What makes a cherry red?

An investigation into flavonoid pathway regulation in sweet cherry (*Prunus avium* L.) fruit

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Abstract

Colour is an important fruit quality indicator because many consumers make their selections based primarily on this trait. Inheritance of colour has been studied within sweet cherry (*Prunus avium* L.) populations and as a result fruit colour is thought to be determined by three genetic factors. A flesh colour factor (*F*) and the major skin colour factor (*A*) are the main determinants of fruit colour, where red pigmentation is incompletely dominant over yellow. A third factor, the minor skin colour factor (*B*), can produce blush skin but is epistatically masked by a dominant *A* allele. The pigments that colour fruit are known as anthocyanins, synthesised via the transcriptionally regulated flavonoid pathway, which also synthesizes the related secondary metabolites, condensed tannins and flavonols. In other fruit and flower species, mutations in flavonoid pathway or regulatory genes can lead to non-functional alleles that explain the inheritance of colour. However the genes encoding the genetic colour factors are not known in sweet cherry. Therefore, this research has endeavoured to study the cherry flavonoid pathway and its transcriptional regulation, with a view to determining the genetic differences responsible for yellow, blush, red and black cultivars.

To achieve this aim, genes encoding flavonoid pathway enzymes and putative regulators of flavonoid synthesis were isolated from the red sweet cherry cultivar 'Lapins'. *PaMYBA1*, an R2R3-MYB factor, possessing a high degree of sequence similarity with characterised anthocyanin regulators and conserved C-terminal motifs common within this type of protein, was identified. Functional characterisation of PaMYBA1 demonstrated its ability to activate transcription from the promoters of *chalcone synthase* (*MdCHS*), which encodes an enzyme that performs the first committed step in the synthesis of flavonoids, and the anthocyanin biosynthetic gene *UDP-glycosyl:flavonoid-3-O-glycosyltransferase* (*MdUFGT*). Furthermore, correlation between anthocyanin accumulation and the expression profile of *PaMYBA1* in developing 'Lapins' fruit and light-treated blush-skinned 'Ranier' fruit suggest that PaMYBA1 might be an important colour factor. Transcript analysis revealed that PaMYBA1 is necessary for the production of colour in cherries; *PaMYBA1* is not expressed in the solid yellow fruit of 'Yellow Glass' that lacks anthocyanins. However, similar levels of expression of *PaMYBA1* in blush, red and black sweet cherry fruit indicate that there are additional factors that contribute to differences in colour intensity.

The intense colour and increased flavonoid levels of the black sweet cherry 'Sam', compared with the blush and red fruits tested, correlated with a large increase in the expression of the putative tannin regulator *PaMYBPA1* in this cultivar. In a functional assay, *PaMYBPA1* could

trans-activate not only the promoters of the tannin genes *anthocyanidin reductase* (*VvANR*) and *leucaonthocyanidin reductase* (*VvLAR*), but also of *MdCHS* and *MdUFGT*. Therefore, it is possible that PaMYBPA1 could regulate both tannin and anthocyanin synthesis, particularly when expressed at high levels.

Taking into consideration the expression of flavonoid pathway genes in different sweet cherry cultivars and tissues, and under different environmental conditions, together with published scientific observations of the genetic factors contributing to fruit colour, we have developed a working model for flavonoid pathway regulation in sweet cherry fruit. Aspects of the model remain to be determined, such as the involvement of two additional anthocyanin-type MYB factors PaMYBA2 and PaMYBA3 in fruit pigmentation. However, it provides a general understanding of differences in the activity of the flavonoid pathway between sweet cherry cultivars, and moves us closer to knowing the identity of the inherited factors that determine skin and flesh colour in sweet cherry fruit.

Declarations

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

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Jessie-Lee Parker

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Abbreviations

Units

°C	degrees Celcius
aa	amino acid
bp/kb	base pairs/kilobase pairs
g	relative centrifugal force
g; mg; µg	gram; milligram; microgram
h	Hour
kPa	Kilopascal
L; ml; µl	litre; millilitre; microlitre
M; mM; µM; nM	molar (moles per L); millimolar; micromolar; nanomolar
m; cm; mm; µm; nm	metre; centimetre; millimetre; micrometre; nanometre
min	Minute
s	Second
U	Unit
µF	Microfarad
V	Volts
w/v	weight per volume

Flavonoid pathway

4CL	4-coumarate CoA ligase
ANR	anthocyanidin reductase
bHLH	basic helix-loop-helix
C4H	cinnamate 4-hydroxylase
CHI	chalcone isomerise
CHS	chalcone synthase
CT(s)	condensed tannin(s)
DFR	dihydroflavonol 4-reductase
DHK	Dihydrokaempferol
DHM	Dihydromyricetin
DHQ	Dihydroquercetin
F3’5’H	flavonoid 3’,5’-hydroxylase
F3’H	flavonoid-3’-hydroxylase
F3H	flavanone-3β-hydroxylase
FLS	flavonol synthase
GT	Glycosyltransferase
LAR	leucoanthocyanidin reductase
LDOX	leucoanthocyanidin dioxygenase
MT	Methyltransferase
PA	Proanthocyanidin
PAL	phenylalanine ammonia-lyase
TTG	transparent testa glabra
UFGT	UDP-glycosyl:flavonoid-3-*O*-glycosyltransferase

General

A, C, G, T	adenine, cytosine, guanine, thymine
AAFC	Agriculture and Agri-Food Research Canada
ABC	ATP binding cassette
AD	activation domain
AGRF	Australian Genome Research Facility
am	ante meridian
ANGIS	Australian National Genomic Information Service
BLAST	basic local alignment search tool
cDNA	complementary DNA
CSIRO	Commonwealth Scientific and Industrial Research Organisation
C-terminal	carboxy-terminal
DABR	days after bag removal
DAFB	days after full bloom
DNA	deoxyribonucleic acid
dNTP	dinucleotide triphosphate
EAR (domain)	ERF-associated amphiphilic repression
EDTA	ethylenediaminetetraacetic acid
EST	expressed sequence tag
Etarget	amplification (target gene)
GPS (buffer)	glycine/phosphate/salt
GUS	ß-glucuronidase
HPLC	high performance liquid chromatography
IPTG	Isopropylthiogalactoside
LB	Luria broth
MAS	marker-assisted selection
MATE (transporter)	multidrug and toxic efflux
MEME	Multiple Em for Motif Elicitation
NCBI	National Center for Biotechnology Information
N-terminal	amino-terminal
oligo	Oligonucleotide
PCR	polymerase chain reaction
Phe	Phenylalanine
PIRSA	Primary Industries and Resources South Australia
QTL	quantitative trait locus
RACE	random amplification of cDNA ends
RNA	ribonucleic acid
rpm	revolutions per minute
SD	standard deviation
SDS	sodium dodecyl sulphate
SEM	standard error of the mean
SG	Subgroup
SNW	sterile nuclease-free water
STE (buffer)	salt/tris/EDTA
TAE	tris-acetic acid-EDTA
TBE	tris-boric acid-EDTA
TE	tris-EDTA
TF	transcription factor
Trp	Tryptophan
TSS	total soluble solids
Tyr	Tyrosine
ubq	Ubiquitin
UTR	untranslated region
UV	ultra violet
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

Chapter One: Introduction

1. INTRODUCTION

Sweet cherries (*Prunus avium* L.) are an appealing summer fruit, grown in temperate regions that experience cool winter temperatures. The colour of the fruit ranges from dark mahogany to pale yellow, and is one of the primary characteristics on which fruit is selected for purchase. Australian consumers prefer the dark coloured fruit, which command a retail price around $15 or more per kilo even at the height of the harvest. The red colour pigments in cherries are anthocyanins, part of a group of secondary metabolites called flavonoids, and are also present in most higher plants. The synthesis of flavonoids has been studied in several model plants and some fruit including apple (*Malus* x *domestica*) and grape (*Vitis vinifera*); however, the biosynthesis of flavonoids in sweet cherry and the molecular genetics behind the range of fruit colour remain to be described.

1.1 FLAVONOIDS AS PLANT SECONDARY METABOLITES

Plant metabolism is generally defined as either primary or secondary metabolism. Primary or basic metabolites are essential to vital plant processes such as respiration and photosynthesis, and include sugars, amino acids and fatty acids. While less crucial to plant survival, secondary metabolites are very important as they enable the plant to interact with, and adapt to, the biotic and abiotic factors within its environment (Seigler, 1998). Some common plant secondary metabolites are encompassed within the alkaloid, terpenoid and phenolic classes of compounds. These metabolites provide benefit to the plant through pigmentation, growth, reproduction, resistance to pathogens and many more mechanisms.

1.1.1 Role of flavonoid compounds in plant and fruit development

Flavonoids are a large class of plant secondary metabolites, representing over 8,000 known compounds (Pietta, 2000). Consequently, flavonoid compounds can play a wide variety of roles *in planta*, including providing protection against herbivory and pathogen attack, and a range of environmental stresses such as ultraviolet (UV) radiation and reactive oxygen species (Gould and Lister, 2006). The involvement of flavonoids in reproduction (Mo *et al.*, 1992), symbiosis (Wasson *et al.*, 2006), and signaling (Buer *et al.*, 2006) has also been demonstrated.

Three flavonoid subclasses are of particular importance in developing fruit; anthocyanins, tannins and flavonols. Anthocyanins are responsible for the red, blue and purple colours seen in many fruits and flowers, and also occur in leaves and sometimes roots. The role of

anthocyanins is to attract animals to flowers and fruit for pollination and seed dispersal (Whitney and Lister, 2004). Condensed tannins (CTs), also known as proanthocyanidins (PAs), are polymers of flavonoid molecules which can become oxidized from a colourless to a brown pigment seen in plant seeds and flesh. The astringent quality imparted by tannins may deter animals from feeding on developing fruit (Feeny, 1970; Forkner *et al.*, 2004). Finally, flavonols are colourless or yellow compounds that function in a number of ways in developing fruit. Flavonols are required for functional pollen in maize and petunia (Deboo *et al.*, 1995), they play role in UV photoprotection (Lois and Buchanan, 1994; Ryan *et al.*, 2002), and they influence anthocyanin colour via co-pigmentation in the vacuole (Lancaster, 1992; Fossen *et al.*, 2000).

1.1.2 Structure of flavonoid compounds

Flavonoids are characterized by a flavan nucleus, consisting of two aromatic rings joined by a three carbon bridge (Yamasaki *et al.*, 1996; Bermudez-Soto and Tomas-Barberan, 2004). The large number of known flavonoid structures arises from the attachment of different functional groups to the flavan nucleus, pictured below in Figure 1.1 (Pietta, 2000).

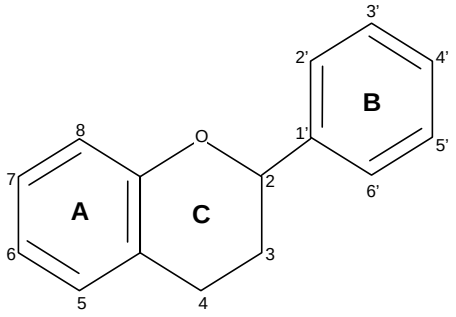


Figure 1.1 - Flavan nucleus characteristic of flavonoid compounds
Carbon atoms within the A, B and C phenolic rings are numbered.

1.1.3 Role of flavonoid compounds in human health

Besides being advantageous to the plant, it is generally accepted that flavonoids belong to a group of phytochemicals that have health-promoting and disease-preventing effects. The bioavailability of flavonoid glycosides has been demonstrated *in vivo* in rats as well as humans (Miyazawa *et al.*, 1999), and epidemiological studies have found an inverse relationship between the intake of fruit, vegetables and cereals (all of which are high in flavonoids) and the incidence of disease and mortality (Hertog, 1996; Hollman *et al.*, 1996; Rice-Evans *et al.*, 1997).

Flavonoids, including anthocyanins, have been shown to protect rat neuronal cells from oxidative stress by hydrogen peroxide in a dose-dependent manner (Kim *et al.*, 2005). The precise mechanisms by which flavonoids promote human health are unclear, but several studies have shown they are very effective free-radical scavengers, acting as antioxidants that prevent cellular damage by oxidative stress (Wattenberg, 1992; Young and Woodside, 2001). Recent research also indicates flavonoids may function through modulation of protein and lipid kinase signaling cascades (Williams *et al.*, 2004; Stevenson and Hurst, 2007). Increasing interest in fruits and vegetables as nutraceuticals (Boots *et al.*, 2008; Lasztity, 2009) necessitates further research to deepen the current understanding flavonoid biosynthesis and its regulation.

1.2 SWEET CHERRIES

Sweet cherry trees are cultivated for their small, edible fruit that is either round or cordate in shape. For a great many years sweet cherry fruit has been consumed due to its appealing taste and texture, long before the nutritional value of the fruit could be quantified. Flavonoid compounds significantly contribute to the quality, and hence to the appeal, of sweet cherry fruit, as will be discussed in the sections to follow.

