural industry giving rise to blue-hued varieties, conferred primarily by the presence of dihydromyricetin-derived anthocyanins, in species that do not naturally produce them. Transgenic expression of a gene-of-interest has been routinely carried out via *Agrobacterium tumefaciens*-mediated genetic transformation in countless plant species, including raspberry. Genetic transformations of β-glucuronidase (GUS) gene (Graham *et al*., 1990; de Faria *et al*., 1997) and *S*-adenosylmethionine hydrolase (SAMase) gene (Mathews *et al*., 1995) have been demonstrated using leaf discs, petioles, and stems as explants.

Here, I tested the hypothesis that the absence of dihydromyricetin-derived anthocyanins in raspberry is due to the lack of F3'5'H activity by genetic complementation of raspberry and apple callus lines with grapevine F3'5'H cDNA. The experimental approaches taken here included establishing callus lines from raspberry and apple, establishing competency of raspberry and apple callus lines for *Agrobacterium*-mediated genetic transformation, testing protocols for anthocyanin induction in raspberry and apple callus lines, constructing a recombinant vector harbouring grapevine F3'5'H cDNA driven by the constitutive CaMV 35S promoter, and genetically complementing the absence of dihydromyricetin-derived flavonoids in raspberry and apple callus lines with grapevine F3'5'H cDNA.

3.2 Materials and Methods

3.2.1 Plant Material

Mature leaves from *Rubus idaeus* cvs. Chemainus and Meeker were collected in summer 2008 from a Pacific Agri-Food Research Centre (PARC) substation in

Abbotsford, British Columbia. During field collections, leaves were kept moist by sandwiching them between damp paper towels and then placing them on dry ice for transport.

Ripe fruit from *Malus* x *domestica* cv. Gala was purchased from a local grocery store. Fruit selection was made based on fruit colour and healthy appearance.

3.2.2 Surface Sterilization

Whole raspberry leaves were surface sterilized with 70% (v/v) ethanol for 1 min, rinsed with sterile Milli-Q water (Millipore, Billerica, MA, USA) for 2 min, submerged in 10% (v/v) commercial bleach (Clorox, Oakland, CA, USA) for 15 min, followed by a quick rinse in sterile Milli-Q water. Whole leaves were then rinsed with sterile dH_2O for 5 min and kept in fresh sterile dH_2O until dissection.

Whole apple fruit was washed in 25% (v/v) commercial bleach with a few drops of Tween 20 (Sigma-Aldrich Canada, Oakville, ON) for 10 min, rinsed twice with sterile Milli-Q water, and kept in fresh sterile Milli-Q water until dissection.

3.2.3 Plant Cell Culture Induction and Maintenance

3.2.3.1 Stock Solutions

Table 3-1. Stock solutions for raspberry and apple cell culture induction and maintenance.

Name	[Final]	[Stock]	Preparation Instructions
2,4-Dichlorophenoxyacetic acid (2,4-D)[a]	9 µM	9 mM	Soluble in 95% ethanol; heat may be required
Indole-3-butyric acid (IBA)[b]	4.9 µM	4.9 mM	Soluble in KOH
6-(γ,γ-Dimethylallylamino) purine (2iP)[b]	4.9 µM	4.9 mM	Soluble in KOH
Nicotinic acid[a]	0.5 mg/L	0.5 mg/mL	Soluble in 1M NaOH
Pyridoxine hydrochloride[a]	0.5 mg/L	0.5 mg/mL	Soluble in water
Thiamine hydrochloride[a]	0.1 mg/L	0.1 mg/mL	Soluble in water

[a]Chemicals purchased from Sigma-Adrich Canada (Oakville, ON).
[b]Chemicals purchased from PhytoTechnology Laboratories (Shawnee Mission, KS, USA).

3.2.3.2 Cell Culture Initiation and Maintenance

Surface-sterilized raspberry cvs. Chemainus and Meeker whole leaves were cut into 1 cm^2 sections. Leaf sections were transferred aseptically with the abaxial surface to the solid medium. Calli (undifferentiated cell growth) were evident after 1-2 wk, emerging from the leaf margins where the excisions occurred. Leaf sections with expanding calli were cultured on solid callus induction medium (CIM), which consisted of Anderson's Basal Salt Mixture (Anderson, 1980) supplemented with 9 µM 2,4-D, 4.9 µM IBA, 4,9 µM 2iP, 100 mg/L myo-inositol, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine hydrochloride, 0.1 mg/L thiamine hydrochloride, 30 g/L sucrose and 3.5 g/L phytagel as described by Borejsza-Wysocki and Hrazdin (1994). Plant growth regulators and organic supplements were prepared according to Table 2-1 and were added after autoclaving. Culture media were adjusted to pH 5.7 with 1 M KOH prior to autoclaving at 121°C and 103 kPa for 30 min. Initially, Plant Preservative Mixture (PPM; Plant Cell Technology, Washington, DC, USA), a broad-spectrum biocide/fungicide for plant tissue culture, was added to the culture medium, but was omitted from culture media after several clean subcultures. Tissue cultures were incubated at 25°C in the dark. Once grown to substantial size, callus masses were separated from leaf explants and subcultured every 4 to 5 wk.

Cell suspension cultures were initiated with 10% (w/v) friable callus in liquid CIM. Cultures were incubated on a rotating shaker at 150 rpm at 25°C in the dark. Cell suspensions were filtered through a 70 µm cell strainer (BD Biosciences, Mississauga, ON, Canada) and transferred in this manner to fresh CIM every 2 wk.

Surface-sterilized whole apple fruit was cut lengthwise away from the core into 1 cm^3 pieces. Callus cultures and cell suspension cultures were initiated and maintained as described above for raspberry leaf explants using the same media. Gala callus was initiated along the cutting lines of fruit sections after 1-2 wk of incubation on CIM. Each fruit section was composed of either flesh only or flesh with skin. Callus initiation was found primarily along the edges of fruit flesh.

3.2.4 Anthocyanin Induction in Raspberry and Apple Cell Cultures

3.2.4.1 Rationale for the Different Types of Inducers Implemented

Light is a required stimulus for the induction of anthocyanin biosynthesis due to the involvement of phytochrome and other UV-specific photoreceptors (Chalker-Scott, 1999). As a result, light (Lithonia Lighting, Conyers, GA, USA) with a constant photoperiod (16hr/8hr) was used in all experiments in combination with other treatments, unless stated otherwise, and periods of UV irradiation (NuAire Inc., Plymouth, MN, USA) were implemented. Jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), are endogenously synthesized by plants in response to various biotic and abiotic stresses and have been shown to induce the accumulation of stress-responsive compounds, including anthocyanins (Blando *et al*., 2005). Sucrose has been shown to act as an osmotic agent and a source of monosaccharide to enhance anthocyanin accumulation (Do and Cormier, 1990). In addition, abscisic acid (ABA) was found to act synergistically with sucrose to stimulate anthocyanin biosynthesis (Loreti *et al*., 2008). It was reported that nutrient availability is inversely related to plant secondary metabolite production (Tuomi *et al*., 1984). Krisa *et al*. (1999) and Edahiro *et al*. (2005)

independently showed that feeding of phenylalanine into the culture medium enhanced accumulation of anthocyanins in cell suspensions cultures, which is in support of the phenylalanine pool hypothesis. Alternatively, the free radical generator, 2,2'-azobis(2-methylpropionamidine) dihydrochloride (AAPH) was found to be a suitable agent for the stimulation of plant secondary metabolism in cell suspension cultures that was mediated through an increase in phenylalanine ammonia lyase (PAL) activity (Ohlsson et al., 1995).

3.2.4.2 Plant Growth Regulator and Elicitor Stock Solutions

Table 3-2. Plant growth regulator and elicitor stock solutions for anthocyanin induction in raspberry and apple cell cultures.

Plant Growth Regulators	[Stock]	Preparation Instructions
Kinetin[b]	100 mM	Soluble in KOH
6-Benzylaminopurine (BAP)[a]	100 mM	Soluble in glacial acetic acid
α-Nathphaleneacetic acid (NAA)[b]	100 mM	Soluble in KOH
Elicitors	**[Stock]**	**Preparation Instructions**
Methyl Jasmonate (MeJA)[b]	10 mM	Miscible with EtOH
(+)-Abscisic acid (ABA)[b]	1 mM	Soluble in KOH
L-Phenylalanine (L-Phe)[b]	100 mM	Soluble in water
2,2'Azobis(2-methylpropionamidine) dihydrochloride (AAPH)[a]	100 mM	Soluble in water

[a]Chemicals purchased from Sigma-Adrich Canada (Oakville, ON).
[b]Chemicals purchased from PhytoTechnology Laboratories (Shawnee Mission, KS, USA).

3.2.4.3 Composition of Media Used

3.2.4.3.1 Callus Cultures

Table 3-3. Composition of culture media used for anthocyanin induction in callus cultures.

Name	AIM0	AIM1	AIM2	AIM3	AIM4	AIM5	AIM6	AIM7	AIM8
Basal Salt Mixture[b] (pack/L)	M524								
Sucrose[a] (g/L)	30								
Organic Supplements									
Myo-Inositol[a] (mg/L)	-								
Nicotinic Acid (mg/L)	-								
Pyridoxine Hydrochloride (mg/L)	-								
Thiamine Hydrochloride (mg/L)	-								
Cytokinins									
2iP (µM)	-								
Kinetin (µM)	-								
BAP (µM)	0.44								
Auxins									
2,4-D (µM)	-								
IBA (µM)	-								
NAA (µM)	5.4								
Elicitors									
MeJA (µM)	-	0.5	5	10	15	20	50	150	450
ABA (µM)	-								
$CaCl_2$[a] (mM)	-								
L-Phe (mM)	-								
AAPH (mM)	-								
Gelling Agent									
Phytagel[a] (g/L)	3.5								

Name	CIM	CIM1	CIM2	CIM3	CIM4	CIM5
Basal Salt Mixture[b] (pack/L)	A267					
Sucrose[a] (g/L)	30				50	90
Organic Supplements						
Myo-Inositol[a] (mg/L)	100					
Nicotinic Acid (mg/L)	0.5					
Pyridoxine Hydrochloride (mg/L)	0.5					
Thiamine Hydrochloride (mg/L)	0.1					
Cytokinins						
2iP (µM)	4.9					
Kinetin (µM)	-					
BAP (µM)	-					
Auxins						
2,4-D (µM)	9					
IBA (µM)	4.9					
NAA (µM)	-					
Elicitors						
MeJA (µM)	-					
ABA (µM)	-	0.1	1	10		
$CaCl_2$[a] (mM)	-					
L-Phe (mM)	-					
AAPH (mM)	-					
Gelling Agent						
Phytagel[a] (g/L)	3.5					

[a]Chemicals purchased from Sigma-Adrich Canada (Oakville, ON).
[b]Chemicals purchased from PhytoTechnology Laboratories (Shawnee Mission, KS, USA).

Table 3-3. Composition of culture media used for anthocyanin induction in callus cultures.

Name	CIM	CIM6	CIM7	CIM8	CIM9	CIM10	CIM11
Basal Salt Mixture[b] (pack/L)	A267						
Sucrose[a] (g/L)	30	50	50	50	90	90	90
Organic Supplements							
Myo-Inositol[a] (mg/L)	100						
Nicotinic Acid (mg/L)	0.5						
Pyridoxine Hydrochloride (mg/L)	0.5						
Thiamine Hydrochloride (mg/L)	0.1						
Cytokinins							
2iP (µM)	4.9						
Kinetin (µM)	-						
BAP (µM)	-						
Auxins							
2,4-D (µM)	9						
IBA (µM)	4.9						
NAA (µM)	-						
Elicitors							
MeJA (µM)	-						
ABA (µM)	-	0.1	1	10	0.1	1	10
$CaCl_2$[a] (mM)	-						
L-Phe (mM)	-						
AAPH (mM)	-						
Gelling Agent							
Phytagel[a] (g/L)	3.5						

Name	CIM	MM	GBM1	GBM2	NDM1	NDM2	NDM3	NDM4
Basal Salt Mixture[b] (pack/L)	A267	-	G398	G398	M407	M531	M571	M571
Sucrose[a] (g/L)	30							
Organic Supplements								
Myo-Inositol[a] (mg/L)	100	-						
Nicotinic Acid (mg/L)	0.5	-						
Pyridoxine Hydrochloride (mg/L)	0.5	-						
Thiamine Hydrochloride (mg/L)	0.1	-						
Cytokinins								
2iP (µM)	4.9	-	-	-				
Kinetin (µM)	-							
BAP (µM)	-		0.44	2.2				
Auxins								
2,4-D (µM)	9	-	-	-				-
IBA (µM)	4.9	-	-	-				
NAA (µM)	-		5.4	26.9				
Elicitors								
MeJA (µM)	-							
ABA (µM)	-							
$CaCl_2$[a] (mM)	-							
L-Phe (mM)	-							
AAPH (mM)	-							
Gelling Agent								
Phytagel[a] (g/L)	3.5	Agar	Agar	Agar				

[a]Chemicals purchased from Sigma-Adrich Canada (Oakville, ON).
[b]Chemicals purchased from PhytoTechnology Labcratories (Shawnee Mission, KS, USA).

3.2.4.3.2 Cell Suspension Cultures

Table 3-4. Composition of culture media used for anthocyanin induction in cell suspension cultures.

Name	CIM	PHE1	PHE2	PHE3	PHE3	AAPH1	AAPH2	AAPH3	AAPH4
Basal Salt Mixture[b] (pack/L)	A267								
Sucrose[a] (g/L)	30								
Organic Supplements									
Myo-Inositol[a] (mg/L)	100								
Nicotinic Acid (mg/L)	0.5								
Pyridoxine Hydrochloride (mg/L)	0.5								
Thiamine Hydrochloride (mg/L)	0.1								
Cytokinins									
2iP (µM)	4.9								
Kinetin (µM)	-								
BAP (µM)	-								
Auxins									
2,4-D (µM)	9								
IBA (µM)	4.9								
NAA (µM)	-								
Elicitors									
MeJA (µM)	-								
ABA (µM)	-								
$CaCl_2$[a] (mM)	-								
L-Phe (mM)	-	0.05	0.1	1	2.5				
AAPH (mM)	-					0.5	2	5	10

Name	CIM	PSM1	PSM2	PSM3	PSM4	PSM5	PSM6	PSM7	PSM8
Basal Salt Mixture[b] (pack/L)	A267								
Sucrose[a] (g/L)	30	150	150	300	300				
Organic Supplements									
Myo-Inositol[a] (mg/L)	100								
Nicotinic Acid (mg/L)	0.5								
Pyridoxine Hydrochloride (mg/L)	0.5								
Thiamine Hydrochloride (mg/L)	0.1								
Cytokinins									
2iP (µM)	4.9								
Kinetin (µM)	-								
BAP (µM)	-								
Auxins									
2,4-D (µM)	9								
IBA (µM)	4.9								
NAA (µM)	-								
Elicitors									
MeJA (µM)	-					0.0005	0.05	0.0005	0.05
ABA (µM)	-								
$CaCl_2$[a] (mM)	-								
L-Phe (mM)	-	0.025	0.05	0.025	0.05	0.025	0.05	0.025	0.05
AAPH (mM)	-								

[a]Chemicals purchased from Sigma-Adrich Canada (Oakville, ON).
[b]Chemicals purchased from PhytoTechnology Laboratories (Shawnee Mission, KS, USA).