Sweet cherry is a naturally self-incompatible, non-climacteric stone fruit belonging to the Rosaceae family, which encompasses rose and other ornamental flowers, apple, pear (*Pyrus communis*), almond (*Prunus dulcis*), peach (*Prunus persica*), plum (*Prunus domestica*), apricot (*Prunus armeniaca*), strawberries (*Fragaria ananasa*) and raspberries (*Rubus idaeus*). Whilst the closely related sour cherry (*Prunus cerasus* L.) is tetraploid, sweet cherry is diploid. On average, sweet cherry trees can grow to between ten and twenty metres in height, are pyramidal to upright spreading in shape and have large, drooping leaves (Marshall, 1954). They are widely grown throughout the world, but prefer geographical areas with a cool climate, particularly cool winters, with good drainage. Sweet cherry is an economically important fruit crop; over two million tonnes are produced annually, with the three main producers being Turkey, United States and Iran (FAO, 2007).

1.2.1 Fruit quality

1.2.1.1 Phenotypic variation in sweet cherry fruit

Hundreds of different sweet cherry varieties exist, displaying differences in flowering and harvest date, and a number of fruit quality parameters including architecture, flavour, firmness and storage properties. Sweet cherry cultivars also vary in skin and flesh colour,

which are closely correlated (Schmidt, 1998). Red fruit, as it will be referred to in this thesis, exhibits colours such as ruby and mahogany whilst black fruit can range from purple to almost black. Blush cherries are often referred to as yellow or white cherries and generally exhibit a red blush on their skin, although their juice is colourless. Figure 1.2 shows some different cherry skin colours.

Figure 1.2 - Sweet cherry fruit displaying different skin colours

Photograph of sweet cherries possessing blush ('Ranier', top left and 'Sue', bottom left), red ('Van', top right) and black ('Sam', bottom right) fruit. Yellow sweet cherries that completely lack red pigmentation also occur but are not represented in this photograph.

1.2.1.2 Consumer perception of fruit quality

The sensory appeal of sweet cherry fruit has been the focus of a number of studies. Skin colour is arguably the most important fruit quality trait for consumers, although preferences for fruit colour can vary between geographical regions. For example, Australian consumers generally look for a deep red colour when purchasing cherry fruit (Turner and Shepherd, 2001), while in the Japanese market, blush fruits are favoured (McCracken *et al.*, 1989). In a controlled trained panel sensory study of dark (black and red) fruit conducted in the United States, perception of sweetness, sourness and cherry flavour were highly correlated to skin colour, total soluble solids (TSS or sugars) and ratio of TSS: titratable acidity at harvest (Crisosto *et al.*, 2002). Cherry flavour is also influenced by a number of factors other than acidity/sugar balance. Among these are flavonoids, with tannins imparting astringency, and volatiles such as benzaldehyde, β-demascenone and eugenol which are known to occur in sour cherry (Schmid and Grosch, 1986; Girard and Kopp, 1998; Poll *et al.*, 2003).

1.2.1.3 Fruit quality improvement through breeding

Fruit quality is of paramount importance to sweet cherry breeders when making their selections. The sweet cherry is thought to have originated between the Caspian and Black Seas of Asia Minor (Marshall, 1954), and has been subjected to centuries of classical breeding. The traits on which breeding efforts have focused include size, shape, texture, colour, taste, resistance to infection and cracking, self-compatibility, and transport and storage properties. More recently, standard breeding procedures have been complemented by marker-assisted selection (MAS). This selection system involves the use of molecular markers to detect genetic variation, used for screening for individuals that exhibit particular traits. With traditional phenotype-based selection, progeny of sweet cherry crosses can not be evaluated for 3-5 years, until they reach physiological maturity and produce fruit (Stockinger *et al.*, 1996). MAS, on the other hand, can provide better information for parental selection and an accurate and early screening of seedlings, thus reducing the need for expensive resources, such as planting space, evaluation time and labour (Luby and Shaw, 2001).

Self-incompatibility in fruiting species reduces the amount of fruit set if conditions are not ideal. Orchard management practices such as row spacing between compatible cultivars (Nunez-Elisea *et al.*, 2008), and the use of pollinating insects (Stern *et al.*, 2005), can enhance fertilisation efficiency but require knowledge about the pollenisation groups (defined by S-alleles, Crane and Lawrence, 1931) that certain cutivars belong to. Increasingly, self-fertile fruit cultivars are being commercialized to circumvent these problems (Iezzoni, 2008). Self-fertility was previously investigated by bagging flowers in the field and recording fruit set, and by microscopic examination of pollen tube growth. Within the last decade, the investigation of sweet cherry pollen compatibility genotypes through DNA-based techniques has assisted in determining the S-alleles present in different cultivars, increasing the efficiency of production and breeding (Granger, 2003; Dirlewanger *et al.*, 2004).

Recently, molecular markers have also been described that could be used to determine the colour of the fruit that apple trees and grapevines will eventually produce. These markers are based on polymorphisms in the MYB genes that regulate colour in these species (Takos *et al.*, 2006a; Walker *et al.*, 2007), and will be described in greater detail in Section 1.4.2. Quantitative trait loci (QTLs) for blooming, ripening and fruit quality characters have been detected in peach and apple (Dirlewanger *et al.*, 2004), and these together with molecular information from segregating sweet cherry populations may enable the development of further molecular markers for MAS in cherry breeding programs.

1.2.2 Genetic control of colour in sweet cherry

Modern genetics traces its roots to Gregor Mendel, who studied the nature of inheritance in the nineteenth century. Through observing the segregation of purple and white garden pea (*Pisum sativum*) flowers within progenies of various crosses, Mendel determined the basis for inheritance of flower colour (Mendel, 1965). Mendel's laws have since been applied to study the segregation of the fruit colour traits amongst sweet cherry populations, enabling the inheritance patterns of the genetic factors that determine skin and flesh colour to been ascertained. It is interesting to note that clearly yellow fruit lacking in anthocyanin has not been included in any of the genetic studies described below. Seedlings bearing solid yellow fruit have not been observed from any crosses between blush and red sweet cherry cultivars, suggesting a genetic pattern independent of the genetically described color factors (Schmidt, 1998). However, the genes that encode these factors are not yet known.

1.2.2.1 Flesh colour is controlled by a single factor

The segregation of flesh colour in the expected monohybrid ratios indicates that this trait is controlled by a single factor in sweet cherry fruit. This observation was first made by Crane and Lawrence (1952), who found that 'blush x red' and 'red x red' crosses consistently yielded progeny with 1:1 and 3:1 ratios of red to blush fruit. Thus, they concluded that white flesh is recessive to red flesh. These findings have since been confirmed in a number of independent studies using different cultivars (Lamb, 1953; Fogle, 1958; Toyama, 1978; Schmidt, 1998; Apostol, 1999).

1.2.2.2 Epistatic factors determine skin colour

Skin colour in sweet cherry fruit is believed to be controlled by two genetic factors, both of which are incompletely dominant. This theory is supported by the 7:1 and 15:1 ratios of red (mahogany through black) to white flesh obtained from 'blush x red' and 'red x red' crosses, respectively (Fogle, 1958). The major factor, *A*, can confer dark skin colour and is thought to be identical with or very closely linked to the flesh colour factor (Fogle, 1975). The separation of these two factors in fruit possessing both light mahogany skin and white flesh, although a rare occurrence, suggests that they are encoded by individual genes (Schmidt, 1998). The minor factor, *B*, is epistatically masked by the major factor. In the presence of the major factor, *B* is believed to intensify skin colour. When the major factor is absent, the heterozygote displays a blush on its skin while the homozygote that is dominant for the trait will have light red skin. Based on these genetic assumptions and numerous test crosses, blush

cultivars such as 'Ranier' would have the genotype *aaBb*, and red (heterozygous) cultivars like 'Lapins' the genotype *AaBb*. Some cultivars bearing dark purple fruit, for example 'Sam', have been described as homozygous for the *A* gene.

1.2.3 Flavonoid compounds in sweet cherry fruit

1.2.3.1 Anthocyanins in sweet cherry

The reddish purple colours visible in cherry skin and flesh are primarily due to the accumulation of anthocyanin glycosides (Mazza and Miniati, 1993). Studied in sweet cherry since the 1970's, anthocyanins identified in this species include 3-rutinoside and 3-glucoside moieties of cyanidin and peonidin, as well as pelargonidin 3-rutinoside and cyanidin 3-sophoroside (Tanchev, 1975; Gao and Mazza, 1995). Within the red fruit of cultivars such as 'Petrovka', 'Bing' and 'Lambert', cyanidin 3-rutinoside and cyanidin 3-glucoside are present in the highest concentrations, constituting 85-92% and 5-16% of total anthocyanins, respectively (Mozetic *et al.*, 2002; Mozetic and Trebse, 2004). Total anthocyanins vary amongst sweet cherry varieties and range from 0.82 to 2.97 mg g^{-1} fresh weight in more intensely coloured varieties ('Bing', 'Lambert', 'Sam', 'Stella', 'Summit', 'Sylvia' and 'Van') and 0.02 to 0.41 mg g^{-1} fresh weight in light-coloured varieties (Gao and Mazza, 1995).

1.2.3.1.1 Anthocyanin accumulation during fruit development

A number of observations have been made regarding anthocyanin accumulation trends following the onset of anthocyanin synthesis at around 50 DAFB. Mozetic *et al*. (2004) reported a linear trend from 54 DAFB until 70 DAFB, whereas Remon *et al*. (2006) found that anthocyanins accumulated exponentially rather than in a linear fashion in 'Burlat' cherry (Remon *et al.*, 2006). Finally, anthocyanin concentration has also been found to consistently decrease during the last part of three consecutive seasons (Poll *et al.*, 2003).

1.2.3.1.2 Anthocyanin accumulation in response to light

Sunlight has long been known to affect anthocyanin content in the skin of blush fruit but not in red and black fruit, in which anthocyanins are synthesised independently of light (Marshall, 1954). In the orchard this is evident by the reduction in colour displayed by fruit grown on shaded limbs of cherry trees. A handful of studies, which will be presented in Chapter 6, have investigated the effect of natural and artificial light on pre- and post-harvest fruit. However, as the flavonoid pathway has not yet been studied at the molecular level in sweet cherry, these reports detail only compositional changes in response to light.

1.2.3.2 Flavonols and tannins in sweet cherry

In addition to the reproductive and protective roles that flavonols and tannins assume within a plant (Section 1.1.1), they may also contribute to the sensory appeal of sweet cherry fruit. Limited information is available regarding these compounds in sweet cherry, as the majority of flavonoid research in this area has involved anthocyanins. Flavonols found in sweet cherry include glycosides of quercetin, kaempferol and isorhamnetin. In other fruit species such as apple and grapes, synthesis of flavonols occurs early in developing flowers and fruit, and again at the ripening stage (Downey *et al.*, 2003b; Takos *et al.*, 2006c). Although the distribution of flavonols in sweet cherry shoots has been studied (Feucht *et al.*, 1986), neither spatial nor temporal accumulation of flavonols nor tannins have been described for sweet cherry fruit.

Tannins occur in fruits and seeds of many plants (Skadhauge *et al.*, 1997). The subunits of tannin synthesis, catechin and epicatechin, have both been identified from sweet cherry leaves and phloem (Feucht *et al.*, 1996). In one study, epicatechin was found to be the main monomer in fruit, with catechin present in smaller amounts in four cultivars tested (Goncalves *et al.*, 2004). An early study found that at their respective commercial harvest dates, acids and tannins were at a higher concentration in an early-ripening sweet cherry variety compared to those of a late-ripening variety, which were richer in sugars and pectins (Agapova *et al.*, 1971). However, it remains unknown whether tannins accumulate to significantly different levels in the fruit of blush, red and black cultivars.

1.2.4 Resources available for genetic studies of sweet cherry

Analysis of the molecular genetics of *Prunus* species may assist in further understanding the mechanisms behind sweet cherry colour. At the inception of this research project, limited genetic resources were available for sweet cherry. Within publicly available databases, such as the Genome Database for Rosaceae (www.bioinfo.wsu.edu/gdr) only a small number of sweet cherry expressed sequence tags (ESTs) existed. However, the genomes of diploid *Prunus* species are essentially co-linear, and a high level of synteny to the related apple (*Rosaceae* family) has been demonstrated (Dirlewanger *et al.*, 2004). Consequently, the sequences and genetic linkage maps available for these related fruit species (Whitepaper, 2006) have been utilised in this study as tools to isolate genes controlling sweet cherry traits. Similarly, the high degree of homology within the apple and pear genomes has recently been

exploited identify flavonoid biosynthetic genes from pear based on published apple sequences (Pierantoni *et al.*, 2009).

The forthcoming release of the genome sequence for the peach haploid cultivar 'Lovell' by the US Department of Energy Joint Genome Institute will greatly assist in the determination of gene sequences from *Prunus* species.

1.3 FLAVONOID BIOSYNTHESIS

1.3.1 Flavonoid biosynthesis requires precursors from the shikimate and phenylpropanoid pathways

The shikimate pathway is a primary metabolic pathway that produces the aromatic amino acids phenylalanine (Phe), tyrosine (Tyr) and tryptophan (Trp), as reviewed by Schmid and Amrhein, 1995. Primary and secondary metabolism converges when Phe is converted by the enzyme phenylalanine ammonia-lyase (PAL) to cinnamic acid, which can be utilised by phenylpropanoid enzymes to synthesise phenolic acids and lignins (Hrazdina, 1994). Cinnamic acid derivatives produced by the general phenylpropanoid pathway may also be converted to chalcones by the enzyme chalcone synthase (CHS), in the presence of carboxylated coenzyme-A (malonyl Co-A). This represents the first committed step in the synthesis of flavonoid compounds. A diagrammatic representation of the direction of metabolites into specific phenylpropanoid pathways is shown in Figure 1.3.