3.2.5 Total Anthocyanin Extraction

2-3 g of induced callus was homogenized in 5 mL extraction buffer (methanol plus 0.1% (v/v) hydrochloric acid) using a handheld homogenizer. Homogenized samples were centrifuged at 3500 *g* for 15 min and the resulting supernatant was filtered using a syringe equipped with a 0.2 μM filter (Pall, Ann Arbor, MI, USA). A 1 mL aliquot of the filtered supernatant was transferred to an amber vial for HPLC analysis.

3.2.6 HPLC-MS Apparatus and Conditions

HPLC-MS analyses were performed on an Agilent 1100 HPLC system equipped with a diode array detector (DAD) and a HP ChemStation software and coupled with an Agilent 1100 Series LC/MSD Trap XCT Plus mass spectrometer. HPLC separation was carried out at a column temperature of 68°C. The mobile phases were acetonitrile with 2% (v/v) formic acid (solvent B) and Milli-Q water with 2% (v/v) formic acid (solvent A) at a flow rate of 1.2 mL/min according to the gradient program listed in Table 3-5. The range of detection spanned 190-600 nm. Phenolic compounds, including flavonoids, were detected at 280, 320, 365, and 520 nm. Electrospray ionization (ESI) was performed in negative ion mode and data were collected over a mass range of *m/z* 50-800.

Table 3-5. HPLC gradient program.

Time (min)	Solvent B (%)	Flow Rate (mL/min)	Pressure (bar)
0.70	5.0	1.200	400
6.00	18.0	1.200	400
6.10	90.0	1.200	400
7.00	90.0	1.200	400
8.00	5.0	1.200	400
9.00	5.0	1.200	400

3.2.7 Plasmids and Vector Constructs

3.2.7.1 pCAMBIA2301

The pCAMBIA2301 vector (Cambia, Brisbane, QLD, Australia) is a GUS intron selection vector with an intron from the castor bean catalase gene that ensures that expression of glucuronidase activity is derived from eukaryotic cells and not from expression by residual *A. tumefaciens* cells (Figure 3-1).

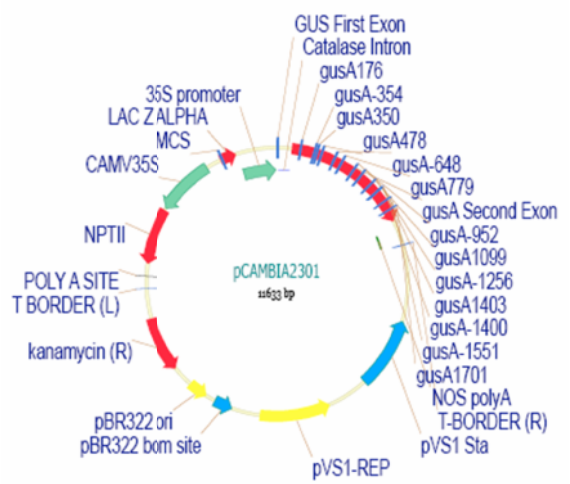


Figure 3-1. Plasmid vector map of pCAMBIA2301. Figure taken from Cambia.

3.2.7.2 pCAMBIA1380::35S::VvF3'5'H cDNA

3.2.7.2.1 pCAMBIA1380 and Vector Digest

The pCAMBIA1380 vector (Cambia) is a minimal selection vector that allows insertion of desired genes for transformation into plants but requires promoter sequences for plant expression of newly cloned genes (Figure 3-2).

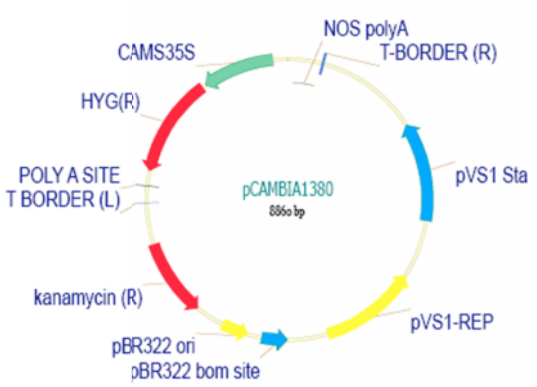


Figure 3-2. Plasmid vector map of pCAMBIA1380. Figure taken from Cambia.

1 µg of pCAMBIA1380 plasmid was double restriction endonuclease (RE) digested with 10 units *SalI* (NEB Canada, Pickering, ON) and 10 units *BglII* (NEB Canada) in NEBuffer 3 containing BSA, then incubated at 37°C for 1 hr. I gel-purified the digested pCAMBIA1380 from 1% TopVision Low Melting Point Agarose (Fermentas Canada Inc., Burlington, ON) using the GeneJET Gel Extraction Kit (Fermentas Canada) as per the manufacturer's instructions.

3.2.7.2.2 Amplification, Subcloning, and Restriction Endonucleases Digestion of Gene Fragments

Oligonucleotide primers were commercially synthesized by Integrated DNA Technologies (Toronto, ON). Sense CaMV 35S promoter PCR products were amplified from transfer vector p118 with sense primer 5'-TCCGTCGACCATGGAGTCAAAGATTCA-3' (*SalI* recognition site is underlined) and antisense primer 5'- CCACTAGTGTCCCCCGTGTTCTCT-3' (*SpeI* recognition site is underlined). Sense VvF3'5'H ORF PCR products were amplified with sense primer 5'-CCACTAGTATGGCCATAGATACAAGCCT-3' (*SpeI* recognition site is underlined) and antisense primer 5'- TCCAGATCTTCAGACTGCATACGCACT-3' (*BglII*

recognition site is underlined). PCRs were carried out with Phusion High-Fidelity DNA Polymerase (Finnzymes, Woburn, Massachusetts). The reaction mixture was set up according to the manufacturer's instructions. PCR conditions were as follows: 1 cycle of 98°C for 1 min, followed by 2 cycles of 98°C for 10 sec, 50°C for 30 sec, and 72°C for 15-30 sec/kb, 33 cycles of 98°C for 10 sec, 55°C for 30 sec, and 72°C for 15-30 sec/kb, and a final extension cycle of 72°C for 5 min.

Blunt-end PCR products were subcloned into pJET1.2/blunt cloning vector using the CloneJET PCR Cloning Kit (Fermentas Canada) according to the manufacturer's instructions, transformed into *E. coli*, and sequenced by the Nucleic Acid Protein Service (NAPS) unit at UBC. Positive clones were inoculated in 4 mL LB containing 30 μg/ml kanamycin (BIO BASIC INC., Markham, ON, Canada) and incubated at 37°C on a rotating shaker at 250 rpm overnight. Plasmid DNA was isolated from recombinant *E. coli* cultures using the GeneJET Plasmid Miniprep Kit (Fermentas Canada) according to the manufacturer's instructions.

1 μg of pJET1.2/blunt cloning vector harbouring CaMV 35S promoter was initially RE digested with 10 units *SpeI* (NEB Canada) at 37°C for 1 hr and purified with GeneJET PCR Purification Kit, followed by a sequential digest of 10 units *SalI* at 37°C for 1 hr. 1 μg of pJET1.2/blunt cloning vector carrying VvF3'5'H cDNA was double RE digested with 10 units *SpeI* and 10 units *BglII* and incubated at 37°C for 1 hr. I gel-purified the digested gene fragments from 1% TopVision Low Melting Point Agarose using the GeneJET Gel Extraction Kit.

3.2.7.2.3 Three Way Ligations

Equal nanogram amounts of each of gel purified *SalI*, *BglII*-digested pCAMBIA1380 plasmids, *SalI*, *SpeI*-digested CaMV 35S promoter PCR products, and *SpeI*, *BglII*-digested VvF3'5'H cDNA gene fragments were added to a reaction mixture containing 10X T4 DNA Ligase Buffer, 5 units T4 DNA Ligase (Fermentas Canada), and nuclease-free water to a total volume of 10 µL and incubated overnight at 16°C.

3.2.7.2.4 Validation of Vector Construct

In silico cloning of gene fragments was performed using the CLC Main Workbench software (CLC bio, Cambridge, MA, USA) to identify restriction sites that could be introduced and to create a complete workflow. Construction of pCAMBIA1380::35S::VvF3'5'H cDNA (Figure 3-3) was experimentally validated by colony PCR, restriction site analysis, and sequencing.

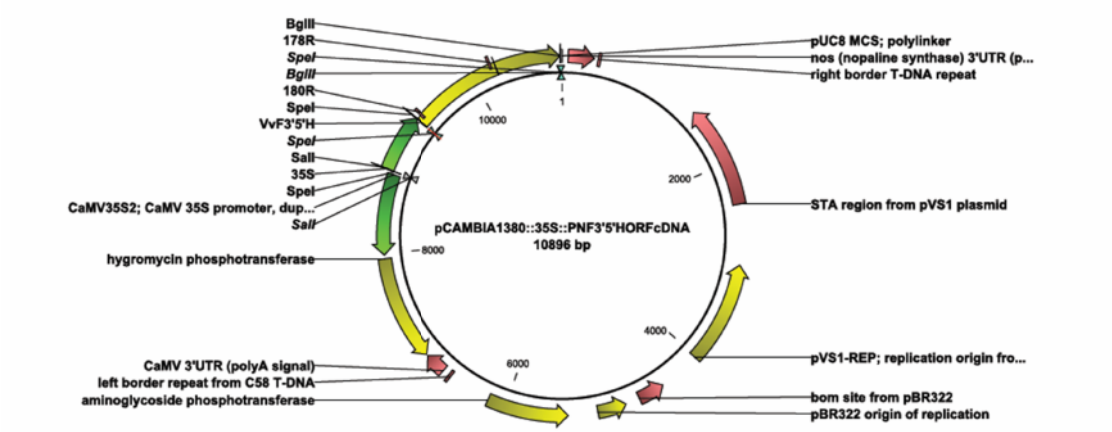


Figure 3-3. Diagram of recombinant vector pCAMBIA1380::CaMV35S::VvF3'5'H cDNA.

3.2.8 Transformation of *Escherichia coli*

Plasmid DNA was transformed into α-Select Chemically Competent Cells Gold Efficiency (Bioline USA Inc., Tauton, MA) according to the manufacturer's suggested transformation procedure.

3.2.9 Transformation of *Agrobacterium tumefaciens*

3.2.9.1 Bacterial Strain and Genotype

Agrobacterium tumefaciens strain EHA105 was kindly donated by Dr. Shawn D. Mansfield, Department of Wood Science, Faculty of Forestry, UBC, Canada. EHA105 is a kanamycin-sensitive derivative of EHA101 (Hood *et al*., 1993).

3.2.9.2 Preparation of Competent Cells

EHA105 competent cells were prepared based on a protocol taken from CellFor Inc. and modified by Thomas Canam. A glycerol stock of EHA105 was streaked on solid YEP medium (10 g yeast extract, 10 g Bacto Peptone, 5 g NaCl, and 15 g Bacto Agar, adjusted pH to 7.0 in a final volume of 1L) and incubated overnight at 28°C. A single colony of EHA105 was inoculated in 5 mL liquid YEP medium overnight at 28°C on a rotating shaker at 250 rpm. 2 mL of overnight culture was added to 50 mL fresh liquid YEP medium in a 250 mL flask and incubated at 28°C at 250 rpm until an OD_{600} value between 0.5-1.0 was reached. The culture was chilled briefly on ice and then centrifuged at 4°C at 8000 rpm for 10 min. The supernatant was discarded and the pellet was resuspended in 1 mL filter-sterilized ice-cold 20 mM $CaCl_2$ by gently pipetting up and

down. 100-200 µL aliquots were dispensed into pre-chilled 1.5 mL Eppendorf tubes, immediately snap-frozen in liquid nitrogen, and stored at -80°C.

3.2.9.3 Transformation of Competent Cells

Transformation of EHA105 competent cells was carried out following a protocol taken from CellFor Inc. and modified by Thomas Canam. EHA105 competent cells were thawed slowly on ice. 1 µg of plasmid DNA was added to the thawed cells and gently mixed by swirling the pipette tip. Cells were incubated on ice for 30 min. Cells were then heat-shocked by incubating in a 37°C heat block for 5 min. 1 mL pre-warmed liquid YEP medium was added to the cells and incubated at 28°C for 2-4 hr. Cells were centrifuged at maximum speed for 1 min and the supernatant was discarded. The pellet was very gently resuspended in 100 µL fresh liquid YEP medium and plated on solid YEP medium containing appropriate antibiotics and 25 mg/L rifampicin (*Phyto*Technology Laboratories). Cells were incubated at 28°C for 2-3 days.

3.2.10 Analysis of Recombinant Clones

Colony PCR was used to quickly screen for the presence of recombinant clones. Individual *E. coli* and *A. tumefaciens* colonies were picked with a pipette tip and resuspended in sterile Milli-Q water and liquid YEP medium, respectively. A PCR master mix was prepared with *Taq* DNA Polymerase (Bioline USA) according to the manufacturer's instructions. 5 µL of bacterial water suspension was added to each 20 µL reaction. PCR conditions were as follows: 1 cycle of 96°C for 7 min, followed by 35 cycles of 96°C for 25 sec, 45°C for 30 sec, and 72°C for 15-30 sec/kb, and a final

extension cycle of 72°C for 5 min. The presence of PCR product(s) was then analyzed on a 1% agarose gel.

3.2.11 Bacterial Glycerol Stock Preparation

4 mL LB medium or YEP medium containing appropriate antibiotics was inoculated with a single colony positive for VvF3'5'H. Liquid culture was allowed to grow overnight at 37°C on a rotating shaker at 250 rpm. In a sterile tube, 1 mL of bacterial culture was added to 1 mL 30% glycerol and mixed by inverting. 15% glycerol stock was stored at -80°C.

3.2.12 *Agrobacterium*-Mediated Genetic Transformation of Plant Callus

3.2.12.1 Agrobacterium-Mediated Genetic Transformation of Raspberry and Apple Callus Lines

3 week old subcultured Chemainus (raspberry) and Gala (apple) calli were independently inoculated and co-cultivated with *Agrobacterium* as described by an in-house protocol adapted from Iocco *et al*. (2001) and Torregrosa *et al*. (2002), as follows. Glycerol stocks of wildtype and vector construct-transformed *Agrobacterium* were streaked on solid YEP medium containing 25 mg/L rifampicin or 30 mg/L kanamycin (*Phyto*Technology Laboratories) and 25 mg/L rifampicin, respectively. A single colony of *Agrobacterium* was incubated overnight in 50 mL of liquid YEP medium containing 25 mg/L rifampicin or 30 mg/L kanamycin and 25 mg/L rifampicin at 28°C on a rotating shaker at 100 rpm. Overnight culture was centrifuged at 2600 *g* for 5 min. The pellet was resuspended in 40 mL liquid hormone-free CIM medium containing 100 μM

acetosyringone (*Phyto*Technology Laboratories) and incubated for an additional 1-2 hr to an OD_{550} value between 0.3-0.6. It was recommended to aerate the culture in the laminar flow hood at least once during the incubation period. 1-3 g of plant callus was added to the *Agrobacterium* suspension and vigorously shaken for 1-2 sec followed by 30 min incubation at 25°C. Callus was collected on a sterile No.2 Whatman filter paper (Whatman Ltd.) by vacuum filtration and transferred onto solid hormone-free CIM medium for co-cultivation in the dark for 2 days. Callus was rinsed with liquid CIM containing 1 g/L timentin (*Phyto*Technology Laboratories), collected via vacuum filtration, and transferred onto solid CIM with 1 g/L timentin for culturing in the dark for 2-3 wk. Thereafter, calli were transferred onto solid CIM containing 1g/L timentin and 80 mg/L hygromycin (*Phyto*Technology Laboratories) and subcultured every 4-5 weeks. Subculturing practice was repeated to generate sufficient transgenic material for downstream genetic and biochemical analyses.

3.2.12.2 Analysis of Putative Transgenic Callus Lines

3.2.12.2.1 Total RNA Extraction

Total RNA was isolated from 0.1-1 g of putative transgenic callus material using the Concert Plant RNA Reagent (Invitrogen, Burlington, Ontario) according to the manufacturer's instructions.

Isolated total RNA was subjected to DNase digestion prior to RNA cleanup using the RNase-free DNase Set (Qiagen, Mississauga, Ontario) according to the manufacturer's instructions. Following which, RNA cleanup was conducted using the RNeasy Mini Kit (Qiagen,) according to manufacturer's instructions and assessed for

quality and quantity via gel electrophoresis and spectrophotometry, respectively (Figure 3-4).

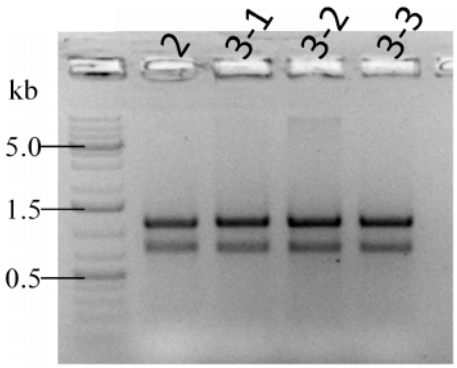


Figure 3-4. Agarose gel electrophoresis of total RNA isolated from four independent 35S::VvF3'5'H cDNA::NOS raspberry putative transgenic callus lines.

3.2.12.2.2 First-Strand cDNA Synthesis

First-strand cDNA was synthesized from 2µg total RNA isolated from putative transgenic callus lines using the SuperScript VILO cDNA Synthesis Kit (Invitrogen).

3.2.12.2.3 Genomic DNA Extraction

Genomic DNA was isolated from the same aforementioned putative transgenic callus lines. An in-house CTAB protocol modified by Dr. Joost Lucker was adopted. 0.2-0.6 g of callus was used for genomic DNA isolation. The protocol is briefly described as follows. Mortar and pestle were washed with 100% ethanol and pre-chilled at -80°C. Putative transgenic calli were ground to a fine powder. 2-3 mL of extraction buffer (2% CTAB (w/v), 100 mM Tris-HCl pH 8, 20 mM EDTA, 1.4 mM NaCl; 4 µL of 2-mercaptoethanol was added to each 1 mL of extraction buffer) was added to the

homogenized sample, collected in a sterile 50 mL falcon tube, mixed by inverting for several minutes, and incubated in a 65°C water bath for 1 hr.

An equal volume of chloroform:isoamyl alcohol (24:1, v/v) was added and mixed by inverting. The sample was then centrifuged at 3500 rpm for 20 min. The upper aqueous phase was collected and transferred to a new sterile 50 mL falcon tube. This extraction was repeated until the dissolution of the phase boundary between aqueous phase and organic phase had been reached.

To precipitate DNA, 0.625 volumes of isopropanol were added to the aqueous phase, incubated at -80°C for 15 min, and centrifuged at 3500 rpm for 30 min. Subsequently, the supernatant was discarded and the pellet was washed with 70% ethanol, centrifuged briefly, and air dried in a 37°C incubator. Extracted DNA was resuspended in autoclaved Milli-Q water and assessed for quality and quantity via gel electrophoresis and spectrophotometry, respectively.

Resuspended DNA was treated with 1 µL RNase A per 100 µL DNA and incubated at 37°C for 1 hr, then 2 µL proteinase K was added and incubated at 37°C for 30 min. Treated DNA was extracted with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1, v/v), mixed by inversion, and then centrifuged at 15000 rpm for 10 min. The upper aqueous phase was collected and extracted repeatedly with an equal volume of chloroform:isoamyl alcohol (24:1, v/v) by invertion to mix and then centrifuged at 15000 rpm for 5 min until the interphase disappeared.

Lastly, DNA was precipitated with 2.5 volume of 100% ethanol and 0.1 volume of 3 M sodium acetate, incubated at -80°C for 1 hr, and centrifuged at 15000 rpm for 20 min. DNA was then washed with 70% ethanol, centrifuged briefly, and air dried in 37°C

incubator. Isolated genomic DNA was resuspended in 10 mM Tris-HCl (pH 8.5) and assessed for quality and quantity via gel electrophoresis (Figure 3-5) and spectrophotometry, respectively.

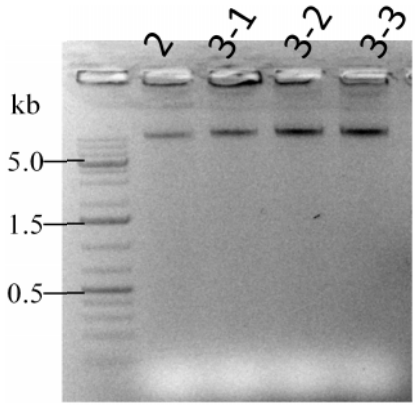


Figure 3-5. Agarose gel electrophoresis of genomic DNA isolated from four independent 35S::VvF3'5'H cDNA::NOS raspberry putative transgenic callus lines.

3.2.12.2.4 PCR Analysis for Transgene Expression and Transgene Presence

Oligonucleotides for standard PCR were designed by CLC Main Workbench software under default parameters. Oligonucleotides were commercially synthesized by Eurofins MWG Operon. cDNA from putative transgenic callus lines were used to detect VvF3'5'H transgene expression with forward primer 5'-TGGTTTTTGCGGATTATGGG-3' and reverse primer 5'TGTAGGTATGGAAGTTTTGG-3'. Genomic DNA isolated from putative transgenic callus lines were used to detect 35S::VvF3'5'H::NOS gene cassette integration with forward primer 5'-ACAATCCCACTATCCTTC-3' and reverse primer 5'-GATAATCATCGCAAGACC-3'. PCR amplifications were carried out with *Pfu* DNA Polymerase (Fermentas Canada). PCR reactions were prepared according to the manufacturer's instructions. PCR conditions were as follows: 1 cycle of 95°C for 3 min,

followed by 35 cycles of 95°C for 30 sec, 50°C for 30 sec, and 72°C for 2 min/kb, and a final extension cycle of 72°C for 5 min.

3.2.13 Histochemical Staining for GUS Activity

3.2.13.1 Preparation of GUS Staining Solution

200 mL GUS staining solution was prepared by combining 150 mL Milli-Q water, 0.744 g of EDTA, disodium salt, dihydrate, 1.76 g of sodium phosphate monobasic, 0.042 g of potassium ferrocyanide, and 0.2 mL of Triton X-100. pH was adjusted to 7.0. 100 mg of X-Gluc dissolved in 2 mL of DMSO was added to 198 mL of solution. GUS staining solution was filter-sterilized and stored at -20°C.

3.2.13.2 Histochemical GUS Assay

Plant calli inoculated with *Agrobacterium* harbouring pCAMBIA2301 were cultured on CIM with 1 g/L timentin without antibiotic selection for two weeks in the dark prior to histochemical staining for GUS activity. 1 mL of GUS staining solution was added to transformed callus and incubated overnight at 37°C. The appearance of blue spots was identified as positives for stable GUS expression. Transformation efficiency was evaluated by counting the total number of blue-coloured spots after the staining procedure.

3.3 Results

3.3.1 Anthocyanin Induction in Callus Cultures

3.3.1.1 Callus Culture Initiation and Maintenance

Raspberry callus lines were established to provide an experimental system for anthocyanin induction and genetic complementation testing for F3'5'H activity. Chemainus and Meeker callus cultures were established in July 2008. There was an observable difference in the friability of the calli produced by the two cultivars. Chemainus calli demonstrated accelerated and robust growth compared to that of Meeker calli. For each cultivar, two types of calli were observed, soft and hard. Chemainus callus was selected for further experimentations due to its rapid growth rate and superior friability (Figure 3-6).

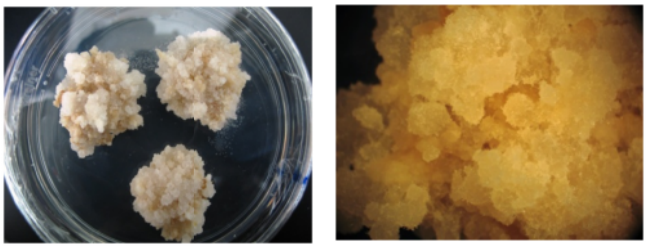


Figure 3-6. *Rubus idaeus* cv. Chemainus callus cultures induced from leaves.

Apple callus lines were established a year later in August 2009 (Figure 3-7). This meant that anthocyanin induction experiments conducted prior to August 2009 did not include apple callus lines. Yellow friable Gala callus exhibited rapid callus growth comparable to that of Chemainus callus but with a consistent puree texture.

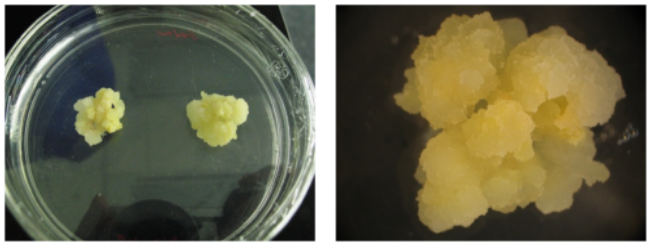


Figure 3-7. *Malus* x *domestica* cv. Gala callus cultures induced from fruit.

Friable Chemainus and Gala calli were selected and plated on different solid anthocyanin induction media. Typically, each treatment consisted of at least two plates with three to four callus clusters on each plate.

3.3.1.2 Manipulation of Culture Medium Composition

3.3.1.2.1 Anthocyanin Induction Medium 1: Effect of Methyl Jasmonate and UV Irradiation

Blando *et al*. (2005) induced anthocyanin production in sour cherry (*Prunus cerasus* L.), a rosaceaous fruit, under light irradiation with and without the addition of jasmonic acid (JA). These authors found that pigmentation appeared after 4-5 days of incubation and reached a maximum after 10-15 days under light exposure alone. The combined effect of jasmonic acid plus light exposure induced anthocyanin production after only 2 days, plateauing after 7 days. Based on these data from cherry, I subcultured raspberry calli on AIM plates (AIM1-8) supplemented with either high concentrations (50, 150, 450 µM) or low concentrations (0.5, 5, 10, 15, 20 µM) of methyl jasmonate (MeJA) at day 0; however, there was no anthocyanin accumulation detected in any of the MeJA-treated raspberry callus cultures after 1 month incubation (data not shown). Selected cultures were subjected to daily 15 min UV irradiation for 3 consecutive days in the laminar flow hood. This implementation of UV treatment also did not promote

anthocyanin production in raspberry callus. While the combination of JA and light was effective for anthocyanin induction in *Prunus* calli (Blando et al., 2005), *Rubus* calli were unresponsive.

3.3.1.2.2 Anthocyanin Induction Medium 2: Effect of Sucrose, Abscisic Acid, and Calcium

Next, I tested the inductive effect of sucrose (5%, 9 % (w/v)), ABA (0.1, 1, 10 μM), and $CaCl_2$ (50 mM) on raspberry callus. Solid CIM (CIM1-11) was supplemented with a single or combination of compound(s) to induce anthocyanin biosynthesis. Because the addition of 50 mM $CaCl_2$ to the culture medium prevented gelation of the agar in the media, it was applied as a solution to individual calli. Decline in callus health was perceived through callus browning and hardening over time and was common to all treated samples.

Elevated levels of sucrose in the culture medium were found to have a negative impact on callus growth. Interestingly, raspberry callus, typically white in colour, acquired a subtle pastel yellow colour in random callus clusters. At day 14, calli incubated on 5% sucrose accumulated higher regional colouration than those incubated on 9% sucrose. As time progressed, a large proportion of callus clusters became yellow-green. This development of colouration and, presumably, the accumulation of induced compounds were visibly more prominent in 5% sucrose-treated samples than in 9% sucrose-treated samples. At the end of 39 days, no anthocyanin accumulation was observed.

Calli incubated on ABA-supplemented CIM showed arrested growth throughout the duration of treatment. Similar to sucrose-treated samples, parts of the calli developed colouration that transitioned from yellow to yellow-green. This was particularly evident in 0.1 and 10 μM ABA-treated samples after 39 days; however, 1 μM ABA did not elicit a similar response in the treated samples. Unexpectedly, after 62 days, limited and localized accumulation of anthocyanins in a single callus cluster that had previously undergone yellow colouration on a 10 μM ABA-treated plate was observed; however, anthocyanin accumulation could not be sustained upon transfer of this individual piece of callus to fresh medium.