1.3.2 Flavonoid biosynthetic pathway

Flavonoid compounds are synthesised via the flavonoid pathway. The enzymes, structural genes and intermediates involved in the pathway have been identified through biochemical and genetic analysis in a number of plant species including maize (*Zea mays*), Arabidopsis (*Arabidopsis thaliana*), petunia (*Petunia* x *hybrida*) and snapdragon (*Antirrhinum majus*). For reviews of the vast literature regarding elucidation of the flavonoid pathway, refer to Dooner *et al.* (1991), Holton and Cornish (1995) and Mol *et al.* (1998). In its entirety, the pathway produces anthocyanins, with tannins and flavonols synthesised at branch points of the pathway (Figure 1.4). Enzymes required to make more than one flavonoid subclass are often referred to as 'shared' enzymes, while those specific to one subclass are called 'branch' enzymes.

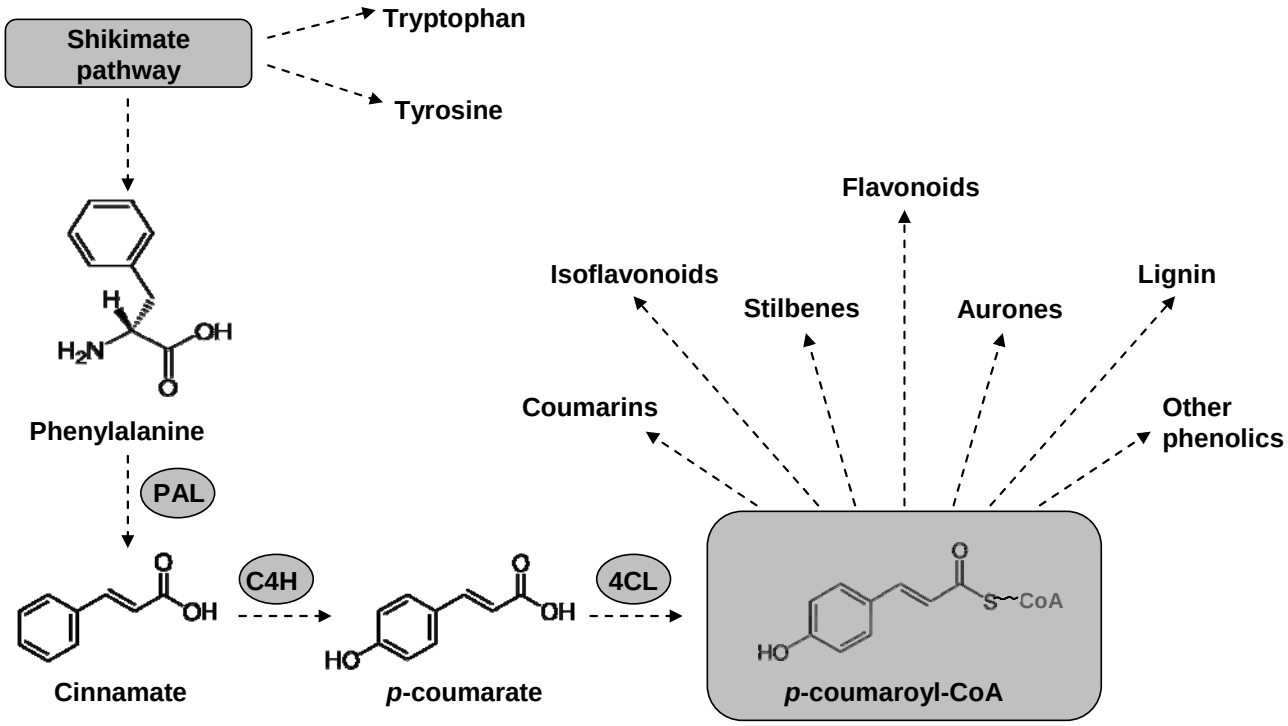


Figure 1.3 – Phenylpropanoid pathways for the production of plant secondary metabolites
Abbreviations for general phenylpropanoid enzymes are shown as grey ovals; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase. The large grey box represents *p*-coumaroyl-CoA, from which numerous phenylpropanoid compounds are synthesised through further enzymatic steps, not shown here. Figure adapted from (Vogt, 2010).

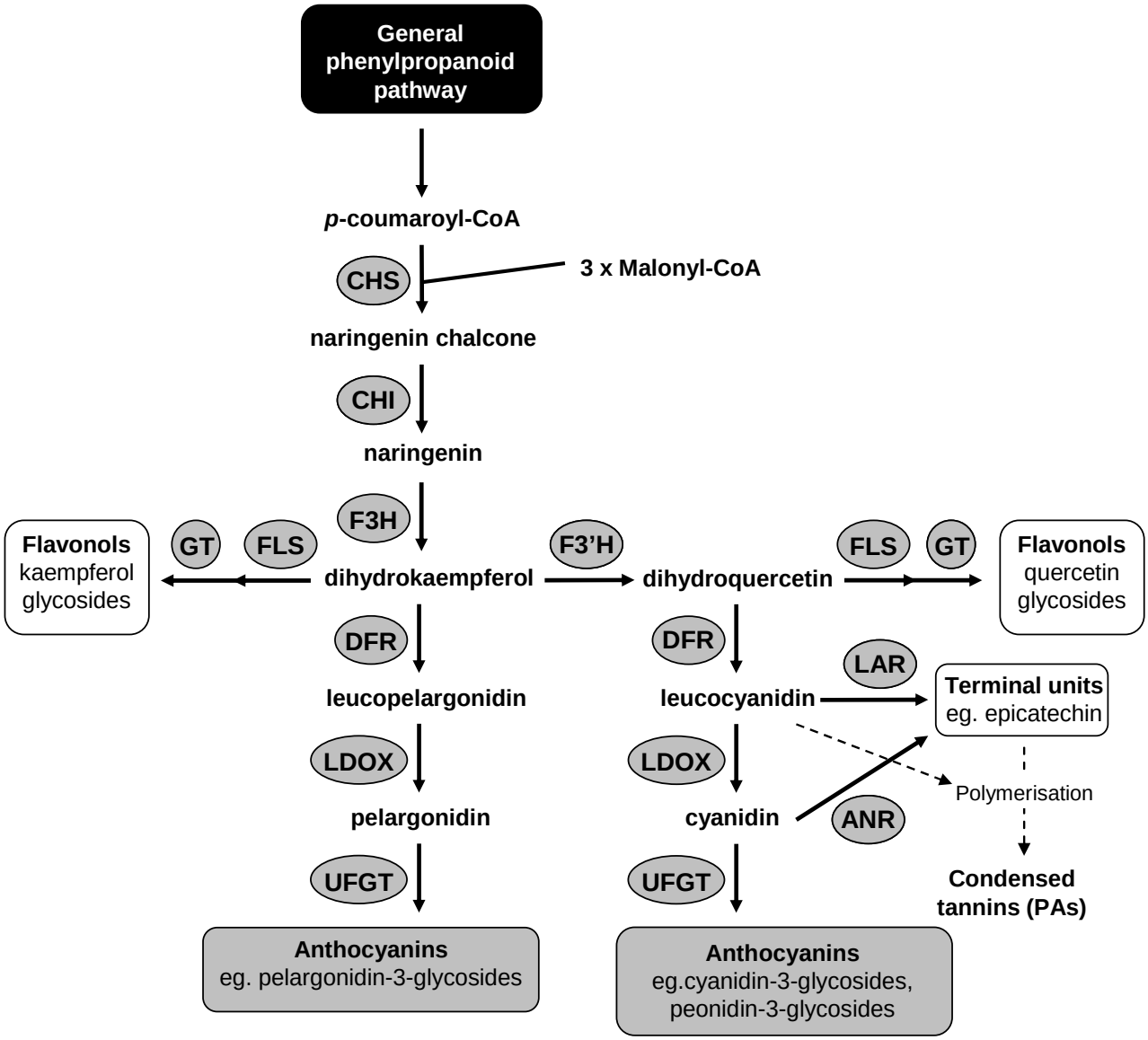


Figure 1.4 - A schematic representation of the sweet cherry flavonoid biosynthetic pathway

Abbreviations for the enzymes involved in the pathway are shown. For anthocyanin synthesis; PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate, CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone-3β-hydroxylase; F3'H, flavonoid-3'-hydroxylase; DFR, dihydroflavonol-4-reductase; LDOX, leucoanthocyanidin dioxygenase; UFGT, UDP glycose:flavonoid-3-*O*-glycosyltransferase. For flavonol synthesis; FLS, flavonol synthase; GT, glycosyltransferase. For condensed tannin synthesis from proanthocyanidin (PA) precursors; LAR, leucoanthocyanidin reductase; ANR, anthocyanidin reductase. F3'H is able to utilise multiple substrates, but these steps have been omitted for clarity.

1.3.2.1 Anthocyanin synthesis

The conversion of colourless dihydroflavonols to anthocyanin pigments involves reduction of the dihydroflavonol to leucoanthocyanidin by dihydroflavonol 4-reductase (DFR), followed by oxidation to anthocyanins by leucoanthocyanidin dioxygenase (LDOX). Anthocyanins are subsequently glycosylated by the enzyme UDP-glycosyl:flavonoid-3-*O*-glycosyltransferase (UFGT). Numerous anthocyanin glycosides can be produced, depending on the dihydroflavonol substrate (Section 1.3.2.3), the aglycone, and the carbon atom to which the aglycone is conjugated. In addition, further modification of anthocyanin glycosides by glycosylation, methylation and acylation increases the number of different anthocyanins that occur in nature.

1.3.2.2 Tannin synthesis

Owing to the structural variation and complexity of CTs, the synthesis of this class of flavonoids is not well understood. The genes *leucoanthocyanidin reductase* (*LAR*) and *anthocyanidin reductase* (*ANR*) encode enzymes for the synthesis of catechin (2,3-*trans*-flavan-3-ol) and epicatechin (2,3-*cis*-flavan-3-ol), respectively, and have recently been isolated from legumes (Tanner *et al.*, 2003; Xie *et al.*, 2003). Catechin and epicatechin can both act as initiating units for the synthesis of condensed tannins, although Arabidopsis lacks LAR and therefore exclusively uses the epicatechin pathway for tannin synthesis (Devic *et al.*, 1999; Abrahams *et al.*, 2003). The mechanism by which extension units are added to produce a polymeric tannin molecule remains unknown (He *et al.*, 2008), although some polymerisation theories have been presented (Dixon *et al.*, 2005; Zhao and Dixon, 2009).

1.3.2.3 Flavonol synthesis

Flavonol synthesis requires the substrate dihydrokaempferol (DHK), a dihydroflavonol produced from naringenin by the activity of the flavanone 3ß-hydroxylase (F3H) enzyme. DHK can be hydroxylated to produce dihydroquercetin (DHQ) or dihydromyricetin (DHM) by flavonoid 3'-hydroxylase (F3'H) or flavonoid 3',5'-hydroxylase (F3'5'H), respectively (Holton and Cornish, 1995). Thus, conversion of the different dihydroflavonols by the enzyme flavonol synthase (FLS) can produce the flavonols kaempferol, quercetin and myricetin. Sweet cherry lacks F3'5'H activity and therefore does not produce myricetin. Recently, Stracke *et al*. (2009) have demonstrated that flavonols can also be synthesised by the activity of LDOX, which was previously thought to be involved only in the production of tannins and anthocyanins. In addition to FLS, modifying enzymes such as glycosyltransferases (GTs) and methyltransferases (MTs) further contribute to the wide range

of flavonol glycosides found in plants (Jones *et al.*, 2003; Yonekura-Sakakibara *et al.*, 2007; Stracke *et al.*, 2009).

1.3.3 Factors affecting flavonoid biosynthesis

Flavonoid compounds perform a variety of roles *in planta* (as introduced in Section 1.1.1); therefore, their synthesis is consequently required in response to a number of different cues. Environmental factors can stimulate flavonoid synthesis through inducing transcription of flavonoid biosynthetic genes. This has been demonstrated for wounding, pathogenic attack, ozone, and temperature (Hutangura *et al.*, 1999; Winkel-Shirley, 2002a; Sgarbi *et al.*, 2003; del Rio and Ortuno, 2004). However, of the external environmental factors, light is one of the most studied with regard to flavonoid synthesis. Sunlight enhances the accumulation of flavonols and anthocyanins in apple skin (Takos *et al.*, 2006b), and the accumulation of flavonols in grape berries (Downey *et al.*, 2004). Investigations into light-induction of the flavonoid pathway will be summarised in Chapter 6.

Genetic factors can dictate flavonoid synthesis, evident in the colour variations of flowers and fruits that occur between cultivars of the same species. Non-red apples have been shown to express genes required for anthocyanin synthesis at significantly lower levels than red apples (Honda *et al.*, 2002), due to the inheritance of non-functional alleles of a regulatory gene (Takos *et al.*, 2006a). Finally, the developmental stage of a plant is of great importance in determining flavonoid production. Synthesis of different classes of flavonoid compounds is often temporally separated, such that the synthesis of flavonols and tannins early in fruit development does not overlap with anthocyanin production during the ripening of strawberry (Cheng and Breen, 1991) bilberry (*Vaccinium myrtillus,* Jaakola *et al.*, 2002) or apple fruit (Takos *et al.*, 2006c). Literature pertaining to developmental and genetic control of flavonoid synthesis will be presented in more depth in Chapters 4 and 6, respectively.