While combinatorial treatments were expected to have an additive effect on anthocyanin induction, similar responses resulted as those elicited by individual inductive treatments. CIM supplemented with 5% sucrose, 10 μM ABA, and 50 mM $CaCl_2$ induced a low incidence of anthocyanin accumulation in raspberry callus after 62 days of incubation in the absence of subculturing practices. It appears that the accumulation of yellow-coloured compounds predates the accumulation of anthocyanins. Typical progressions of phenotypic changes in response to inductive treatments are shown in Figure 3-8. Despite the fact that negligible anthocyanin accumulation was observed in treated raspberry callus, the lengthy time period required to reach this endpoint as well as the low percentage of cells exhibiting anthocyanin production made this induction treatment highly unfeasible as a standard procedure.

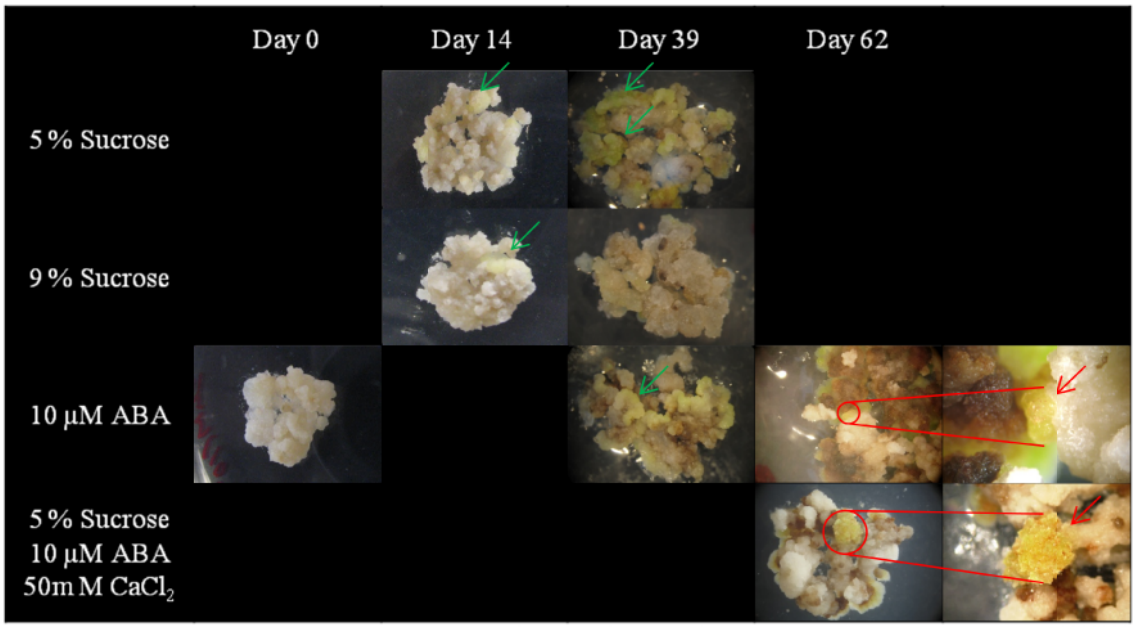


Figure 3-8. Effect of sucrose, ABA, and $CaCl_2$ on anthocyanin induction in raspberry callus cultures.

3.3.1.2.3 Anthocyanin Induction Medium 3: Effect of Nutrient Depletion

Given that the exogenously applied stimuli tested thus far for anthocyanin induction were ineffective in raspberry calli, a minimalist approach was taken. Minimal medium (MM), consisting of only sucrose and a gelling agent, was used to study the effect of nutrient depletion on anthocyanin induction in raspberry callus. Granulated Agar (Fisher Scientific) was substituted for Phytagel (Sigma-Aldrich Canada) because Phytagel requires the presence of divalent cations for gelling to occur. It was found that raspberry calli were able to tolerate minimal growth conditions while exhibiting regional browning and arrested growth (Figure 3-9); however, I did not observe any anthocyanin accumulation in the nutrient-stressed raspberry callus.

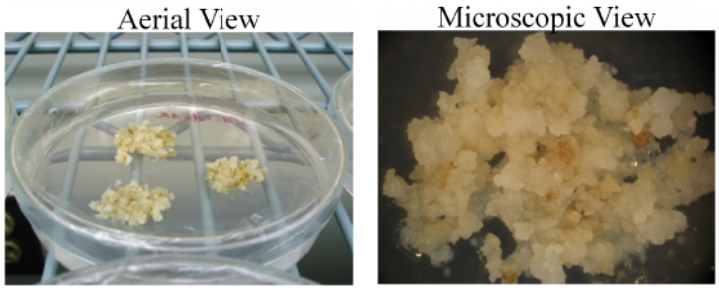


Figure 3-9. Effect of nutrient depletion on anthocyanin induction in raspberry callus cultures after 57 days of incubation.

3.3.1.2.4 Anthocyanin Induction Medium 4: Effect of Gamborg B5 Basal Medium

First developed for the initiation and growth of soybean suspensions, Gamborg B5 basal medium (Gamborg *et al*., 1968) has since been commonly used to replace Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) in order to increase secondary metabolite production in strawberry suspension cultures (Mori and Sakurai, 1994), cranberry callus cultures (Madhavi *et al*., 1995), ohelo cell cultures (Fang *et al*., 1999), and grapevine suspension cultures (Curtin *et al*., 2003).

Gamborg B5 basal media were supplemented with either 0.1 mg/L 6-benzylaminopurine (BAP) and 1.0 mg/L naphthaleneacetic acid (NAA) or 0.5 mg/L BAP and 5.0 mg/L NAA, named as GBM1 and GBM2, respectively. I found that GBM1 and GBM 2 promptly induced the accumulation of yellow to yellow-green coloured compounds after 22 days of incubation (Figure 3-10). The rate at which this was achieved was considerably faster than that of sucrose, ABA, and $CaCl_2$-treated samples. Additionally, the intense colouration in these treated samples suggested greater accumulation of induced compounds.

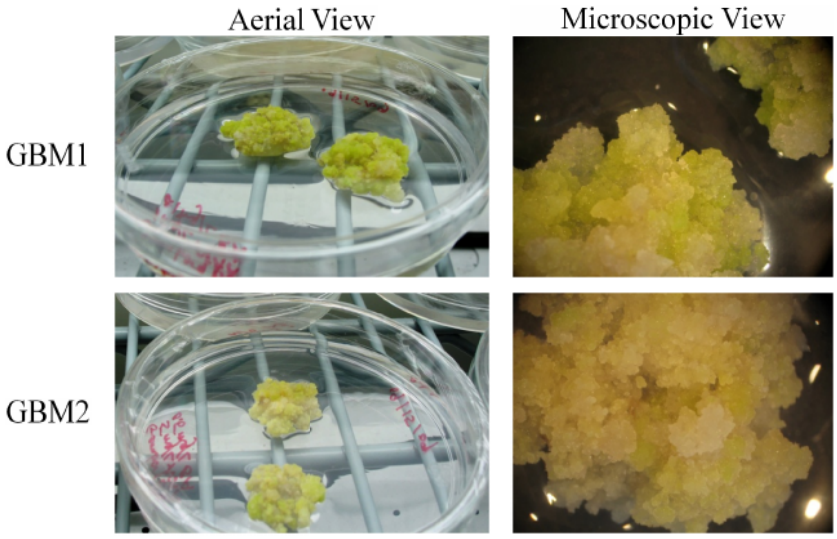


Figure 3-10. Effect of Gamborg B5 basal medium on anthocyanin induction in raspberry callus cultures after 22 days of incubation.

3.3.1.2.5 Anthocyanin Induction Medium 5: Effect of Nitrogen Deficient Media

Three types of nitrogen deficient basal salt mixture were used to test the effect of nitrogen stress on anthocyanin induction in callus cultures: NDM1 (without nitrogen, phosphate, and potassium), NDM2 (without nitrogen), and NDM3 (without ammonium nitrate). Raspberry calli incubated on NDM1, NDM2, and NDM3 did not show any signs of cell proliferation nor anthocyanin accumulation. While previous treatments induced the accumulation of yellow to yellow-green coloured compounds, nitrogen-stressed raspberry callus became grey in appearance (Figure 3-11).

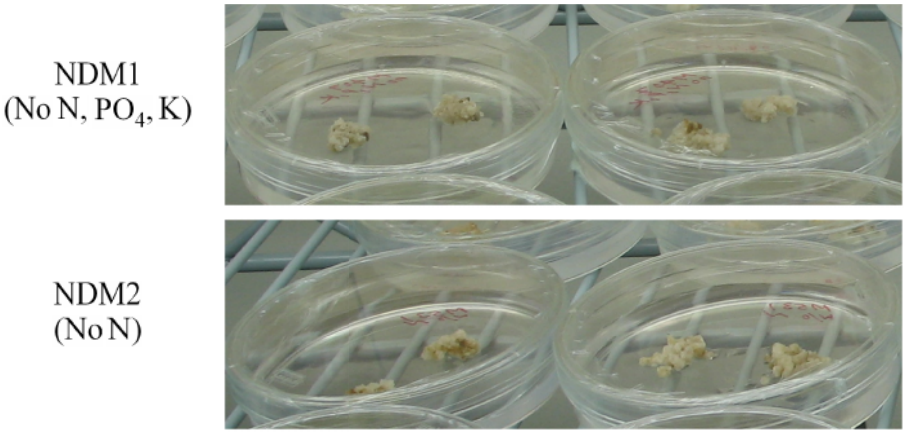


Figure 3-11. Effect of nitrogen deficient media on anthocyanin induction in raspberry callus cultures.

3.3.2 Introduction of Apple as a Comparative Model

3.3.2.1 Anthocyanin Induction Medium 6: Effect of Nitrate and 2,4-D Deficient Media

Given the unexpected lack of anthocyanin inducibility of raspberry calli, apple callus was introduced as a comparative model to test whether this was a more broad limitation of the Rosaceae or specific to raspberry. Independently, raspberry and apple calli were incubated on NDM4 under continuous light to investigate the combined effect of nitrate and 2,4-D deficient media. A marked difference in the elicited response between raspberry and apple callus cultures was detected over a two week span.

Phenotypically, raspberry callus remained relatively unchanged. Interestingly, some callus clusters also developed yellow colouration reminiscent of those sucrose- and ABA-treated samples, albeit less pronounced (Figure 3-12). Given the length of the experimental period, callus browning was not substantial.

In stark contrast to raspberry callus, apple callus cultures began accumulating anthocyanins accumulation two days following incubation on solid NDM4. Anthocyanin

accumulation gradually increased over time and plateaued between 10-14 days (Figure 3-12). The rate of anthocyanin accumulation was not uniform across individual callus clusters with some responding more rapidly and/or slowly than others. While anthocyanin production was found primarily on the topical surface of each callus mass under direct light exposure, the distribution was widespread.

Neither raspberry nor apple callus accumulated anthocyanins when incubated in continuous dark; however, upon transfer to continuous light, apple callus began accumulating anthocyanins (Figure 3-12). These results strongly emphasize the importance of light for inducing anthocyanin accumulation. I have shown that the nitrate and 2,4-D deficient media can rapidly and effectively induce anthocyanin production in apple callus in less time than light alone, but not in raspberry callus.

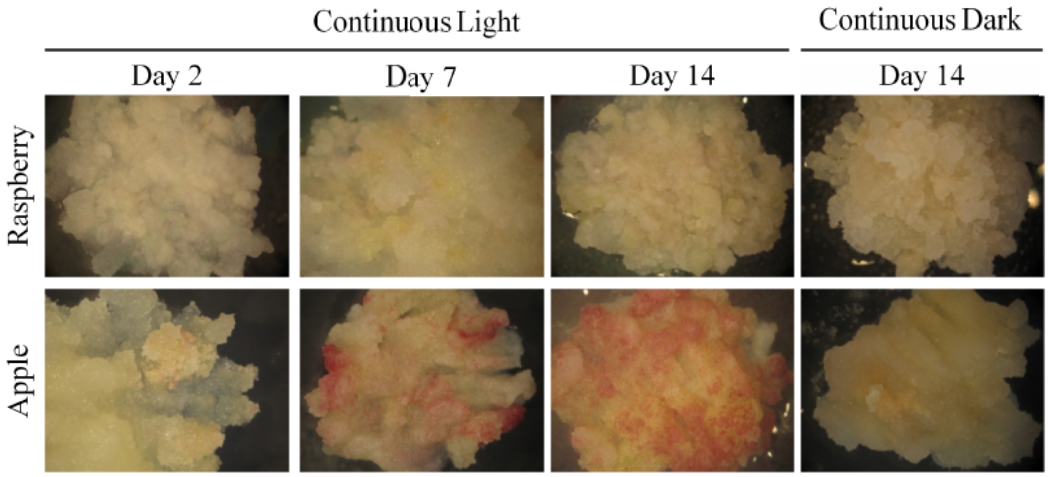


Figure 3-12. Effect of nitrate and 2,4-D deficient media on anthocyanin induction in raspberry and apple callus cultures.

3.3.2.2 HPLC-MS "Fingerprinting" Analysis of Induced Callus Cultures

To investigate the tentative identity of flavonoid compounds produced under elicitation, raspberry and apple calli cultured on NDM4 under continuous light were

extracted with acidified methanol 12 days after induction was initiated. Three separate control incubation treatments were included to account for metabolic differences that may be attributed to nitrate and 2,4-D deficient media under continuous light: 1) CIM under continuous darkness, 2) NDM4 under continuous dark, and 3) CIM under continuous light. Each treatment was done in three plates with three callus clusters each. For each treatment, an HPLC chromatogram of each replicate was overlaid and the mass spectrum of each replicate was thoroughly scanned to confirm reproducible data. One representative replicate was selected from each treatment for tentative compound identification. HPLC chromatograms at 320 nm for each treated sample are shown in Figures 3-13 and 3-14 for raspberry callus and apple callus, respectively.