1.3.4 Cellular location of flavonoids

Immuno-localization experiments suggest that flavonoid enzymes are loosely bound to the endoplasmic reticulum within cells, possibly in a multi-enzyme complex (Saslowsky and Winkel-Shirley, 2001). Whilst synthesised in the cytoplasm, anthocyanins and tannins have been found to accumulate in the vacuole (Lepiniec *et al.*, 2006). Vacuolar sequestration is still poorly understood but evidence suggests that transportation occurs via ATP binding cassette (ABC), glutathione S-transferase (GST) and multidrug and toxin extrusion (MATE) transporter proteins (Debeaujon *et al.*, 2001; Kitamura *et al.*, 2004; Zhao and Dixon, 2009).

The roles of flavonols in UV protection and anthocyanin co-pigmentation indicate that they may also be sequestered to the vacuole (Koes *et al.*, 1994; Mol *et al.*, 1998); however, the localisation and potential transport of flavonols remains unconfirmed.

1.4 TRANSCRIPTIONAL REGULATION OF FLAVONOID SYNTHESIS

Regulation of gene expression by transcription factors (TFs) is one way that plants can control the production of secondary metabolites in response to environmental and developmental cues. TFs are proteins that bind to specific DNA sequences, thereby enhancing or preventing recruitment of RNA polymerase and subsequently modulating the initiation of target gene transcription. Many TF families exist, and are classified based on their DNA binding motifs. One of the most abundant classes of plant TFs is the *myb* family, which includes a subfamily containing the two-repeat R2R3 DNA binding domain. Within Arabidopsis there are over 125 R2R3-type *MYB* genes, many of which have been found to function as regulators of a wide variety of processes (Stracke *et al.*, 2001), including secondary metabolism (Borevitz *et al.*, 2000; Jin *et al.*, 2000; Nesi *et al.*, 2001; Baudry *et al.*, 2004), cell morphogenesis (Oppenheimer *et al.*, 1991; Lee and Schiefelbein, 1999; Baumann *et al.*, 2007), floral and seed development (Penfield *et al.*, 2001) and control of the cell cycle (Hirayama and Shinozaki, 1996).

1.4.1 Transcriptional control of flavonoid biosynthesis by MYB factors

The flavonoid pathway is considered one of the best systems available for studying regulation of plant gene expression (Koes *et al.*, 1994; Davies and Schwinn, 2003). The co-ordinate expression of particular flavonoid biosynthetic genes in response to developmental (Dooner, 1983; Martin and Gerats, 1993; Pelletier *et al.*, 1999) and environmental cues (Kubasek *et al.*, 1992; Sparvoli *et al.*, 1994; Kim *et al.*, 2003) has long been indicative that these genes are regulated at the level of transcription. The first plant MYB gene to be isolated, *COLOURLESS1* (*C1*) from maize, was characterised as a TF regulating expression of the genes required for anthocyanin synthesis (Paz-Ares *et al.*, 1987). Since then, control of the flavonoid pathway has been extensively studied in a variety of plant species including snapdragon, petunia and Arabidopsis, as reviewed by Mol *et al.* (1998) and Koes *et al.* (2005). In contrast to maize, many species regulate anthocyanin synthesis through discrete control of specific gene subsets (Almeida *et al.*, 1989; Martin *et al.*, 1991; Quattrocchio *et al.*, 1993), or individual flavonoid genes such as *UFGT* in grape (Boss *et al.*, 1996a; Kobayashi *et al.*, 2002; Walker *et al.*, 2007).

The spatial and temporal accumulation of different flavonoid end products necessitates the independent regulation of flavonoid pathway branches. Through PCR-based and T-DNA tagging approaches, R2R3-MYB proteins that specifically regulate anthocyanin (PRODUCTION OF ANTHOCYANIN PIGMENT1, AtPAP1), (Borevitz *et al.*, 2000) flavonol (AtMYB11, AtMYB12, AtMYB111) (Mehrtens *et al.*, 2005; Stracke *et al.*, 2007) or tannin synthesis (TRANSPARENT TESTA2, AtTT2) (Nesi *et al.*, 2001) have been identified from Arabidopsis.

More recently, the potential product quality and human health benefits presented by flavonoid compounds has encouraged the regulation of their synthesis to be studied in fruit species. In apple, three *MYB* sequences have been isolated from different cultivars by independent groups, all of which are capable of regulating anthocyanin genes when functionally tested in heterologous systems. Of these sequences, *MdMYB1* (Takos *et al.*, 2006a) and *MdMYBA* (Ban *et al.*, 2007) encode identical proteins that differ in 3 residues to the protein encoded by *MdMYB10* (Espley *et al.*, 2007). Transcriptional control of genes involved in anthocyanin biosynthesis has also been demonstrated in grape berries by VvMYBA1 (Goto-Yamamoto *et al.*, 2002; Kobayashi *et al.*, 2004; Walker *et al.*, 2007) and in mangosteen (*Garcinia mangostana*) fruit by GmMYB10 (Palapol *et al.*, 2009). Further research in grape has involved the discovery of more R2R3-MYB transcription factors, including VvMYBPA1 and VvMYBPA2 which control tannin biosynthesis in berry seeds and flesh respectively (Bogs *et al.*, 2007; Terrier *et al.*, 2009), VvMYBF1 as a specific regulator of flavonol biosynthesis (Czemmel *et al.*, 2009), and VvMYB5a and VvMYB5b which have a broad effect on a number of phenylpropanoid genes (Deluc *et al.*, 2006; Deluc *et al.*, 2008).

1.4.2 Anthocyanin MYBs segregate colour for apple and grape

A large number of complementary genetic factors can contribute to colour in plants. In petunia, for example, there are about 35 genes that are responsible for the biosynthesis of pigments in different parts of the plant (Wiering and de Vlaming, 1984). Colour factors include genes that encode enzymes required for pigment biosynthesis, as well as the transcription factors that control their expression. Colour variants or mutants can result from the inheritance of non-functional alleles of these colour factors. The heritable factors that determine fruit colour in apple and grape are MYB transcription factors, in which mutations produce MYBs that are deficient in the transcriptional activation of anthocyanin biosynthetic genes. An allele of the apple anthocyanin regulator, *MdMYB1-1*, was found to be expressed

in red apple skin, whilst green or yellow apple skin possessed the mutated *MdMYB1-2* and *MdMYB1-3* alleles (Takos *et al.*, 2006a). Similarly, the lack of pigmentation in white grape berries is due to the inheritance of inactive alleles of the anthocyanin regulatory genes *VvMYBA1* and *VvMYBA2*. The white berry allele of *VvMYBA1* results from the insertion of a transposon in its promoter (Kobayashi *et al.*, 2004), while *VvMYBA2-white* arose from two non-conservative mutations (Walker *et al.*, 2007). Flavonoid biosynthetic genes or other regulatory genes, such as *bHLH* genes, can also determine colour, and will be discussed in greater detail in Section 5.1.

1.4.3 Structure of R2R3-MYB factors

MYB TFs are generally described as possessing a MYB DNA-binding domain and a C-terminal or activation domain (AD). MYB domains, characteristic of MYB proteins, are regions of approximately 50-53 amino acids that bind DNA in a sequence-specific manner (Jin and Martin, 1999). R2R3-MYB proteins contain two such imperfect repeats that adopt a helix-turn-helix conformation for DNA intercalation. The C-terminal helix of each repeat is the recognition helix for DNA binding (Ogata *et al.*, 1994). Within the repeats are regularly spaced tryptophan residues that form a characteristic tryptophan cluster (Ogata *et al.*, 1992; Konig *et al.*, 1996). In addition to the tryptophan residues, there is generally a high degree of conservation in the DNA-binding domain, with 25/53 positions in the R2 and 31/51 positions in the R3 repeat occupied with one or two very similar residues in over 80% of Arabidopsis R2R3-MYB proteins (Stracke *et al.*, 2001). Some R2R3-MYB proteins, which require a basic helix-loop-helix (bHLH) partner to function (Section 1.4.4), have a conserved interaction motif $[DE]LX_2[RK]X_3LX_6LX_3R$ located on helices 1 and 2 of the R3 repeat (Zimmermann *et al.*, 2004).

In contrast to the DNA-binding domain, the C-terminal regions of MYB proteins are not strongly conserved. Nonetheless, conserved C-terminal motifs have been identified that may contribute to function, and are the basis for the division of *MYB* family genes into subgroups (Kranz *et al.*, 1998; Stracke *et al.*, 2001). MYB proteins may regulate transcription by either promoter activation or repression, as will discussed in the sections to follow. The regions that encompass features of activation or repression domains are also contained within the C-terminus (Martin and Paz-Ares, 1997; Ohta *et al.*, 2001). The structural features of R2R3-MYB factors are summarised in Figure 1.5.

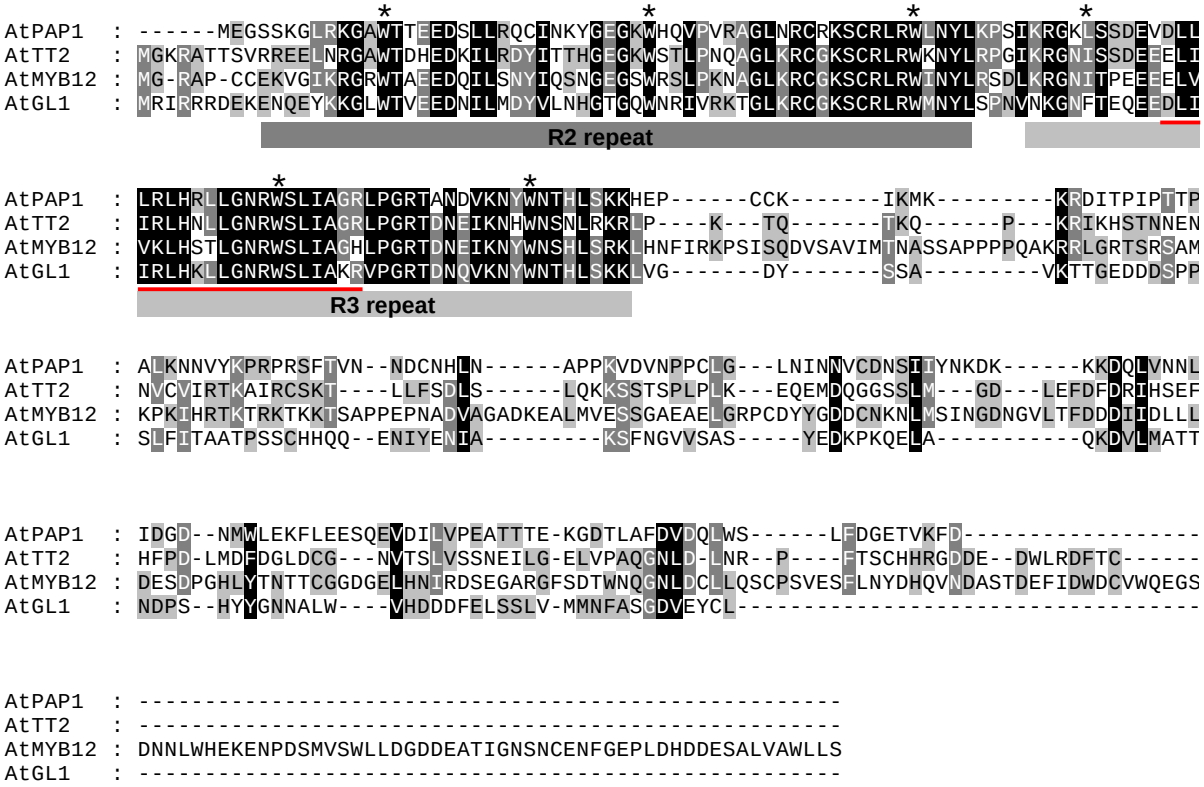


Figure 1.5 – Structural features of R2R3-MYB type transcription factors

Alignment of Arabidopsis R2R3-MYB proteins for regulation of anthocyanin synthesis (AtPAP1, AAG42001), tannin synthesis (AtTT2, CAC40021), flavonol synthesis (AtMYB12, ABB03913) and trichome formation (GLABROUS1, AtGL1, NP_189430), showing some features common to this type of transcription factor. Black blocks -100% conservation; Dark grey blocks -80% conservation; Light grey blocks -60% conservation. Grey blocks beneath the sequences depict R2 and R3 repeats. Regularly spaced tryptophan residues are labeled with an asterisk. The bHLH binding motif is underlined in red.

1.4.4 A protein complex is required for initiation of transcription

In some cases, the binding of a MYB factor to a target gene promoter is sufficient to activate transcription of the gene. The Arabidopsis flavonol regulator AtMYB12, for example, is capable of transcriptional activation that is independent of a bHLH partner (Mehrtens *et al.*, 2005). However, more commonly a protein complex is required for transcriptional activation of target genes (Larkin *et al.*, 1999; Grotewold *et al.*, 2000). In species analyzed to date, regulatory proteins isolated include those from the *MYB*, *bHLH* and *WD40* gene families. Figure 1.6 depicts a model of the role of these regulators in transcriptional activation of a structural pigmentation gene. Yeast one- and two-hybrid assays have indicated these proteins can interact with each other, and that MYB domains can bind to the promoters of target genes (Payne *et al.*, 2000; Aharoni *et al.*, 2001; Zimmermann *et al.*, 2004; Koshino-Kimura *et al.*, 2005). It is hypothesized that some R2R3-MYB transcription factors can even activate transcription of some bHLH genes (Quattrocchio *et al.*, 1998; Nesi *et al.*, 2001).

Functional specificity of these regulatory complexes appears to reside with the MYB factors, which display different binding specificities as determined by sequences within the DNA-binding domain (Carr *et al.*, 1996). Target differences, together with tissue-specific expression, account for the existence of a large number of MYB factors with somewhat overlapping functions. On the other hand, proteins belonging to the bHLH and WD40 (Walker *et al.*, 1999) families are more highly conserved and are consequently often involved in regulation of several processes (Koes *et al.*, 2005). For example within the flavonoid pathway, the Arabidopsis WD40 protein TRANSPARENT TESTA GLABRA1 (TTG1) works together with AtPAP1 (MYB) and GLABRA3 (AtGL3, bHLH) or ENHANCER OF GL3 (AtEGL3) to regulate anthocyanin synthesis (Zhang *et al.*, 2003; Zimmermann *et al.*, 2004; Gonzalez *et al.*, 2008), and also works in combination with AtTT2 (MYB) and TRANSPARENT TESTA8 (AtTT8, bHLH) to control tannin production (Baudry *et al.*, 2004). Furthermore, in addition to anthocyanin synthesis, GL3 and TTG1 are also required by the MYB factor GL1 and WEREWOLF (AtWER) for control of the formation of leaf and root hairs, respectively, in Arabidopsis (Larkin *et al.*, 1994; Lee and Schiefelbein, 1999; Walker *et al.*, 1999; Payne *et al.*, 2000).

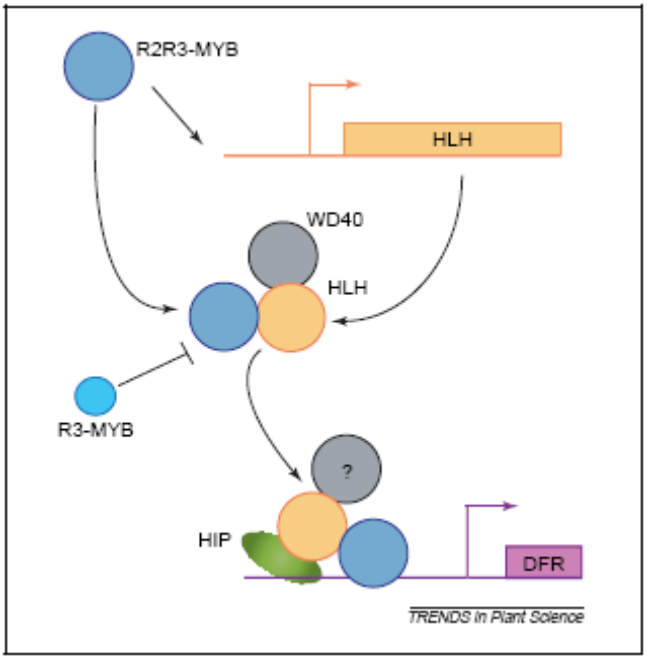


Figure 1.6 - Model depicting the role of MYB, bHLH and WD40 regulators in transcriptional activation of a flavonoid pathway gene (*DFR*)

A model for plant transcriptional regulation, as presented by Koes *et al*. (2005). Proteins are displayed as circles, genes by rectangles. (Some) of the MYBs control transcription of (some) of the bHLH factors, and subsequently form a complex that also involves the WD40 protein. The MYB factors contact the DNA directly, whereas the HLH proteins probably bind indirectly via a hypothetical HLH Interacting Protein (HIP). The small R3-MYB acts as an inhibitor, probably by sequestration of the bHLH protein into an inactive complex.

1.4.5 Repression of transcription by negative regulators of flavonoid biosynthesis

In addition the trans-activation of target genes, transcriptional regulators may modulate metabolite biosynthesis through gene repression. Evidence suggests this may be achieved either by competitive inhibition, as seen in Arabidopsis where the truncated gl3-1 bHLH interacts with GL1 and TTG1, thereby reducing the availability of these proteins to interact with the functional GL3 and initiate trichome synthesis (Payne *et al.*, 2000). Investigation of trichome patterning in Arabidopsis has also revealed that repression may also be achieved by the direct binding of a negative regulator to target DNA. An example of this is the recruitment of the R3-MYB TRICHOMELESS1 (TCL1) to the cis-acting regulatory elements of *GL1*, suppressing transcription of *GL1* and subsequent trichome development (Wang *et al.*, 2007). MYB factors have been described that negatively regulate lignin biosynthesis (Goicoechea *et al.*, 2005; Fornale *et al.*, 2006; Legay *et al.*, 2007), trichome formation (Schellmann *et al.*, 2002), root hair formation (Wada *et al.*, 2002) and phenolic acid synthesis (Tamagnone *et al.*, 1998; Jin *et al.*, 2000). In fruit, the repression of flavonoid biosynthesis by MYB factors has also been reported. Ectopic expression and suppression of the persimmon (*Diospyros kaki*) *MYB4* gene in kiwifruit (*Actinidia deliciosa*) has revealed it is a negative regulator of tannin synthesis (Akagi *et al.*, 2009). The strawberry R2R3-MYB transcription factor *FaMYB1* is capable of lowering the expression of anthocyanin and flavonol biosynthetic genes in transgenic tobacco (*Nicotiana tabacum,* Aharoni *et al.*, 2001). Furthermore, a number of Arabidopsis R3-type, single domain MYB factors, including AtMYBL2, AtMYB60 and CAPRICE (AtCPC), can inhibit anthocyanin synthesis in transgenic plants (Matsui *et al.*, 2008; Park *et al.*, 2008; Zhu *et al.*, 2009).

Many MYB factors that function as transcriptional repressors share conserved sequences in their C-terminal regions. Some belong to subgroup 4 due to the presence of the C2 motif LNL[E/D]L and the presence of a putative zinc-finger domain (Kranz *et al.*, 1998; Stracke *et al.*, 2001). The C2 motif has become known as the ERF-associated amphiphilic repression (EAR) domain (Ohta *et al.*, 2001). However, some MYB factors have been characterised as repressors despite the absence of an EAR domain. A novel repression domain R/KLFGV was recently identified (Ikeda and Ohme-Takagi, 2009), assisting in the classification of repressors that do not possess any of the previously described functional motifs, such as AtMYBL2 and *Eucalyptus gunnii* MYB2 (Goicoechea *et al.*, 2005; Matsui *et al.*, 2008).

1.4.6 Engineering of flavonoids within plant species

Knowledge obtained from the study of flavonoid pathway regulation has been applied in an attempt to enhance the visual and nutritional qualities of plants. Early metabolic engineering efforts focused on the over-expression and silencing of flavonoid structural genes, as reviewed by Shimada (2000), Schijlen *et al.* (2004) and Tanaka and Ohmiya (2008). However, the manipulation of MYB TFs has presented a more effective way to alter the expression of flavonoid genes and control pathway flux (Grotewold, 2008). Whilst a plethora of transgenic plants have been generated, some notable efforts have been made in tomatoes (*Solanum lycopersicum*). Tomato fruit containing very high levels of flavonols were obtained from the expression of *AtMYB12* under a strong constitutive promoter (Luo *et al.*, 2008). Secondly, the generation of a purple tomato was achieved by introducing the snapdragon MYB and bHLH genes *ROSEA1* (*AmRos1*) and *DELILA* (*AmDel*), driven by a fruit-specific promoter, into tomato (Butelli *et al.*, 2008).

Another excellent example of genetic modification is the blue rose. Plant species belonging to the Rosaceae family, like sweet cherry and rose (*Rosa hybrida*), lack the F3’5’H activity required to produce the tri-hydroxylated delphinidin-based anthocyanins that give a blue hue. In recent years, through the introduction of pansy (*Viola* spp.) F3’5’H genes, highly sought after blue roses have been obtained (Brugliera *et al.*, 2004; Katsumoto *et al.*, 2007). Although blue roses are not currently commercially available, blue/purple coloured carnations, engineered by Florigene (Australia, www.florigene.com.au) to introduce petunia and snapdragon F3’5’H genes, have been available on the cut-flower market for almost 15 years.

1.5 SUMMARY AND PROJECT AIMS

Flavonoids are important secondary metabolites that have implications in terms of plant protection, reproduction, fruit quality and human health. The biochemical pathway by which flavonoid compounds are synthesised in plants has been well characterised. However, significant differences in the targets of regulators of flavonoid synthesis have been reported amongst different plant species. Variations in skin and flesh colour between varieties, and the light-dependent pigmentation of blush-skinned fruit, suggest that sweet cherry exhibits complex control of flavonoid biosynthesis. However, limited information is available regarding the molecular biology behind flavonoid biosynthesis and its regulation in sweet cherry fruit. Currently, there are no databases containing flavonoid gene sequences from this species.

1.5.1 Aims

The aim of this research project is to investigate the flavonoid biosynthetic pathway and its regulation in sweet cherry fruit. Within this thesis, the following scientific questions will be addressed:

- Is flavonoid synthesis temporally regulated in developing sweet cherry fruit, and how is this achieved?
- How does sunlight affect the expression of flavonoid pathway genes in various sweet cherry cultivars?
- What is the genetic basis of sweet cherry fruit colour?

To answer these questions, the specific aims of the research are:

- Isolate flavonoid structural and regulatory genes from sweet cherry
- Determine copy numbers of cloned genes in the sweet cherry genome
- Analyse the expression of the flavonoid-related genes in response to developmental stage and sunlight
- Compare gene sequences and expression amongst phenotypically different sweet cherry cultivars
- Correlate flavonoid metabolite accumulation with gene expression
- Identify putative regulators of flavonoid synthesis through molecular characterisation
- Demonstrate that putative MYB transcription factors function as pathway regulators by promoter-specific binding and transient trans-activation of flavonoid structural genes

Chapter Two: General methods

2. GENERAL METHODS

Methods that vary from their published form or manufacturers' protocol are outlined. Reagents, buffers, solutions, and media (and their suppliers) are listed in Appendix 1 and are underlined in the text.

2.1 SWEET CHERRY VARIETIES OF IMPORTANCE TO THIS RESEARCH

Worldwide, there are a vast number of commercially grown sweet cherry varieties. Of particular importance in this study are 'Yellow Glass', 'Ranier', 'Lapins' and 'Sam', which are described below. These varieties represent four different colour phenotypes displayed on the skin of sweet cherries; yellow, blush, red and black. Pictures of fruit from these varieties are shown in Chapter 5.

2.1.1 Yellow Glass

Fruit is small and sweet-tasting, with golden skin and clear flesh. A very hardy cultivar, it grows extremely well in cold regions but requires a pollinator. The origin of 'Yellow Glass' has not been documented.

2.1.2 Ranier

An extremely hardy, very attractive, large yellow cherry with clear flesh and a bright red blush. The fruit is firm, and the flesh is sweet and finely textured. 'Ranier' originated from a cross between the two red sweet cherry varieties 'Bing' and 'Van' at Washington State University Research Station in 1952. Since its introduction 'Ranier' has become one of the highest value sweet cherries produced in the United States.

2.1.3 Lapins

In 1968, the Summerland breeding program released the self-fertile 'Stella' sweet cherry (Lambert x John Innes Seedling 2420), which has assisted with breeding across incompatible pollen groups (Iezzoni, 2008). The cultivar 'Lapins' resulted from a cross between 'Stella' and 'Van', and is late maturing, highly cracking-resistant and self-fertile. A large, high yield, mahogany-red cherry that exhibits excellent firmness and flavour. 'Lapins' was released out of the Agriculture and Agri-Food Canada (AAFC) Research Centre in Summerland, British Columbia (BC), Canada in 1983.

2.1.4 Sam

'Sam' bears fully black fruit that is medium in size and firmness with a coarse texture. The tree is a vigorous grower and is resistant to bacterial canker, while the fruit shows good resistance to cracking. 'Sam' results from the open pollination of Vineland selection V-160140, which was generated through open pollination of 'Windsor'. It was a release from Summerland in 1953.

2.2 SAMPLE PROCESSING AND PREPARATION

2.2.1 Fruit developmental series

Sweet cherry trees used for this study were located in the Adelaide Hills, at Primary Industries and Resources South Australia's (PIRSA) Lenswood station (34° 55' S, 138° 49' E). Sweet cherry buds, flowers and fruit from the cultivar 'Lapins' were sampled at 10 am at weekly intervals during September to December 2006. Early growth stages were defined according to a reference chart from Michigan State University Extension (Figure 2.1). Fruit samples were identified by when they were sampled, in terms of number of days after full bloom (DAFB). 'Lapins' full bloom, when all or most of the flowers on the tree are open, occurred on September 11 2006. Each sample contained 10-12 individual fruit, where possible. Following random selection of fruit, field measurements were taken. Calipers were used to determine fruit diameter (mm) and deformability (mm). Fruit mass (g) was determined with electronic scales and volume (ml) was measured by displacement of water in a measuring cylinder. Soluble sugar content (°Brix) was measured with a hand-held refractometer (Reichert, USA). For storage, all samples were pitted, pooled and snap frozen on liquid nitrogen within 20 minutes of being removed from the tree. Samples were stored at -80°C.