Flavonoid compounds were tentatively determined by UV-visible absorption spectra and mass spectra. Absorption maxima were manually detected. These measurements were matched against findings from Jurd (1957), Mabry *et al*. (1970), Harbone (1989), Rice-Evans *et al*. (1996) and Cerovic *et al*. (2002) for the tentative classification of flavonoid types. The tentative identification of each UV peak for NDM4 under continuous light-treated samples is summarized in Tables 3-6 and 3-7 for raspberry callus and apple callus, respectively. Common characteristics associated with the UV profiles of flavonoids were considered. Typically, flavonoids have two UV absorption maxima in the 200-400 nm region: Band II (210-290 nm) and Band I (300-400nm) (Mabry *et al*., 1970). Band I is due to the absorption of the benzoyl structure (A ring) and Band II is associated with the hydroxycinnamoyl structure (B and C ring). In most cases, glycosylation causes a shift of Band I maximum towards the shorter wavelength and, often, Band I is a minor band for isoflavones and flavanones (Cerovic *et al*., 2002).

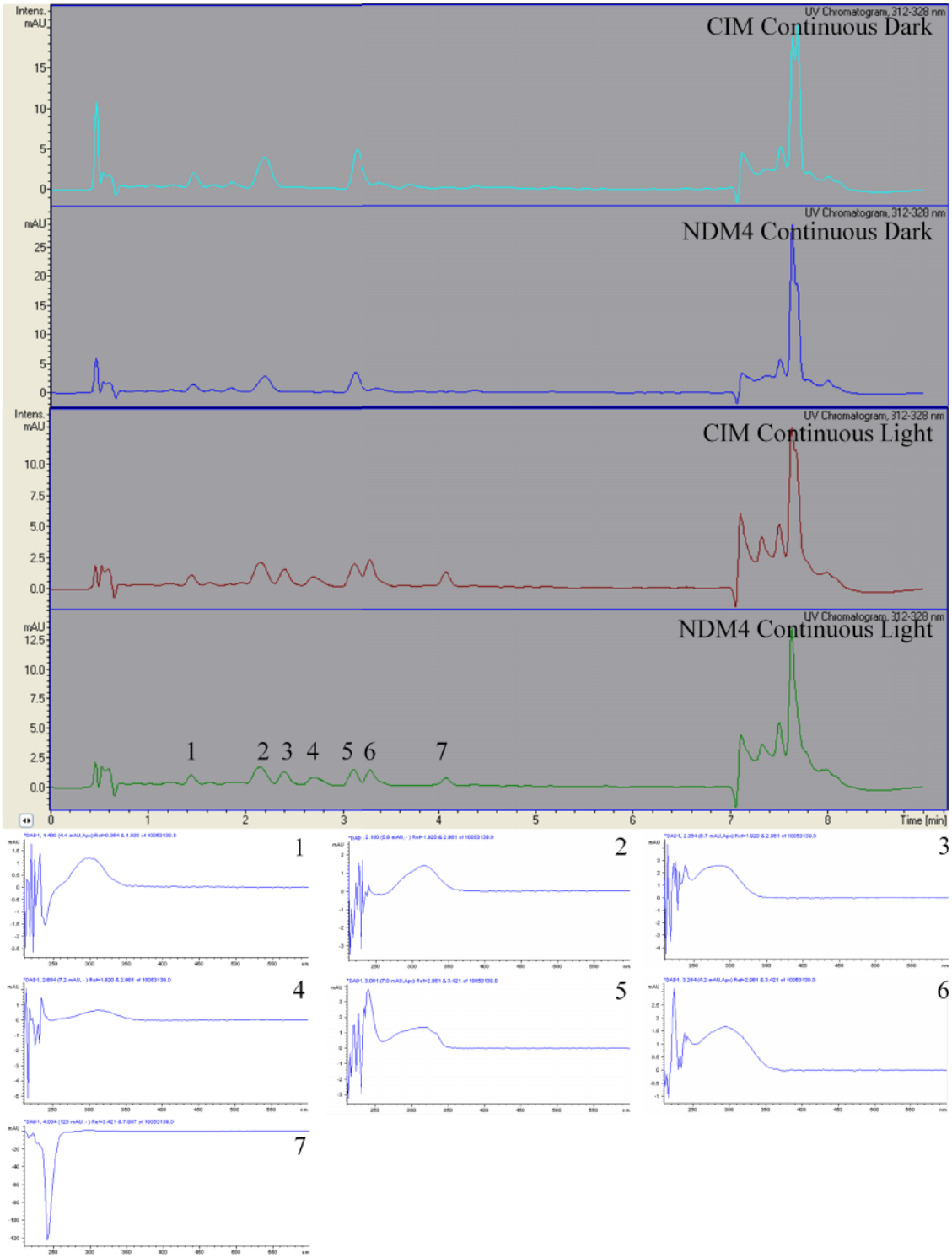


Figure 3-13. HPLC chromatogram at 320 nm of acidified methanol extract of treated raspberry callus for anthocyanin induction and UV spectrum of each peak from NDM4 under continuous light-treated callus.

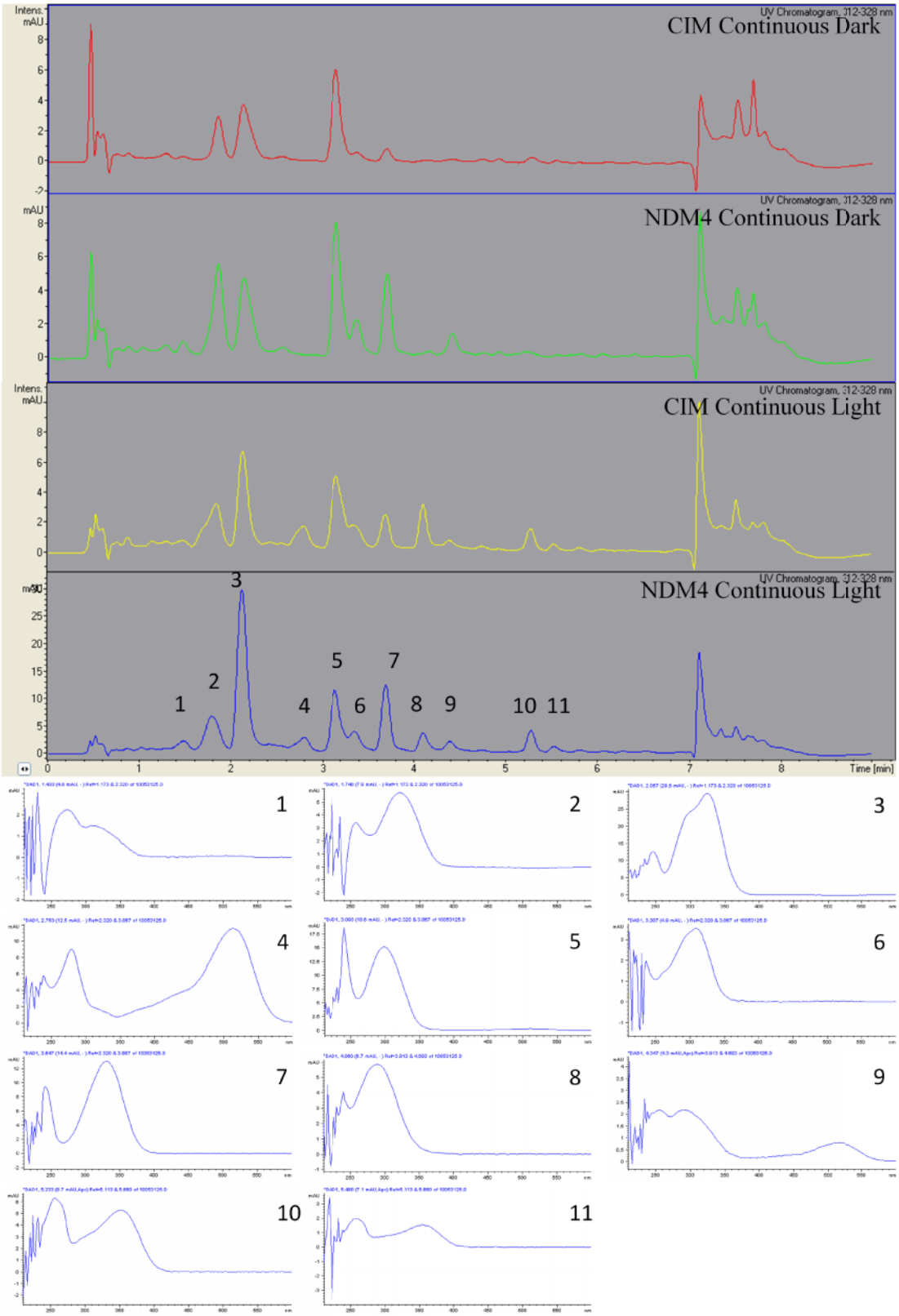


Figure 3-14. HPLC chromatogram at 320 nm of acidified methanol extract of treated apple callus for anthocyanin induction and UV spectrum of each peak from NDM4 under continuous light-treated callus.

Interpretation of UV absorbance spectrum and mass spectrometry data was challenging. Peak annotation in raspberry calli was complicated by a lack of definitive characteristic UV absorption pattern, given low quantity and co-elution of possibly one or more compounds, as evident from the presence of multiple molecular ions. Collectively, seven peaks were detected in each of NDM4 and CIM under continuous light-treated raspberry callus. The tentative identity of each of the seven peaks relevant to flavonoid class is summarized in Table 3-6. There was no difference between NDM4 and CIM-treated samples; however, light irradiation specifically induced the accumulation of peaks 3, 4, 6, and 7 that were absent in culture conditions without light (Figure 3-13); however, preliminary data showed that, given sufficient incubation time on NDM4, flavonols were detected in treated raspberry callus lines (data not shown).

Table 3-6. Tentative peak identification of HPLC chromatogram at 320 nm of acidified methanol extract of NDM4 under continuous light-treated raspberry callus for anthocyanin induction based on UV-visible absorption spectra of different flavonoid types.

Peak No.	Time (min)	λ_{max} (nm)			Flavonoid Class
		Band II	Shoulder	Band I	
1	1.400	-	250	300	Hydroxycinnamic acid derivatives
2	2.100	-	290	315	Hydroxycinnamic acid derivatives
3	2.354	285	-	-	Flavanones
4	2.654	235	-	315	Hydroxycinnamic acid derivatives
5	3.061	240	-	320	Hydroxycinnamic acid derivatives
6	3.254	225	-	295	Hydroxycinnamic acid derivatives
7	4.034	-	260	300	Hydroxycinnamic acid derivatives

As shown by the presence of peaks 4 and 9 in Figure 3-14, anthocyanins were induced in apple calli. A total of eleven compounds were detected in NDM4 continuous light-treated calli. Similar polyphenolic profiles were found in CIM continuous light- and NDM4 continuous light-treated samples. Light stimulus was instrumental in the induction

of anthocyanin and flavonol biosynthesis, as shown by the appearances of peaks 4, 9, 10, and 11. Peak 3, tentatively identified as chlorogenic acid, was the most abundant phenolic species found in apple callus and most responsive to the treatments.

Table 3-7. Tentative peak identification of HPLC chromatogram at 320 nm of acidified methanol extract of NDM4 under continuous light-treated apple callus for anthocyanin induction based on UV-visible absorption spectra of different flavonoid types.

Peak No.	Time (min)	λ_{max} (nm)			Flavonoid Class
		Band II	Shoulder	Band I	
1	1.433	270	-	310	Flavanones
2	1.740	260	-	320	Flavones
3	2.067	-	295	325	Hydroxycinnamic acid derivatives
4	2.753	280	-	510	Anthocyanins
5	3.093	240	-	300	Hydroxycinnamic acid derivatives
6	3.307	-	295	310	Hydroxycinnamic acid derivatives
7	3.647	240	-	330	Hydroxycinnamic acid derivatives
8	4.060	-	-	290	Hydroxycinnamic acid derivatives
9	4.347	290	-	520	Anthocyanins
10	5.233	255	-	350	Flavonols
11	5.480	260	-	355	Flavonols

HPLC-MS "fingerprinting" analysis of induced callus cultures provided insight into the efficacy of the given treatments and the tentative identity of induced metabolites present in treated callus versus wildtype callus.

3.3.3 Anthocyanin Induction in Cell Suspension Cultures

3.3.3.1 Suspension Culture Initiation and Maintenance

Cell suspensions were established as an alternate experimental system to callus cultures for anthocyanin induction to test the effect of the culture medium phase as well as to broaden the type of elicitors used. Raspberry and apple suspensions demonstrated cell multiplication and browning was not observed due to frequent subcultures. Raspberry

cell suspensions were found to form small to medium sized aggregates. Comparatively, apple cell suspensions were considerably finer and denser with diffuse cells.

3.3.3.2 Manipulation of Culture Medium Composition

3.3.3.2.1 Anthocyanin Induction Medium 7: Effect of Exogenous Phenylalanine

Stock phenylalanine (L-Phe) solution was added to raspberry or apple cell suspensions to a final concentration of 50 μM, 100 μM, 1 mM, or 2.5 mM (PHE1-4) to study the effect of single substrate feeding at inoculation on anthocyanin accumulation. I found that the addition of L-Phe to apple cell suspensions did not induce anthocyanin production nor promote cell growth; however, L-Phe feeding evoked a range of concentration-dependent responses in raspberry cell suspensions (Figure 3-15). High L-Phe concentrations (1 and 2.5 mM) caused severe browning, particularly evident in large cell aggregates. At intermediate L-Phe concentration (100 μM), there was no discernible difference from untreated cultures. The addition of 50 μM L-Phe resulted in yellow-green colouration of a small portion of cell aggregates. This was similar to what was previously observed in sucrose-, ABA-, and GBM-treated callus cultures.

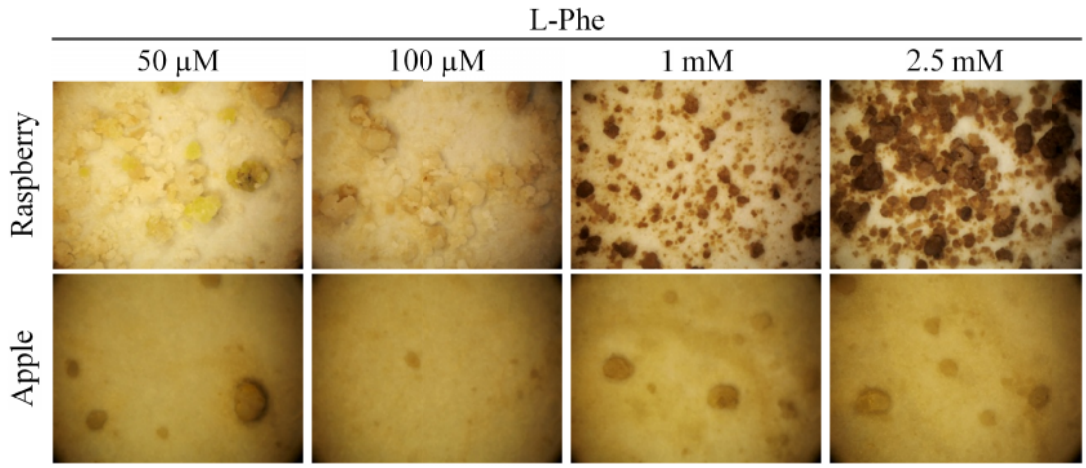


Figure 3-15. Effect of precursor feeding of exogenously applied L-Phe on anthocyanin induction in raspberry and apple cell suspension cultures after 14 days of incubation.