2.2.2 Varietal comparison series

'Ranier', 'Lapins' and 'Sam' fruit were collected at three different developmental stages, from PIRSA Lenswood, as described for the developmental series. Green, immature fruit were sampled between 35 and 37 DAFB, after the onset of pit-hardening so the endosperm could be removed. Similarly, colouring and ripe fruits were also pitted. Colouring fruit were sampled within the week that red colour began to appear on the skin of the particular variety, corresponding with 56-65 DAFB. Finally, ripe fruit were sampled within days of their commercial harvest dates, with collection falling between 86 and 93 DAFB for each variety.

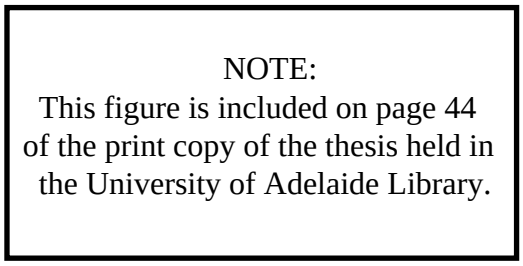


Figure 2.1 – Sweet cherry fruit growth stage reference

Photographs and descriptors for stages of fruit growth in the sweet cherry variety ‘Heldenfingen’. Not all stages are represented by pictures; numbers in top left corner of pictures correspond to descriptor numbers. Adapted from Michigan State University Extension’s website (Longstroth, 2009). Photographs are courtesy of Mark Longstroth and are not to scale.

2.2.3 Fruit bagging series

Sweet cherry trees used in this study were located in the Okanagan Valley, at Agriculture and Agri-Food Canada's Summerland research centre (49° 36' N, 119° 40' W). For the dark-grown fruit treatment, foil-lined bags were placed over bunches of green 'Yellow Glass', 'Ranier', 'Sam' and 'Lapins' fruit on June 19 2008 (39-48 DAFB) to exclude sunlight from the fruit (Figure 2.2). Bags contained slits for air flow and drainage and were positioned randomly throughout the canopy. Bags were removed from mature fruit on July 21 2008, leaving some bags as a baseline control at the completion of the experiment. Upon bag removal dark-grown bunches, referred to hereafter as unbagged fruit, were marked with flagging tape. Unbagged fruit, together with control fruit (grown under normal light conditions) from the same branch, were randomly sampled at 10 am on 0, 1, 2, 4, and 7 days after bag removal (DABR). Bagged fruit remained protected from light throughout the experiment and was sampled 7 DABR. For each sample ten fruit were selected and field measurements were taken. Fruit mass (g) was determined with an electronic scale. Colour of each individual fruit was also measured on both cheeks with a Colormeter (Konica Minolta) using the CIELab colour system. Samples were then divided into biological triplicates of three individual fruit per replicate. Following dissection and snap freezing of skin containing <5mm flesh, samples were stored at -80°C.

Figure 2.2 – Sweet cherry cv. Lapins 'bagged' fruit

Photograph demonstrating the covering of sweet cherry fruit in early developmental stage with foil-lined bags to block sunlight.

2.3 ANALYSIS OF FLAVONOID COMPOSITION

For analysis of flavonoid composition, tissue was ground into a fine powder. This was performed under liquid nitrogen in a chilled grinding mill (IKA®, Germany) and then with a chilled mortar and pestle. Powdered tissue was then returned to -80°C storage. All HPLC analysis was kindly performed by Dr Samuel Brooke (CSIRO, Plant Industry).

2.3.1 Determination of anthocyanin and flavonol content

2.3.1.1 UV-VIS spectrophotometry

For determination of anthocyanin concentration, 1ml of 3% (v/v) HCl in methanol was added to 0.1g of finely ground plant material and the anthocyanins extracted by sonication for 20 min. Samples were then centrifuged at 13,000*g* for 10 min and the absorbance of the supernatant at 520nm was measured using a 10mm path length quartz cuvette in a Shimadzu UV-1601 spectrophotometer (Shimadzu Scientific Instruments, Australia). Samples were diluted in extraction solvent in order to fall within the working range of the spectrophotometer. Anthocyanin concentration was calculated using the method described in (Iland *et al.*, 2000). Each sample was prepared in triplicate.

2.3.1.2 High Performance Liquid Chromotography (HPLC) analysis

Anthocyanins and flavonols were extracted by adding 1mL of 50% (v/v) methanol in high purity water to 0.1g aliquots of frozen plant material and sonicating for 20 min in an ice-water bath. Samples were centrifuged for 10 mins at 13,000*g*, and a 200µl aliquot of the supernatant transferred to auto-sampler vials for analysis by HPLC. Separation of sweet cherry anthocyanins and flavonols was performed using a Hewlett Packard 1100 HPLC system with a Wakosil C18 analytical column (3µm, 150mm x 4.6mm, SGE, USA) protected by an C18 guard column (SGE), as described by Downey *et al.* (2003b). The separation used 10% (v/v) formic acid (solvent A) with methanol (solvent B). The gradient conditions and flow rate were as published by Czemmel *et al*. (2009). Anthocyanin concentrations were determined using standard curves prepared from malvidin-3-glucoside (Extrasynthese, France). Anthocyanin peaks were tentatively identified based on elution order of grape anthocyanins compared to the retention time of commercial standards, and also on the reported abundance of sweet cherry anthocyanins (Tanchev, 1975; Gao and Mazza, 1995; Mozetic *et al.*, 2002). All anthocyanins were expressed as malvidin-3-glucoside equivalents. Flavonol concentrations were determined using standard curves prepared from quercetin-3-glucoside, quercetin-3-rutinoside, kaempferol-3-rutinoside and syringetin-3-glucoside (Extrasynthese,

France), myricetin and quercetin (Sigma Chmicals, Australia) and quercetin-3-glucuronide isolated from coriander seeds and kindly provided by Dr David Jeffery. Details of the extraction and isolation are described in Jeffery *et al.* (2008). Uncalibrated flavonols were identified based upon their characteristic UV spectra and expressed as quercetin-3-glucoside equivalents.

2.3.2 Determination of tannin content

2.3.2.1 High Performance Liquid Chromotography (HPLC) analysis following phloroglucinol cleavage

Sweet cherry condensed tannins were extracted into 70% (v/v) acetone with 0.1% (w/v) ascorbate from 0.1g of ground and frozen plant material. Following centrifugation, one 200µl aliquot and one 500µl aliquot of each supernatant were transferred to fresh tubes and dried down under vacuum at 35°C for 60 mins. The 500µl aliquot was resuspended in 100µl 50% (v/v) methanol, centrifuged (13,000*g* for 10 mins), and 80µl transferred to an auto-sampler vial for the analysis of free monomers. The other aliquot was subjected to acid-cleavage in the presence of excess phloroglucinol following the method of Kennedy and Jones (2001), as described by Downey *et al.* (2003a). Briefly, the dried sample was resuspended in 100µl of phloroglucinol buffer and incubated at 50°C for 20 mins, then neutralised with 100µl of 200mM sodium acetate and centrifuged (15 mins at 13,000*g*). 100µl of the supernatant was then transferred to HPLC auto-sampler vials. Samples were run on a reverse-phase HPLC device (HP-1100) with a LiCrospher C18 analytical column (5µm, 250mm × 4mm, Merck, Germany) protected by a C18 guard column (SGE). The separation used 0.2% (v/v) acetic acid (solvent A) with methanol (solvent B). The gradient of solvent B was: 0 min, 1%; 40 min, 1%; 120 min 30%; 120.1 min, 100%; 130 min, 100%; 132 min, 1%. 25 µL of sample was injected, and run at a column temperature of 25°C with a flow rate of 1ml min^{-1}. Concentrations of free monomers and hydrolysed terminal subunits were determined from standard curves prepared from commercial standards of catechin and epicatechin (Extrasynthese, France). The concentration of extension subunit-phloroglucinol adducts was calculated from published molar extinction co-efficients (Kennedy and Jones, 2001).

2.4 EXTRACTION OF SWEET CHERRY NUCLEIC ACIDS

For extraction of nucleic acids (RNA and DNA), finely ground plant material was prepared as described in Section 2.3.

2.4.1 RNA extraction

RNA extractions were performed on the majority of samples using a modified hot borate method. However, over-ripe fruit samples (100 and 107 DAFB) are very high in soluble pectins, a characteristic unique to cherry (Choi *et al.*, 2002). Therefore, extraction using a mini cellulose column to bind RNA was required. Total RNA isolated by both methods was DNase-treated with a Turbo DNA-free™ kit (Ambion, USA).

2.4.1.1 Hot borate method

RNA was extracted from 1g sweet cherry material using a modified hot borate method (Wan and Wilkins, 1994). The final RNA pellet was resuspended in 200µl Tris-EDTA (TE) (pH 8.0), quantified by UV spectrometry (Section 2.5.3) and the quality of the RNA checked on a 1% (w/v) agarose Tris-borate-EDTA (TBE) gel.

2.4.1.2 Mini cellulose method

RNA was extracted from sweet cherry plant material using a small scale modification (by Dr Paul Wiersma) of a previously published cellulose-binding method (FilsLycaon *et al.*, 1996). 500mg of powdered sweet cherry tissue in two 2.0ml micro-centrifuge tubes was shaken at room temperature for 20 min with (per tube): 0.6ml GPS buffer; 33µl 20% (w/v) SDS; 33µl 2-mercaptoethanol; and 0.6ml equilibrated phenol (pH 8.0). 200µl of chloroform:isoamyl alcohol (24:1) was added and mixed briefly before centrifugation for 20 min at 14,000*g* at room temperature. The aqueous upper layer was carefully removed and 95% ethanol was added to bring the final ethanol concentration to 30% (462µl 95% ethanol per ml of supernatant). The RNA/30% ethanol extract was poured into a Bio-Rad Poly-Prep column that was mounted on a vacuum manifold and packed with 1ml of Whatman CC41 cellulose slurry in 30% STE. The vacuum was applied to draw the extract through the cellulose and wash the column with approximately 40ml 30% STE. Residual buffer was expelled with air. Total RNA was eluted with RNase-free water, then precipitated with sodium acetate/ethanol at +4°C and washed with cold 70% ethanol. The RNA was resuspended in 40µl RNase-free TE and quantified as described in Section 2.5.3.

2.4.2 DNA extraction

DNA was extracted from 1g sweet cherry leaf material (young leaves) using a nuclear isolation method adapted from Thomas *et al.* (1993). Initial resuspension of the pellet in extraction buffer was performed at 55°C. The final DNA pellet was resuspended in 150µl TE and quantified by UV spectrometry.

2.4.3 Plasmid DNA isolation

Bacterial colonies (Section 2.5.4) were used to inoculate 2ml of LB medium + 100mg/ml Ampicillin in 12ml culture tubes and incubated overnight at 37°C in a Ratek Orbital Mixer Incubator (Ratek, Australia), shaking at 200rpm. Plasmid DNA was isolated from bacterial cells using a QIAprep Mini Kit (QIAGEN, Germany), following the manufacturers' protocol.

2.4.4 First strand cDNA synthesis

First strand cDNA was synthesised from 4µg total RNA. Oligo $dT_{(18)}$ was used in the reaction, together with SuperScript™ III RNase H- Reverse Transcriptase and RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, USA), following the manufacturers' protocol. Working concentration of cDNA for Real Time PCR was obtained by performing a 1:15 dilution with sterile nuclease-free water (SNW).

Once synthesised, cDNA was checked to ensure it contained no genomic DNA. This was achieved by standard PCR (Section 2.5.2) of cDNAs using *PaMYBA1* intron probe F and R primers designed to amplify the 599bp second intron of *PaMYBA1*.

2.5 ISOLATION AND MOLECULAR ANALYSIS OF FLAVONOID PATHWAY GENES

Prior to 2006, when this study began, a single flavonoid-related gene sequence from sweet cherry was present in the GenBank™ database. The *Prunus avium phenylalanine ammonia-lyase* (*PaPAL1*, AF036948) sequence was deposited by Dr Paul Wiersma of AAFC, who became a collaborator. Dr Wiersma had isolated partial sequences from anthocyanin-related flavonoid genes, and thus individual contributions to the isolation of the flavonoid pathway genes are summarised in Table 2.1.

2.5.1 Multiple sequence alignment and primer design

For each gene a Basic Local Alignment Search Tool (BLAST, Altschul *et al.*, 1997) program available from the National Centre for Biotechnology Information (NCBI) website (http://www.ncbi.nlm.nih.gov/) was applied to identify sequences with similarity to a characterized sequence, preferably fruit such as apple or grape but often Arabidopsis. Resulting peptide sequences from several different plant species were aligned (Section 2.5.5) and degenerate primers were designed in areas of highly conserved sequence. Primers were designed based on the following criteria: 22-27bp in length, degeneracy of less than 128,

maximum of 5 inosine bases but none in the last 5 bases and the final bases should encode a conserved amino acid such methionine (M) or tryptophan (W). Primers are listed in Appendix 2a.

Table 2.1 – Contributors to the isolation of flavonoid pathway gene sequences

Genes were isolated by Paul A Wiersma (PAW) or Jessie-Lee Parker (JP). Additionally, the accession numbers for the promoter regions and 5'UTRs of some genes are listed in Appendix 4.

Gene	GenBank accession	Isolated by
PaPAL1	AF036948	PAW
PaC4H	GU990522	PAW
Pa4CL	GU990523	PAW
PaCHS1	GU990524	PAW
PaCHS2	GU938683	JP
PaCHS3	GU938684	JP
PaCHI	GU990525	PAW
PaF3H	GU990526	PAW
PaF3'H	GU990527	PAW
PaFLS	GU938685	JP
PaDFR	GU990528	PAW
PaLAR1	GU938686	JP
PaLAR2	GU938687	JP
PaLDOX	GU990529	PAW
PaANR	GU938688	JP
PaUFGT	GU990530	PAW
PaMYBA1	GU938680	JP
PaMYBA2	GU938681	JP
PaMYBA3	GU938689	JP
PaMYBPA1	GU938690	JP
PaMYBF1	GU938682	JP
PaTT8	GU938693	JP
PaTT12	GU938692	JP
PaMYBR	GU938691	JP

2.5.2 Polymerase chain reaction (PCR)

50µl PCR reactions were performed in 0.2ml PCR tubes, in a Hybaid PCR Express thermocycler (Hybaid, UK). Each reaction contained 30ng genomic DNA or 2µl diluted cDNA as template, 10x PCR buffer, 1.5mM $MgCl_2$, 200nM dNTP mix, 500nM of each primer, 0.25U Platinum® Taq DNA polymerase (Invitrogen) and SNW. Standard thermocycling conditions were: initial denaturation of 94°C for 3 min; 35 cycles of 94°C for 30s, 55°C for 30s, 72°C for 2 min; and final extension of 72°C for 10 min. For amplification with degenerate primers, betaine (1M final concentration) was added to the reaction as a PCR enhancer and a touchdown PCR with the following conditions was used: initial denaturation of 94°C for 1 min; 2 cycles of 94°C for 45s, 37°C for 2 min, 72°C for 3 min; 33 cycles of

94°C for 30s, 52°C for 30s, 72°C for 2 min; and final extension of 72°C for 7 min. Where necessary, PCR conditions were optimised for annealing temperature with a gradient thermocycler, or the magnesium concentration altered if required. PCR templates, parameter variations and primer sets used are summarised in Appendix 3.

2.5.3 Visualisation and quantification of nucleic acids

Nucleic acids were resolved on a 1% (w/v) agarose TBE gels (in 1x TBE buffer), using standard protocol (Sambrook *et al.*, 1989). Nucleic acids were visualised by ethidium bromide staining and photography of gels under UV light (240nm). Size and quantity of nucleic acid bands were estimated by comparison to 1kb Plus DNA Ladder (Invitrogen) and Hyperladder (Bioline, UK) molecular markers, respectively. If necessary, quantification of nucleic acids was performed using a Nanodrop spectrophotometer (Thermo Scientific, USA). Absorbance of 1.0 is equivalent to 50µg ml^{-1} DNA and 40µg ml^{-1} RNA.

2.5.4 Cloning procedures

Nucleic acid bands were visualised under a dark reader and excised from a Tris-acetate-EDTA (TAE) 1% (w/v) agarose gel with a sharp scalpel blade. DNA fragments purified from the gel using a QIAquick Gel Extraction Kit (QIAGEN, Germany) were ligated to a pDRIVE (QIAGEN, Germany) cloning vector. 5µl pDRIVE ligation reactions contained a 5 molar excess of insert, 25ng vector and 2x master mix, were incubated for 2h at 16°C, and the reaction terminated with 70°C heat for 10 min.

For transformation of electro-competent *Escherichia coli* cells (XL-1 Blue, Stratagene, USA), 2µl of the ligation and 45µl thawed cells were placed in a pre-cooled 0.1cm electroporation cuvette (BioRad, USA) and an electrical pulse was applied at a field strength of 1.8V, capacitance of 25µF, resistance of 200Ohms and a pulse length of 4 - 4.5s. Cells were transferred into SOC media, incubated at 37°C for 1h, and plated onto Ampicillin/IPTG/X-Gal (AIX) + agar plates. Following incubation of plates overnight at 37°C, single white colonies were streaked onto fresh AIX plates, incubated overnight and used for inoculating overnight cultures as described in Section 2.4.3.

To confirm the presence of the DNA insert in the isolated plasmid clones (Section 2.4.3), 3µl of DNA solution extracted by a miniprep method was digested in a 10µl reaction with 10U *Eco*R1 (Roche, Switzerland) for 1h at 37°C. The digested plasmid was resolved on a 1% (w/v) agarose TBE gel, visualised and photographed.

2.5.5 Sequencing and sequence analysis

PCR products from genomic DNA or cDNA templates and clones containing DNA inserts from standard PCR and PCR with degenerate primers were sequenced by the Big Dye Terminator (ABI, Version 3.1) reaction (AGRF, Brisbane). Reactions contained ~1000ng plasmid and 6.4pmol SP6 or T7 primer or ~100ng of 200-500bp PCR product and 5pmol gene-specific primer. Sequences were aligned and checked in BioManager (Australian National Genomic Information Service (ANGIS, http://www.angis.org.au/), using the CLUSTALW program (Thompson *et al.*, 1994). Alignments for presentation were generated using GeneDoc Version 2.7 (Nicholas and Nicholas, 1997).

2.5.6 Determination of 5' and 3' gene sequences

The partial gene sequences obtained by degenerate PCR were used to design gene-specific forward and reverse nested primers for the determination of 5' and 3' gene sequences (primers sequences listed in Appendix 2, primer sets used in Appendix 4). 3' random amplification of cDNA ends (RACE) was performed under standard PCR conditions using 'Lapins' young leaf cDNA as the template for most reactions. Any variations in template and conditions are listed in Appendix 3. The 5' coding sequences and untranslated regions (UTRs) of the genes were determined by PCR from 'Lapins' genomic DNA libraries generated with a Universal Genome Walker™ Kit (CLONTECH Laboratories), as per the manufacturers' protocol. Genome Walker PCR conditions were standard with the exception of the extension temperature of 68°C, due to the use of the Platinum® Taq HiFi (Invitrogen) polymerase enzyme. Using sequence information obtained, primers were designed to amplify the full-length coding sequences of the genes, which were sequenced for gene confirmation.

2.5.7 Identification of amino acid motifs

Following isolation of sweet cherry MYB genes, the GENESCAN web server at http://genes.mit.edu/GENSCAN.html (Burge and Karlin, 1997) was used to obtain predicted peptide sequences. Predicted sequences, together with other highly similar protein sequences, as identified by BLAST search (Section 2.5.1), were analysed for the presence of conserved motifs using the program Multiple Em for Motif Elicitation (MEME Version 4.1.1 at http://meme.sdsc.edu Bailey *et al.*, 2006). Putative MYB C-terminal motifs were supported by BLAST searches of both nucleotide and EST databases, using the motif peptide sequences as queries.

2.6 DETERMINATION OF GENE COPY NUMBER

2.6.1 Digestion of genomic DNA

Genomic DNA from 'Yellow Glass', 'Ranier' (or 'Dame Nancy'), 'Lapins' and 'Sam' was digested with the restriction enzymes that did not have cut sites within the probe sequence for the gene of interest. Some enzymes used include *Eco*RV, *Bgl*II, *Bam*HI, *Eco*RI, *Nco*I and *Xba*I (Roche). Digestion reactions contained 2µg DNA, 10U of the appropriate enzyme, and its corresponding buffer in a total volume of 40µl. Digests were incubated for 5h at 37°C, electrophoresed on a 0.7% (w/v) agarose TBE gel overnight at 15V, stained with ethidium bromide and photographed under UV light.

2.6.2 Southern blotting procedure

The 0.7% (w/v) agarose TBE gel (Section 2.6.1) was depurinated for 10 min in 0.25N HCl, neutralised for 30 min in 0.4N NaOH/0.6M NaCl and washed for 30 min in 1.5M NaCl/0.5M Tris-HCl pH 7.5. The DNA was blotted to a GeneScreen Plus® membrane (NEN, USA) overnight using a TurboBlotter™ Rapid Downward Transfer System (Schleicher and Schuell, Germany). Transfer solution was 10x SSC. Following the transfer, the DNA on the membrane was denatured in 0.4N NaOH, neutralised in 0.2M Tris-HCl pH 7.5/1x SSC and dried on Whatman paper (Whatman, UK), then the DNA fixed to the membrane using a UV Stratalinker (Stratagene). Prehybridisation of the membrane was carried out in GeneScreen hybridisation buffer in a rotor oven at 65°C.

Approximately 500bp DNA fragments generated using standard PCR (Section 2.5.2) were used as probes. A list of gene probes and the primers used to generate them is provided below (Table 2.2). The purified and sequenced DNA was labelled with [^{32}P]-dCTP (3000Ci/mmol), using a Rediprime™ II DNA Labelling System (Amersham Biosciences, UK). Unincorporated nucleotide was removed from the probe solution using Illustra™ MicroSpin S-200 HR columns (GE Healthcare, USA). The denatured labeled probe was added to the hybridisation buffer and allowed to hybridise to the membrane in a rotor oven overnight at 65°C. The membrane was washed twice in 2x SSC and three times in 0.1x SSC containing 0.1% (w/v) SDS. Each wash was executed at 65°C for 15 min. Finally, the membrane was blotted dry and exposed to BioMax MS film (Kodak, Australia) for 3h at -70°C. Subsequently, membranes were boiled for 30 min in a solution of 0.1x SSC containing 3% (w/v) SDS to strip the probe and allow multiple hybridisations to be performed on each membrane.

Table 2.2 – Primers to generate DNA fragments for use as oligonucleotide probes in Southern blots

DNA fragments were generated using 'Lapins' young leaf cDNA as a template, except where 'Lapins' genomic DNA was used (denoted by #). Due to the conserved nature of MYB genes, probes were located either in the 5' UTR (*) or in the more divergent activation domain (^). Primer sequences are listed in Appendix 2.

Gene	Primers	Probe size (bp)
CHS	*CHS* probe F/R	411
FLS	*FLS* probe F/*FLS* QRT R1	409
DFR	*DFR* probe F/R	700
LAR	*LAR* probe F/R	501
ANR	*ANR* probe F/R	531
LDOX	*LDOX* probe F/R	484
UFGT	*UFGT* probe F/R	504
MYBA1#	*AN2-1* 5'UTR F/*MYBA1* 5' probe R*	574
	MYBA1 frameshift F/*AN2* QRT R1^	669
MYBA2#	*MYBA2* 3' F2/*MYBA2* QRT R1^	569

2.7 EXPRESSION ANALYSIS OF FLAVONOID GENES

2.7.1 Determination of primer efficiency

Real Time PCR primers specific for each flavonoid gene were designed to amplify a 150-300bp product, flanking the stop codon where possible. The specificity of the PCR product generated from each primer set was confirmed by detection of a single band of the correct size on an agarose gel, sequencing, and a single peak in fluorescence from a melt curve (Section 2.7.2). The amplification efficiency (E_{target}) of each primer set was calculated by the method of Muller *et al*. (2002), using the cycle threshold values obtained from Real Time PCR of a series of dilutions of 'Lapins' cDNA. Primer sequences are listed in Appendix 2, while Appendix 5 shows an example of an amplification efficiency plot and E_{target} values for each primer set.

2.7.2 Real Time PCR

Real Time PCR was performed in a Rotor-Gene™2000 instrument (Corbett Research) with 5µl of diluted cDNA used as template in a reaction containing 266nM of each primer and Absolute™ QPCR SYBR® Green buffer (ABgene®, UK). Thermocycling conditions were as follows: an initial activation of 15 min at 95°C, followed by 35 cycles of denaturation at 95°C

for 30s, annealing at 58°C for 30s and extension at 72°C for 30s, followed by a melt gradient starting from 50°C and heating to 96°C at a rate of 0.2°C s^{-1}. Reaction fluorescence was measured with an excitation wavelength of 470nm and a detection wavelength of 510nm after each extension step and at each 1°C increment of the melt profile. For comparison of gene expression between cDNAs, for each gene Real Time PCR was conducted on all cDNAs in a single batch.

2.7.3 Normalisation and analysis of gene expression

Expression levels of the various genes analysed were calculated by applying the amplification efficiency values described in Section 2.7.1 to the equation in Figure 2.3. For all analyses the signal obtained for the gene of interest (target) was normalised against the signal from the constitutively expressed sweet cherry *ubiquitin* (*PaUBI*, reference) gene. Where relative changes in gene expression are shown, transcript abundance was plotted as a fold increase above the expression of a particular time point or treatment.

$$NE = \frac{(E_{ref})^{CT\ ref}}{(E_{target})^{CT\ target}}$$

Figure 2.3 – Formula for the calculation of normalised gene expression

NE, normalized gene expression; E_{target}, PCR amplification efficiency of the target gene; E_{ref}, PCR amplification efficiency of the reference gene; CT_{target}, threshold cycle of the PCR amplification of the target gene; CT_{ref}, threshold cycle of the PCR amplification of the reference gene. The CT is defined as the cycle at which the fluorescence rises appreciably above the background fluorescence. Figure taken from Muller (2002).