3.3.3.2.2 Anthocyanin Induction Medium 8: Effect of Free Radical Generator, AAPH

Next, raspberry and apple cell suspensions were treated with varying concentrations of AAPH (0.5, 2, 5, and 10 mM) at inoculation. The free radical scavenging activity of berry extracts has been correlated to high anthocyanin content (Bao *et al*., 2005; Radovanovic and Radovanovic, 2010). It was, thus, of interest to test whether the introduction of free radicals could induce anthocyanin accumulation. Treatment with AAPH (AAPH1-4) did not induce anthocyanin accumulation in raspberry or apple cell suspensions. AAPH did promote slight browning with increasing concentrations of AAPH leading to greater browning in raspberry cell suspensions (Figure 3-16).

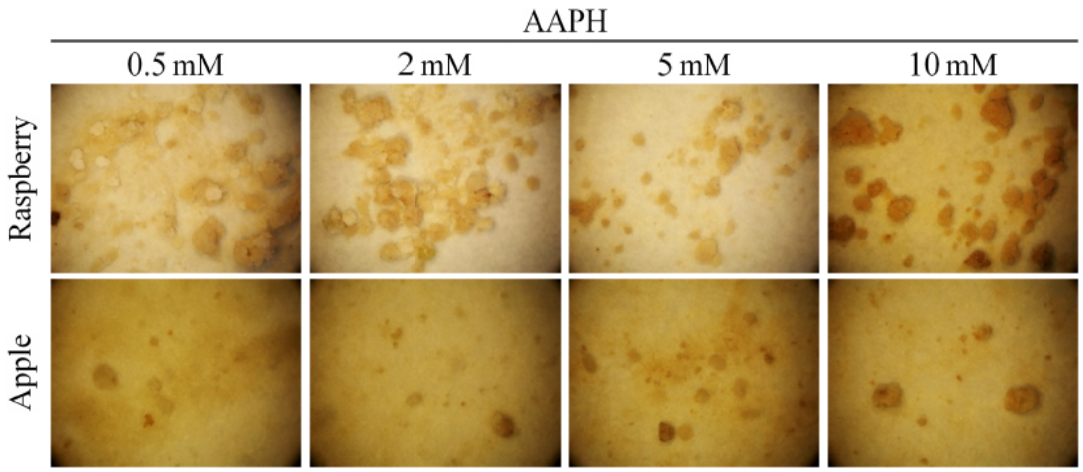


Figure 3-16. Effect of free radical generator, AAPH, on anthocyanin induction in raspberry and apple cell suspension cultures after 14 days of incubation.

3.3.3.2.3 Anthocyanin Induction Medium 9: Effect of Phenylalanine, Sucrose, and Methyl Jasmonate

Based on the results obtained from the L-Phe feeding experiment, I explored the effect of a double treatment with L-Phe (0.05 and 0.025 µM) and either sucrose (15, 30 %) or MeJA (0.5, 50 µM) (PSM1-8) on raspberry cell suspensions. I found that all suspensions underwent severe browning when treated with 50 µM MeJA, regardless of exogenous L-Phe concentration (Figure 3-17). The combined effect of L-Phe and sucrose, as well as L-Phe and low concentrations of MeJA, induced the production of coloured compounds in a few clusters of cells. None of the treatments induced anthocyanin accumulation in individual cells or cell clusters.

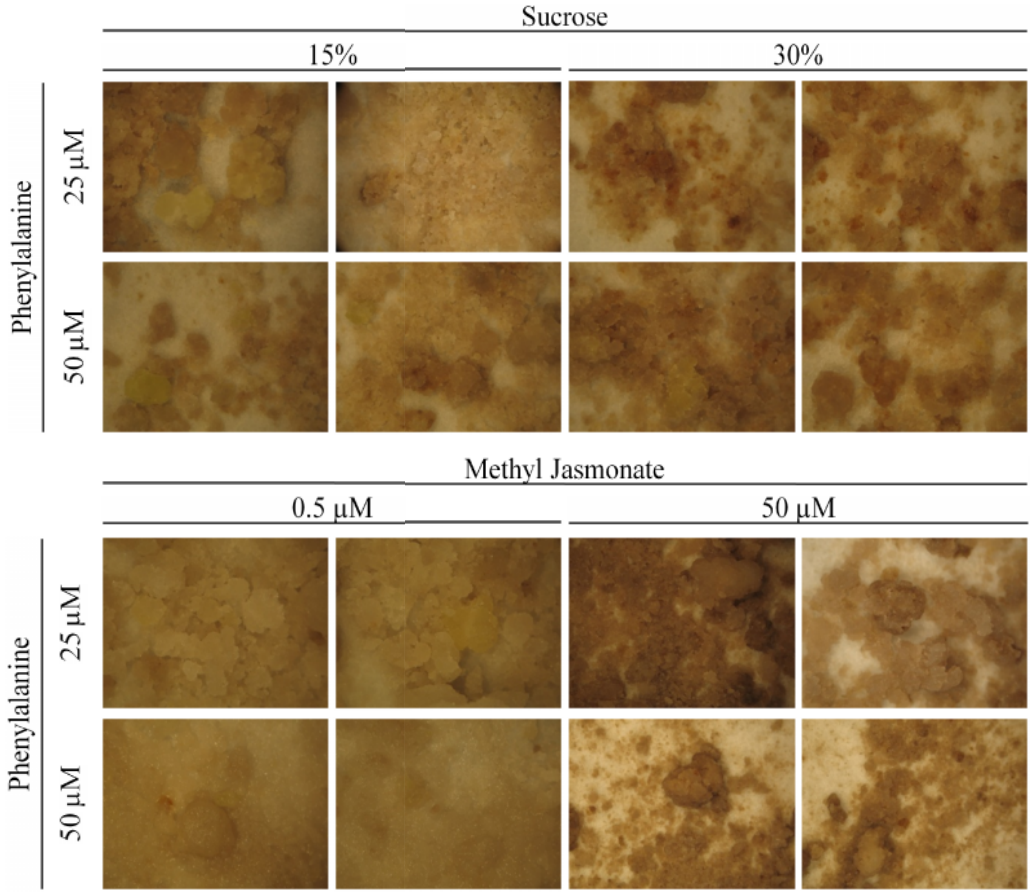


Figure 3-17. Effect of (A) phenylalanine and sucrose and (B) phenylalanine and methyl jasmonate on anthocyanin induction in raspberry cell suspension cultures.

3.3.4 *Agrobacterium*-Mediated Genetic Transformation of Plant Callus Lines

3.3.4.1 Establishing Antibiotic Selection Pressure Threshold for Screening Transformants

Small clusters of wildtype raspberry and apple callus were incubated in the dark on solid CIM containing different concentrations of hygromycin (10, 20, 30, 40, 50, and 100 mg/L) for a period of 5 weeks without subculturing. I found that intermediate to high levels of hygromycin arrested callus growth in both raspberry and apple lines (Figure 3-18). While browning was uniformly distributed at all concentrations tested, I did not observe any significant dose-dependent response. Low concentrations of hygromycin (10

and 20 mg/L) were considered ineffective since some callus growth was detected in both raspberry and apple calli.

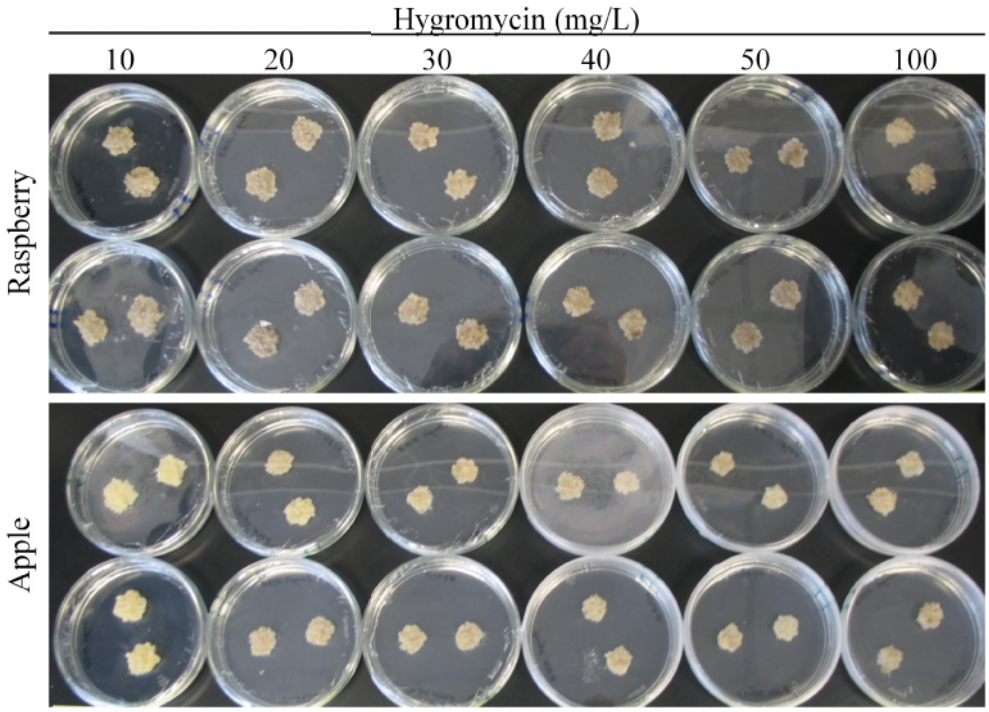


Figure 3-18. Effect of hygromycin selection pressure against wildtype raspberry and apple callus cultures after 5 weeks of incubation.

3.3.4.2 Establishing *Agrobacterium*-Mediated Genetic Transformation Competency in Raspberry and Apple Callus Lines

Transgenic plant calli harbouring the β-glucuronidase (GUS) gene under the control of the constitutive CaMV 35S promoter exhibited stable GUS expression after two weeks of incubation on solid CIM containing 1 g/L timentin as shown in Figure 3-19. Transformation experiments were done in triplicate (3 plates with 2-3 g of callus per plate) per species and transformation trials were repeated more than twice. Histochemical assay of wildtype callus and callus transformed with wildtype *Agrobacterium* did not result in the appearance of localized blue staining. The level of GUS expression was

found to vary between raspberry and apple callus lines and between transformation experiments indicating differences in transformation efficiency. Additionally, there is an inverse relationship between the level of GUS expression and the rate of cell growth post-transformation.

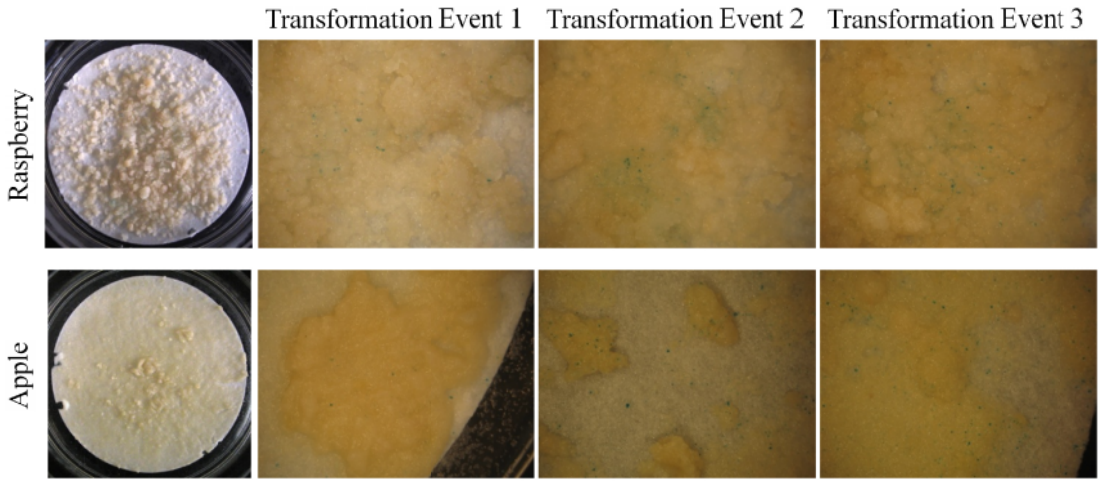


Figure 3-19. *35S::GUS* expression in *Agrobacterium*-mediated genetic transformation of raspberry and apple callus lines.

3.3.4.3 Establishing Transgenic Callus Lines for Genetic Complementation of the Absence of Dihydromyricetin-Derived Flavonoids

Three independent callus lines each from raspberry and apple were inoculated with *Agrobacterium tumefaciens* harbouring a recombinant vector with the *hpt* gene as the selectable marker and the 35S::VvF3'5'H cDNA::NOS gene cassette. Transformations of No Vector Control (NVC) and pCAMBIA2301 (35S::GUS) into separate calli were included as negative control and positive control. I screened for transgenic plant calli expressing hygromycin resistance by selecting actively proliferating cell masses against those that exhibited browning and arrested growth. Clusters of new

growth were transferred and maintained on fresh selection medium consisting of CIM containing 1 g/L timentin and 80 mg/L hygromycin every 4-5 weeks.

I found that the rate of post-transformation callus growth in response to hygromycin selection pressure was significantly reduced compared to wildtype calli grown on solid CIM. After 15 weeks of incubation on selection medium, there was insufficient growth of putative transgenic apple calli for any downstream molecular or biochemical analyses; however, PCR analysis was attainable for raspberry putative transgenic callus lines to test for VvF3'5'H transgene expression. It was found that F3'5'H transcripts were readily detected in all four raspberry transgenic callus lines tested (Figure 3-20).

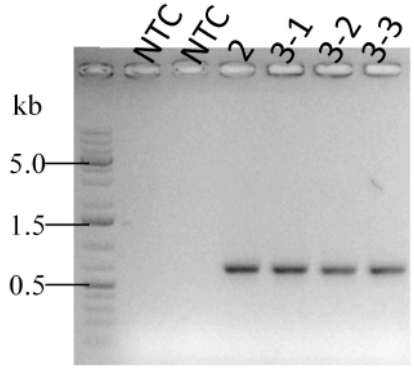


Figure 3-20. PCR analysis for detecting F3'5'H gene expression in four independent 35S::VvF3'5'H cDNA::NOS raspberry putative transgenic callus lines.

Additionally, genomic DNA was isolated from the same four raspberry transgenic callus lines to test for integration of 35S::VvF3'5'H cDNA::NOS gene cassette into the raspberry genome. PCR analysis showed amplification of full-length gene fragment flanked by CaMV 35S promoter on one side and NOS terminator on the other (Figure 3-21). This PCR product was sequence verified to be VvF3'5'H. Transgene copy number was not determined.

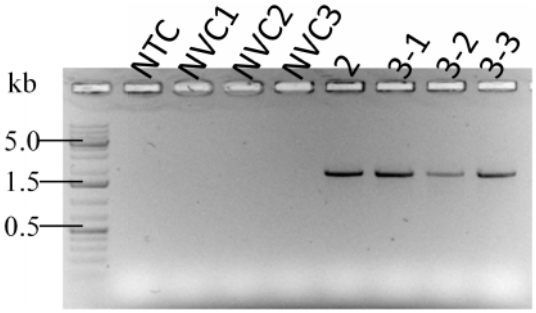


Figure 3-21. PCR analysis for detecting 35S::VvF3'5'H cDNA::NOS gene cassette insertion in raspberry putative transgenic callus lines.

Although the isolation of total RNA and genomic DNA from raspberry NVC callus lines was attempted, it was found that the selection pressure imposed by hygromycin obviated high quality RNA or DNA isolation from these callus lines. It was not surprising to find the lack of nucleic acids as both DNA and RNA are rapidly degraded in dying cells, which further supports antibiotic resistance conferred by 35S::VvF3'5'H cDNA::NOS vector construct in actively proliferating callus cells. Collectively, these results showed constitutive expression of VvF3'5'H transgene in stably transformed raspberry callus lines.

3.4 Discussion

3.4.1 Choice of Plant Experimental System

Genetic complementation tests provide an analytical tool to decipher gene function in plants and other organisms. Additionally, complementation tests are facilitated by an annual growth habit and rapid time from seed to seed. Raspberry is a woody perennial plant with biennial canes in which vegetative canes (primocanes) grow one year and become fruiting canes (floricanes) the next (Jennings, 1988; Graham and Jennings, 2009). Although direct and indirect plant regeneration in *Rubus* has been

reported, studies have shown reduced incidence of organogenesis in raspberry explants after co-cultivation with *Agrobacterium* (Hassan *et al*., 1993; Mathews *et al*., 1995). Taken together, the regeneration of transgenic raspberry plants was simply not feasible for the scope of this M.Sc. thesis; therefore, I elected to conduct genetic complementation tests in a cell culture system.

3.4.2 Establishing *Agrobacterium*-Mediated Genetic Transformation Competency in Raspberry and Apple Callus Lines

I have shown that calli from both the Chemainus raspberry cultivar and the Gala apple cultivar are amenable to *Agrobacterium*-mediated genetic transformation, based on GUS reporter assays. There have been a number of publications on *Agrobacterium*-mediated transformation in rosaceous fruits with principal interests in *Fragaria*, *Malus*, *Prunus*, and *Pyrus* species (Aldwinckle and Malnoy, 2009). To my knowledge, this thesis is the first published report of *Agrobacterium*-mediated genetic transformation in raspberry and apple callus.

Aldwinkle and Malnoy (2009) provided a comprehensive review of the genetic engineering resources available in Rosaceae to study the function of annotated genome sequence data. *Agrobacterium*-mediated gene transfer has been documented in 30 different apple cultivars using young expanded apple leaves as explants. The most common strains of *A*. *tumefaciens* used in apple transformation are EHA105 and EHA101. While kanamycin is more widely used as a selectable marker in the regeneration of apple transgenic plants, hygromycin has also been used (Dolgov *et al*., 2000; Dolgov *et al*., 2004). Typically, transformation efficiency is defined as the

percentage of explants that produce a transgenic shoot (Aldwinkle and Malnoy, 2009). On average, the reported transformation efficiency of Gala and sports of Gala is between 0.4-20% (Yao *et al*., 1995; Puite and Schaart, 1996; Norelli *et al*., 1999). Alternately, only 5 raspberry cultivars, Comet, Candy, Chilliwak, Meeker, and SCRI8242E6, have been tested for *Agrobacterium*-mediated transformation (Graham *et al*., 1990; Mathews *et al*., 1995; de Faria *et al*., 1997). Explants, such as leaf disk, petiole, and stem, have been successfully transformed in raspberry. Similar to gene transfer practices in apple, EHA105 is a commonly used *Agrobacterium* strain and both kanamycin and hygromycin have been used as selectable markers depending on the cultivar. The published transformation efficiencies amongst these cultivars ranged from 0.37 to 49.6%. Although carbenicillin and cefatoxime had been the popular antibiotic choice for the suppression of *Agrobacterium* after co-cultivation, timentin was found to be an inexpensive alternative that was equally, if not more, effective (Cheng *et al*., 1998).

The difference in transformation efficiency between raspberry and apple can be attributed to a myriad of factors including cell number, cell density, and the metabolic state of the cell (Maximova *et al*., 1998). Additionally, the effect of explant characteristics, such as source, age, and genotype, can influence the susceptibility of host cells to *Agrobacterium* transfection.

3.4.3 Testing Protocol for Anthocyanin Induction in Raspberry and Apple Cell Lines

Unlike calli from *Vitis vinifera* L. cv. Gamay Freaux (Cormier et al., 1994), no raspberry callus genotype constitutively produces anthocyanins *in vitro*. Here, it was important to first establish that leaf-derived raspberry calli are competent for anthocyanin biosynthesis in order to generate a visible phenotype in a timely manner. In nature, secondary metabolites, including anthocyanins, are produced in response to biotic and abiotic environmental stresses. Similarly, I aimed to simulate those stressors by manipulating the compositions of culture media.

To the best of my knowledge, there has not been any reported case of anthocyanin induction in raspberry cell cultures. Also, there is a lack of literature on inducing anthocyanins from previously non-anthocyanin-producing cell cultures; however, other members belonging to the Rosaceae family, such as sour cherry (Blando *et al*., 2005) and apple (Li *et al*., 2004), have been shown to be capable of accumulating anthocyanins in callus cultures.

3.4.3.1 Effect of Various Induction Factors for Anthocyanin Biosynthesis *In Vitro*

In my experiments with raspberry calli, the individual effects of sucrose and ABA treatments on anthocyanin production were evaluated against the combined effect of sucrose, ABA, and $CaCl_2$. While I expected an additive effect from the combined treatment, the course of colour development was similar in all treated samples; however, the endpoint of yellow-green pigmentation was not reached in sucrose, ABA, and $CaCl_2$-treated raspberry calli, as it was in sucrose- and ABA- treated raspberry calli. Although

limited anthocyanin production was detected in 10 μM ABA-treated Chemainus callus, it was considered an isolated and rare event that could not be replicated.

Jasmonic acid and its methyl ester, methyl jasmonate, play an integral role in the intracellular signalling cascade that activates transcription of specific genes involved in secondary metabolism. They are frequently used in cell culture experiments for the induction of anthocyanin biosynthesis. While the effect of jasmonic acid as an elicitor was successfully exploited by Blando *et al*. (2005), raspberry callus cultures did not respond accordingly.

The incorporation of carbohydrates in *in vitro* cultures is necessary to provide a source of carbon for cell respiration and structure (George *et al*., 2008). High sucrose concentrations have been shown to positively influence anthocyanin accumulation. In addition to its nutritional role as a carbon source, this can be attributed to its physical role as an osmotic agent and its breakdown products of simple sugars, glucose and fructose, where glucose can be utilized by glucosyltransferases to stabilize the flavylium cation in the last step of the anthocyanin biosynthetic pathway (Do and Cormier, 1990; Do and Cormier, 1991; Rajendran *et al*., 1992). Nagarajan *et al*. (1989) and Mori and Sakurai (1994) tested the effects of different carbon sources on cell growth and anthocyanin production in carrot and strawberry, respectively, and reached opposing conclusions. While experimental data from Mori and Sakurai (1994) suggested that glucose, sucrose, and fructose are effective at enhancing anthocyanin accumulation in cell suspensions, Nagarajan et al. (1989) found that sugar combinations, such as galactose-glucose and galactose-sucrose promoted anthocyanin production more effectively than any single sugar. This may suggest a species-specific response to sugars; however, sucrose is

generally considered to be the best carbon source in terms of carbohydrate nutrition in plant tissue culture, followed by glucose and maltose (George *et al*., 2008).

While the positive effect of sugars on anthocyanin production appears consistent among different species, the effect of ABA is less so. Both the suppression and stimulation of anthocyanin biosynthesis caused by exogenously applied ABA have been reported in intact plant organs and in *in vitro* culture systems. For example, in radish seedlings, ABA inhibited anthocyanin synthesis that could not be restored by enrichment with precursors (Guruprasad and Laloraya, 1980). Similar findings were observed in petunia flowers (Weiss *et al*., 1995) and in carrot suspension cultures (Ozeki and Komamine, 1986). In contrast, ABA induced anthocyanin accumulation in Arabidopsis (Loreti *et al*., 2008), regenerated torenia shoots (Nagira *et al*., 2006), rice seedlings (Hung *et al*., 2008), grapevine leaf and fruit tissues (Pirie and Mullins, 1976), apple pulp (Kondo *et al*., 2001), and strawberry fruit (Jiang and Joyce, 2003). Some of these studies showed that ABA acted synergistically with sucrose in promoting anthocyanin biosynthesis (Pirie and Mullins, 1976; Nagira *et al*., 2006; Loreti *et al*., 2008). Although the mechanism of ABA action on anthocyanin biosynthesis is not yet defined, it has been suggested that exogenous ABA may influence the level of endogenous ABA, which affects ethylene production leading to up-regulation of PAL activity. Note the evidence that plants from the Rosaceae family were competent for the stimulatory effect of ABA on anthocyanin production, as shown in apple and strawberry.

The involvement of calcium and calcium channel modulators has been previously demonstrated in grapevine suspension cultures (Vitrac *et al*., 2000) and carrot callus cultures (Sudha and Ravishankar, 2003). Depletion of extracellular calcium ions

effectively reduced anthocyanin accumulation (Vitrac *et al*., 2000). Sudha and Ravishankar (2003) found that treatment with a calcium ionophore enhanced cell growth and anthocyanin production; treatment with calcium-channel blockers reduced cell growth and anthocyanin production. Furthermore, anthocyanin accumulation was elevated with increasing calcium concentrations in apple callus cultures (Li *et al*., 2004). It has been suggested that calcium is a component of phytochrome signalling that helps to facilitate the expression of genes required for anthocyanin biosynthesis in response to light exposure (Neuhaus *et al*., 1993; Bowler *et al*., 1994).

Nitrogen is essential for cell growth. Generally, nitrogen sources in plant cell culture media are present in two forms, ammonium (NH_4^+) and nitrate (NO_3^-), the form and concentration of which can influence secondary metabolism (Do and Cormier, 1991). Moreover, the ratio of ammonium to nitrate can affect anthocyanin production and composition (Mori and Sakurai, 1994). Specifically, nitrogen deficiency or low nitrate concentration has been shown to enhance anthocyanin production in Arabidopsis (Lillo *et al*., 2008), carrot (Rajendran *et al*., 1992), grapevine (Do and Cormier, 1991), and strawberry (Mori and Sakurai, 1994). I tested the effect of nitrogen deprivation on anthocyanin production in raspberry calli. While nitrogen deficient media did not yield the desired end product, Gamborg B5 basal mediumwith no added elicitors was particularly effective at inducing yellow-green pigmentation. In strawberry cell suspension cultures, Mori and Sakurai (1994) tested the effects of twelve different ratios of ammonium to nitrate on cell growth and anthocyanin production. These authors found that the most effective ratio of ammonium to nitrate was 2:28, which is closest to the

composition of B5 media that I used (ratio of 2:25). In addition, B5 medium contains half of the total nitrogen concentration than that of regular MS medium.

3.4.3.2 Introduction of Apple as a Comparative Model

Because of the lack of anthocyanin inducibility of leaf-derived raspberry callus cultures, I introduced a second experimental system, fruit-derived apple callus cultures. Like raspberry, apple does not accumulate DHM-derived anthocyanins and, thus, could serve as an alternate system for complementation testing. With apple, I also established cell suspension cultures from callus tissue to determine whether less clumping and aeration in the suspension positively impacted competence for anthocyanin production.

I implemented a series of precursor feeding experiments using both the raspberry and apple culture systems to induce anthocyanin accumulation. Precursor feeding has been shown to induce and enhance anthocyanin production in cell suspension cultures. Kakegawa *et al*. (1995) found that the level of endogenous free-state phenylalanine becomes elevated immediately before anthocyanin accumulation. This is supported by L-Phe feeding experiments in grapevine (Krisa *et al*., 1999; Qu *et al*., 2005) and strawberry (Edahiro *et al*., 2005). Other precursors such as DHQ and leucocyanidin similarly promoted anthocyanin synthesis (Dedaldechamp and Uhel, 1999; Almeida *et al*., 1989). I found that single precursor feeding of low levels (25 µM and 50 µM) of L-Phe to raspberry suspensions was more effective at inducing changes in the colour phenotype than to high levels (100 µM, 1 mM, and 2.5 mM) of L-Phe, whereas apple suspensions were unaffected. The appearance of novel colours in some raspberry cell aggregates suggested the uptake of exogenous L-Phe. It would be interesting to measure the changes

in the endogenous pool of free-state L-Phe after adding L-Phe to the culture medium (Kakegawa *et al*., 1995) and to follow the incorporation and distribution of isotopically (^{13}C) labelled L-Phe in the plant cell (Krisa *et al*., 1999).

AAPH is a water-soluble free radical generator that forms two carbon-centred radicals upon decomposition in water, which react with oxygen to form peroxyl radicals (Ohlsson *et al*., 1995). Typically, AAPH is used to induce oxidative damage for the evaluation of antioxidant efficacies of anthocyanin and other extracts (Blando et al., 2004; Gabrielska and Oszmianski, 2005; Elisia and Kitts, 2008); however, Ohlsson *et al*. (1995) found that in the presence of AAPH, there was an increase in PAL activity and an increased excretion of phenolic substances into the culture medium. I found that the exposure of AAPH to raspberry and apple suspensions did not promote anthocyanin biosynthesis. This is in agreement with Kitamura *et al*. (2002) who found that AAPH repressed growth and decreased the anthocyanin content of anthocyanin-producing *Glehnia littoralis* cells. In this case, AAPH may be a more suitable agent for the stimulation of other polyphenolic compounds.

Various parameters have been discussed thus far for anthocyanin induction in raspberry cell cultures, including the plant growth regulator, ABA. The effects of other plant growth regulators on anthocyanin production have been shown in the literature to be largely variable. For example, 2,4-D, a synthetic auxin analog commonly supplemented in plant cell culture media, has been shown to both promote and inhibit anthocyanin production in callus and suspension cultures. The inhibitory effect of 2,4-D has been demonstrated in *Oxalis reclinata* callus (Makunga *et al*., 1997), as well as carrot (Takeda, 1988), strawberry (Nakamura *et al*., 1998), and sweet potato (Nozue *et al*.,

1997) cell suspensions. A number of mechanisms has been proposed. Makunga *et al*. (1997) speculated that 2,4-D causes a reduction in the intracellular phenylalanine pool by increasing its incorporation into amino acids. In *Ajuga reptans*, 2,4-D negatively acts on CHS, which presents the entry point into the flavonoid biosynthetic pathway (Seitz and Hinderer, 1988). Also, 2,4-D has been shown to inhibit the expression of a vacuolar protein, which plays a role in intravacuolar trapping of anthocyanins, as shown in sweet potato (Nozue *et al*., 1997). Alternatively, 2,4-D changes the physiological state of the cells from light-sensitive to light-insensitive in carrot. In other words, the omittance of 2,4-D in the culture media renders the cells light-sensitive and competent for anthocyanin biosynthesis by irradiation. This corroborates my findings in apple callus cultures in which the combination of light and nitrate with 2,4-D deficient media rapidly and effectively induced anthocyanin accumulation. Furthermore, apple callus cultures incubated in the absence of light did not produce anthocyanins. Interestingly, similarly treated raspberry callus cultures did not accumulate anthocyanins, but did induce the synthesis of yellow-coloured compounds, which are reminiscent of the colours produced in response to various induction treatments previously reported. Chalcones, aurones, flavonols and/or flavones are plausible candidates for the identities of these yellow compounds.

HPLC-MS 'fingerprinting' analysis of NDM4-treated calli has shed some light on the flavonoid pathway in *in vitro* cultured raspberry and apple. The predominant presence of hydroxycinnamic acids and their derivatives in raspberry calli strongly suggests active phenolic metabolism. While hydroxycinnamic acids occur primarily as glycosides (Haskins and Kosuge, 1965), the generalized route leading to the formation of

hydroxycinnamic acids is as follows: phenylalanine → cinnamic acid → *p*-coumaric acid (Harbone and Corner, 1961); however, these substrates are not being funnelled down towards flavonoid biosynthesis. It has been suggested that soluble esters of hydroxycinnamic acids present in cell vacuoles may serve as a pool of precursors for flavonoids, among other compounds (Liu *et al*, 1995).

3.4.3.3 Control of Anthocyanin Biosynthesis in Raspberry Callus

Factors that cause a bathochromatic or hypsochromatic shift in the absorption maxima of anthocyanins include pH of the culture medium and copigmentation; however, these do not adequately explain the absence of anthocyanin biosynthesis in raspberry callus cultures. It is widely acknowledged that a close relationship exists between the state of plant cell differentiation and secondary metabolism (Endress, 1994). Secondary metabolism is said to be completely shut off when a cell is dedifferentiated, yet an advanced differentiation state imposes an offsetting quality to cell growth. Thus, the challenge lies in attaining the optimal differentiation state for secondary metabolism whilst maintaining maximal cell growth. This difference in differentiation state could account for the markedly contrasting response observed in raspberry and apple callus cultures in their capacity to synthesize anthocyanins *in vitro*. Typically, undifferentiated cells are larger and of random shape, whereas more differentiated cells are smaller and of more spherical shape with increasing cytoplasmic density (Deroles, 2009). Earlier published work in *Nicotiana* spp. suggested that high auxin content contributes to the growth of undifferentiated cells (Kehr and Smith, 1953; Schaeffer and Smith, 1962). This

supports my observation in which the omittance of 2,4-D induced anthocyanin accumulation in apple callus, possibly through increased cell differentiation.

The absence of anthocyanins and the likely presence of flavonols in induced raspberry callus cultures could suggest a lack of DFR activity *in vitro* or could imply a competition effect between DFR and FLS for the dihydroflavonol substrate. English ivy (*Hedera helix* L.) presents an interesting case in that juvenile phase plants accumulate anthocyanins whereas the genetically equivalent plants of the mature phase do not; however, comparable levels of flavonols are detected in leaf discs of both phases (Murray and Hackett, 1991). Murray *et al*. (1994) determined that the lack of DFR activity is regulated at the level of epigenetic transcriptional silencing. Epigenetic gene silencing in a plausible explanation for the lack of anthocyanin accumulation in raspberry calli, considering the variable differentiation states of these cells. The formation of anthocyanin from its aglycone structure through the activity of UDP:glucosyltransferase (UFGT) provides the first stable coloured intermediate in the anthocyanin biosynthetic pathway. As such, the lack of UFGT activity could result in the lack of anthocyanin accumulation as in *bronze1* mutants in maize which is reflected in the near complete absence of cyanidin-3-glucoside (Larson and Coe, 1977) and in several white grapevine cultivars Riesling, Muscat Gordo, Semillon, Chardonnay, and Sultana, which lack UFGT expression (Boss *et al*., 1996). Alternatively, the inability of anthocyanins to be transported into the vacuole due to the lack of anthocyanin-specific glutathione *S*-transferase (GST) as in *bronze2* mutants in maize (Larson and Coe, 1977) could similarly contribute to the absence of anthocyanin accumulation in raspberry calli.

Ectopic expression of apple (*Malus* x *domestica*), pear (*Pyrus communis*), strawberry (*Fragaria vesca* and *Fragaria* x *ananassa*) and kiwifruit (*Actinidia deliciosa*) MYB10 transcription factors in both homologous and heterologous systems have shed some light on the lack of anthocyanin accumulation in raspberry calli. Dr. Andrew Allan and his group (Plant and Food Research, Auckland, NZ) found that the cisgenic overexpression of MYB10 orthologs, such as apple, pear, and kiwifruit, efficiently enhanced ectopic expression of anthocyanins throughout transgenic plant development; however, this did not immediately hold true for strawberry in that the onset of anthocyanin accumulation occurred after the regeneraton of whole plants from callus. Additionally, apple- and kiwifruit-derived MYB10 transgene expression in either strawberry species tested (*Fragaria vesca* and *Fragaria* x *ananassa*) failed to induce anthocyanin biosynthesis at any developmental stages, neither spatially nor temporally, resulting in the complete lack of anthocyanin accumulation (A. Allan, unpublished data). Considering that, of the species examined here, strawberry (*Fragaria*) is the most phylogenetically closely related species to raspberry (*Rubus*), both belonging to the Rosoideae subfamily, this outcome should come as less of a surprise. Allan's team's results with strawberry corroborate my inability to induce anthocyanin production in raspberry calli and may indicate that anthocyanin biosynthesis is subject to tighter stage- and/or tissue-specific regulation in the Rosoideae.

3.4.4 Establishing Transgenic Callus Lines for Genetic Complementation of the Absence of Dihydromyricetin-Derived Flavonoids

To elucidate the genetic basis for the absence of dihydromyricetin-derived flavonoid biosynthesis in raspberry, I asked whether this deficiency can be complemented by the introduction of grapevine F3'5'H gene. Functional conservation of F3'5'H genes in plants has been demonstrated in petunia, rose, and tobacco by ectopic expression of grapevine F3'5'H, viola F3'5'H, and *Campanula medium* F3'5'H, respectively (Bogs *et al*., 2006; Katsumoto *et al*., 2007; Okinaka *et al*., 2003). As a result, transgenic lines carrying F3'5'H accumulated high levels of delphinidin-based anthocyanins, but this has not been published for any fruit phenotype to date.

To screen for transgenic calli, I subjected transformed cells to 80 mg/L hygromycin antibiotic selection. I had unsuccessfully experimented with 30 mg/L and 50 mg/L hygromycin for selecting transformants, as the majority of the cells continued to proliferate despite the imposed selection pressure (data not shown). Firoozabady and Robinson (1997) genetically transformed rose calli by co-cultivation with *Agrobacterium* and selected for transformed cells with 20-80 mg/L hygromycin. This was, thus, adapted for this study, as this is the first instance I have found where Rosaceae callus cells were utilized for *Agrobacterium*-mediated transformation. Also, rose plants and raspberry are even more phylogenetically closely related than are raspberry and strawberry (Potter *et al*., 2007; Shulaev *et al*., 2008).

Other forms of transformant screening were considered in order to test for transformation efficiency. These include the integration of a green fluorescent protein (GFP) cassette, which carries a distinct GFP from the jellyfish *Aequorea coerulescens*

(AcGFP) driven by CaMV 35S promoter; however, preliminary biolistic transformation of raspberry callus with an AcGFP-containing plasmid revealed strong autofluorescence by the raspberry callus cells (data not shown), so this method was abandoned. Also, I considered inserting a gene cassette containing GUS into the transformation vector. This was deemed unfeasible as the colorimetric signal generated by GUS could potentially mask the appearance of delphinidins and their derivatives, as well as the detrimental effect of staining for GUS activity on cell viability.

Through PCR analysis, it was determined that F3'5'H transgene expression was detected in four out of four raspberry putative transgenic callus lines tested. This result demonstrated 100% selection efficiency indicating hygromycin as a suitable selective agent; however, it is possible that these putative transgenic lines are chimeric, containing both transformed and non-transformed cells. While raspberry putative transgenic callus lines demonstrated growth proliferation, apple putative transgenic callus lines showed growth arrest in the absence of cell necrosis. This could be the result of an increased sensitivity of transformed apple callus cells to a relatively high dose, non-lethal of hygromycin. It has been suggested that the initial cell density strongly influences the relative growth rate of plant cells *in vitro* (Matsubayashi *et al*., 2004). Matsubayashi and Sakagami (1996) had previously shown that phytosulfokine, a peptide plant hormone, promotes cell proliferation and development in low-density plant cell cultures. Given the considerably reduced growth rate of apple putative transgenic callus, some agglomeration of cells that showed promising prospects for survival on selection medium were perhaps subcultured away from the remaining cells prior to a critical level of cell density has been

reached. Improving transformation and selection efficiency would be key to obtaining higher percentage of transformants.

While I have successfully shown that the absence of F3'5'H gene expression can be genetically complemented by *Agrobacterium*-mediated transformation, the immediate future work stemming from this research project should determine whether these raspberry putative transgenic callus lines can be biochemically complemented through the induction of flavonol biosynthesis and, subsequently, the accumulation of dihydromyricetin-derived flavonols.

4 CONCLUSION

Current research in the field of fruit crop genomics has placed its emphasis primarily on some members of the Rosaceae family, such as apple and peach; however, raspberry has attracted significant interest due to its commercialization of consumer driven demand of "super fruits" as a component of a healthy diet. While ellagitannins, a class of hydrolysable tannins, are considered the biggest contributor to antioxidant activity, anthocyanins contribute to 25% of the total antioxidant activity in raspberry. It has been suggested that the difference in anthocyanin composition could play a role in determining the degree of antioxidant potential. Interestingly, raspberry, together with other Rosaceae species such as apple, strawberry, and peach, preferentially accumulate dihydroquercetin-derived anthocyanins, while lacking dihydromyricetin-derived anthocyanins. The molecular basis underlying this differential accumulation of anthocyanins is currently unknown.

In this dissertation, I investigated whether the absence of dihydromyricetin-derived anthocyanins in raspberry can be attributed to the lack of F3'5'H activity and whether this deficiency can be functionally complemented by the introduction of grapevine F3'5'H gene. In addition, I examined whether the lack of F3'5'H activity is due to aberrations in genomic sequences and/or gene expression. Numerous PCR efforts did not detect any F3'5'H transcripts nor F3'5'H gene presence in the raspberry genome. Additionally, apple and peach whole genome sequence data mining also did not identify F3'5'H. Taken together, I showed that PCR, RT-PCR, anthocyanin profiling, and comparative genomics data are corroborated and collectively make a strong case for the raspberry genome lacking an F3'5'H ortholog. I found that although both raspberry and

apple callus lines are amendable to *Agrobacterium*-mediated genetic transformations as evident through histochemical GUS assay, their capacity for anthocyanin induction *in vitro* is markedly different. While cell cultures derived from carrot, grapevine, and strawberry have been commonly utilized for the production of secondary metabolites, the induction of anthocyanin biosynthesis in raspberry and apple cell lines is considered relatively novel. I tested various protocols for anthocyanin induction in raspberry and apple callus lines and found that light together with nitrate and 2,4-D deficient culture media rapidly and efficiently induced anthocyanin accumulation in apple callus. While anthocyanins and flavonols are readily detected based on HPLC-MS-UV profiles in treated apple callus lines, only hydroxycinnamic acid derivatives and early pathway intermediates were detected in treated raspberry callus lines; however, given sufficient time on the same induction medium, I did detect flavonols in treated raspberry callus lines. Successful genetic transformation of VvF3'5'H transgene expression in raspberry callus lines was demonstrated through PCR analyses.

This dissertation made extensive use of the tissue culture system and adequately exploited the inductive power of comparative genomics; however, the limitations of cell culture are such that the *ex vivo* nature of cell culture reflects that the cells are not in their normal physiological state and environment, as well as being heterogeneous as a population of cells. It is possible that the state of callus is simply not developmentally advanced enough for the manifestation of an anthocyanin producing phenotype.

The goal of this investigation was to advance our basic understanding of the metabolic flux leading to anthocyanin production in raspberry and to build on current resources concerning raspberry genomics. It would be of interest to explore the potential

for metabolic engineering in raspberry to produce high nutritional value fruits through manipulation of the flavonoid biosynthetic pathway.

With the release of peach (cv. Lovell) and, now, apple (cv. Golden Delicious) whole genome sequence data, cracking the woodland strawberry genome is imminent with an international effort from the Strawberry Genome Sequencing Consortium. This will strengthen the findings uncovered here as both raspberry and strawberry belong to the same Rosoideae subfamily, unlike apple and peach which cluster more distantly. Future works are required to advance findings presented here; specifically, these include establishing whole plant regeneration in raspberry VvF3’5’H transformants, evaluating antioxidant capacity of transgenic versus wildtype raspberry fruit extracts, and targeting other flavonoid structural enzymes to better understand structure-activity of flavonoids in determining antioxidant capacity.

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