2.8 FUNCTIONAL CHARACTERISATION OF PUTATIVE MYB REGULATORS

2.8.1 Preparation of constructs

Full-length MYB cDNAs were isolated by PCR with a high fidelity polymerase enzyme, using primers with restriction sites incorporated into them. Following confirmation of the PCR product sequence, the MYB cDNA was ligated to the multiple cloning site of the pART7 vector (Gleave, 1992). Plasmids from clones containing full-length MYB inserts with the correct sequence were prepared using a HiSpeed™ Plasmid Midi Kit (QIAGEN), as per the manufacturer's protocol. MYB cDNAs in pART7 and gene promoter fragments in pLuc from

apple, grape and Arabidopsis were provided by previous lab members Dr Adam Takos, Dr Jochen Bogs and Dr Felix Jaffé.

2.8.2 Functional assay

Gold particles were coated by the method of Ramsay *et al*. (2003) with a mixture of 150ng each of the constructs; a sweet cherry, grape, apple, or Arabidopsis MYB in pART7, AtEGL3 in pFF19 (Ramsay *et al.*, 2003), a sweet cherry, grape, apple, or Arabidopsis promoter fragment in the firefly (*Photinus pyralis*) luciferase (LUC) plasmid pLuc, and 3ng of the *Renilla reniformis* LUC plasmid pRluc (Horstmann *et al.*, 2004). A suspension culture of grapevine 'Chardonnay' petiole callus was grown in Grape Cormier (GC) medium (Do and Cormier, 1991) to a log phase and filtered onto sterile Whatmann® discs (5.5cm) on the surface of GC-agar plates. The grape cells were bombarded with DNA-coated gold particles at 350kPa helium in a vacuum of 75kPa and a distance of 14cm as described by (Torregrosa *et al.*, 2002). Cells were incubated in the dark for 48h at 28°C, harvested and lysed by grinding on ice in 150µl of Passive Lysis buffer (Promega). Enzyme activities of both *P. pyralis* and *R. reniformis* LUC were determined using the Dual-Luciferase Reporter Assay system (Promega). Light emission was measured with a TD-20/20 Luminometer (Turner Biosystems).

Chapter Three: Isolation of flavonoid pathway genes from sweet cherry

3. ISOLATION OF FLAVONOID PATHWAY GENES FROM SWEET CHERRY

3.1 INTRODUCTION

Flavonoid pathway studies provide meaningful insight into the complex control by plants over secondary metabolite production. Moreover, knowledge of flavonoid biosynthesis and its regulation has provided plant growers with crop management suggestions (Reynolds *et al.*, 2006; Takos *et al.*, 2006b), breeders with molecular tools (Takos *et al.*, 2006a; Walker *et al.*, 2007), and scientists with metabolic engineering targets (Tanaka and Ohmiya, 2008). Ultimately, these will enable the quality improvement of plant products like flowers, fruits and vegetables. The flavonoid pathway has been well studied in a number of plant species, in particular maize, petunia and snapdragon (Holton and Cornish, 1995). However, genes involved in flavonoid production in sweet cherry fruit, an important high-value product, have not been described.

The genes encoding flavonoid biosynthetic enzymes and transcription factors were initially elucidated from various plant species through a combination of purified enzyme analysis, differential screening, genetic mapping, and characterisation of natural and induced mutations (Section 1.3.2). The important roles of flavonoid compounds in seed dispersal, reproductive viability and evasion of herbivory have meant that the sequences of flavonoid-related genes are generally well conserved amongst different plant species. The conserved domains and motifs present within R2R3-MYB transcription factors were presented in Chapter 1. As a consequence of such conservation, known sequences have been utilised to identify flavonoid-related genes from different species, using predominantly PCR-based methods, and analysis of sequencing projects. Molecular probes based on known sequences have also been used in identifying corresponding genes from a range of plant species. The first flavonoid biosynthetic gene identified, *CHS* from parsley (*Petroselinum hortense*) (Kreuzaler *et al.*, 1983), was used as a probe to isolate sequences of two different *CHS* genes from petunia and the single *CHS* gene from snapdragon (Reif *et al.*, 1985; Sommer and Saedler, 1986).

The correlation between expression of flavonoid structural genes, regulatory genes, and accumulation of metabolites has proven to be useful in the identification of candidates controlling specific branches of the flavonoid pathway, particularly in Arabidopsis (Borevitz *et al.*, 2000; Nesi *et al.*, 2001; Mehrtens *et al.*, 2005). Therefore, the broad aim of this study

was to identify candidate flavonoid pathway regulators from sweet cherry, and to develop gene-specific quantitative PCR primers that will facilitate their molecular characterisation, as detailed in Chapters 4, 5, and 6 of this thesis. To achieve this aim, the available genetic resources (Section 1.2.4) were exploited to isolate and subsequently determine the copy numbers of flavonoid structural and regulatory genes from sweet cherry.

3.2 RESULTS

Accession numbers for sequences referred to in this thesis are listed in Appendix 6.

3.2.1 PCR-based approach to isolate flavonoid genes from sweet cherry

In the absence of sweet cherry flavonoid sequences within public databases, the Prunus EST database was searched (within the Genome Database for Rosaceae, http://www.bioinfo.wsu.edu/cgi-bin/gdr/gdr_EST_search.cgi), but initially (Version 2) failed to yield any ESTs with similarity to flavonoid biosynthetic genes or characterised flavonoid-regulating MYB factors. Consequently, regions of amino acids displaying a very high level of conservation amongst a variety of plant species were exploited, in order to design PCR primers to the consensus degenerate code for characterised flavonoid structural and regulatory genes. The degenerate primers utilised in the isolation of MYB factors, and the reference sequences on which they were based, are listed in Table 3.1. Additionally, Appendix 2 lists the degenerate primers used to isolate the sweet cherry structural genes. Specific details of the isolation of all of the flavonoid-related genes described in this study by degenerate primer PCR and RACE, (Sambrook *et al.*, 1989) methods are summarised in Chapter 2.

3.2.2 Identification of candidate regulators of flavonoid synthesis

3.2.2.1 Putative homology-based assignment of function

The six sweet cherry transcription factor genes resulting from this research all encode putative R2R3-type MYB factors, where the N-terminal MYB DNA-binding domain consists of two repeats. The number and position of introns within the MYB sequences were generally conserved when compared with other species. Based on the bioinformatic analyses discussed in this chapter, the deduced PaMYB proteins were assigned putative functions.

Table 3.1 – Primers for isolation of flavonoid pathway-related regulatory sequences from sweet cherry

Gene	Reference sequences		*Prunus avium* source^		5' primer	3' primer
	Species	Name	Cultivar	Tissue		
*MYBA**	*Arabidopsis thaliana*	PAP1	Stella	Ripe fruit	TGYATHRAYAARTAYGGIGARGGIAARTGG	GTRTTCCARTARTTYTTIACRTCRTTNGC
	Petunia x *hybrida*	AN2	Lapins	Young leaf (gDNA)		
	Antirrhinum majus	VENOSA				
MYBPA	*Arabidopsis thaliana*	TT2	Lapins	Young leaf	CCTTGTTGTTCTAAGGTTGGTTTG	GACTCGAGTCGACATCGAT[17]
	Vitis vinifera	MYBPA1				
MYBF	*Arabidopsis thaliana*	MYB11	Lapins	Open cluster	GAYAAYGARATAAARAAYTAYTGG	TGTTCTTCTTCATGGCCCTDC
	Arabidopsis thaliana	MYB12				
	Arabidopsis thaliana	MYB111				
MYBR	*Arabidopsis thaliana*	MYB4	Lapins	Stage 1 fruit	GGIAAYMGITGGWSIYTIATHGC	CCARTIRTTITTIATITCRTTRTC
	Vitis vinifera	MYB4				
TT8	*Arabidopsis thaliana*	TT8	Lapins	shuck split	TTYTGGCARYTITGYCCICARCA	GTISWRTGYTCISWIARIGCIGGYTTNGG
	Petunia x *hybrida*	AN1				
TT12	*Arabidopsis thaliana*	TT12	Lapins	Tight cluster	ATGCTTTGCCTTGAGATTTGG	TATCATACCTCCCCAAATTCC
	Prunus persica	Prunus_v2_ contig 1162				

Reference sequences are proteins that were aligned to design primers, except for Prunus EST contig. Accessions for all sequences are listed in Appendix 6

* *PaMYBA1, PaMYBA2, PaMYBA3* were all isolated with the same degenerate primers

^ All genes isolated from cDNA unless otherwise specified

Notes on primers:

PaMYBPA1 was isolated using B26 as the 3' primer (3'RACE)

For degenerate primers D = A/G/T, H = A/C/T, I = inosine, M = A/C, N = A/C/G/T, R = A/G, S = C/G, W = A/T, Y = C/T

TT8 and *MYBA* primers supplied by Dr Mandy Walker

Subsequently, the putative regulators of anthocyanin, tannin (proanthocyanidin) and flavonol synthesis were named PaMYBA(1 to 3), PaMYBPA1 and PaMYBF1, respectively. A potential transcriptional repressor was also isolated from sweet cherry fruit and designated PaMYBR.

3.2.2.1.1 Anthocyanin-type regulators PaMYBA1, PaMYBA2 and PaMYBA3

The two related genes PaMYBA1 and PaMYBA2 encode predicted proteins of 223 amino acids and 244 amino acids respectively. The PaMYBA1 and PaMYBA2 predicted proteins display 91.3% amino acid identities and 94.2% similarities within the R2R3 (DNA-binding) domain, and 73% identity throughout their entire length (Table 3.2). The closest characterised protein to both PaMYBA1 and PaMYBA2, as determined by a BlastP search, was MdMYB1, a regulator of anthocyanin biosynthesis in the related Rosaceous fruit species, apple (Takos *et al.*, 2006a). During the course of this PhD, scientists from HortResearch (New Zealand) deposited the sequence of a putative anthocyanin regulator, PaMYB10 (ABX71493) in the GenBank database. This sequence most closely matches PaMYBA2, with the predicted proteins sharing 97% identity, both in the R2R3 domain and whole sequence (Table 3.2). The cultivar from which *PaMYB10* was isolated is not stated; however, differences between the two very similar sequences may indeed be varietal and/or allelic.

Additionally, a sequence almost identical to *PaMYBA1* was detected from genomic analysis of *PaMYBA1*. The coding sequence differed from *PaMYBA1* in just two nucleotides located within the C-terminal end of the R2R3 repeat (Figure 3.2), suggesting these mutations were a recent genetic event arising after a gene duplication. The detection of the *PaMYBA1*-like sequence in cDNA from the fruit of each cultivar analysed (as will be shown in Chapter 6) discounts it as either a pseudogene or an allele of *PaMYBA1*. Hence, this sequence was deemed to be a third *MYBA* gene, *PaMYBA3*.

Table 3.2 – Amino acid comparisons between predicted PaMYBA proteins and closely related anthocyanin regulators

	% amino acid identities / % amino acid similarities					
	PaMYBA1		PaMYBA2		PaMYBA3	
	R2R3	whole	R2R3	whole	R2R3	whole
PaMYBA1						
PaMYBA2	91.3 / 94.2	73.0 / 77.9				
PaMYBA3	90.4 / 90.4	45.3 / 45.3	81.7 / 84.6	37.7 / 38.9		
MdMYB1*	84.6 / 90.4	68.0 / 73.4	86.5 / 92.3	69.1 / 78.5	76.0 / 81.7	35.0 / 37.4
PaMYB10	90.4 / 95.2	72.5 / 78.3	97.1 / 99.0	97.5 / 98.4	80.8 / 85.6	37.3 / 39.3

Amino acid identities and similarities based on global alignment using EMBOSS-Align (Needle program, BLOSUM62 matrix, gap open/gap extend penalties 25/0.5)

* Most closely related, characterised protein as determined by a BLAST search

Accession numbers are provided in Appendix 6

Figure 3.2 – Sequence polymorphisms between PaMYBA1 and PaMYBA3 result in different length proteins

Section of nucleotide sequence for *PaMYBA1* and *PaMYBA3* with corresponding residues below. Locations of C to T substitution, A deletion and the resulting premature stop codon are indicated with blue arrows. Black/grey nucleotides and residues in PaMYBA1 sequence indicate the end of the R2R3 domain/beginning of the activation domain.

The first difference between PaMYBA1 and PaMYBA3 is a substitution of cytosine with thymine, although this change does not alter the encoded residue. However, the deletion of an adenosine nucleotide, with respect to PaMYBA1, is a mutation that shifts the reading frame at the 44th amino acid of the R3 repeat (Stracke *et al.*, 2001). Subsequently, the predicted protein is terminated after the addition of another 11 amino acids to be just 113 amino acids in length, and therefore lacks the entire C-terminal domain. Similarly, Arabidopsis and grape also possess truncated anthocyanin regulators, although the truncations occur within the C-terminal domain rather than the R2R3 domain; AtPAP4 (also called AtMYB114, AAG38381) is 139 amino acids in length and VvMYBA3 (ABL14067) is slightly longer with 158 amino acids. Whilst VvMYBA3 was unable to activate anthocyanin synthesis in a transient assay (Walker *et al.*, 2007), the mutant colour phenotype of some grape sports have been attributed to polymorphisms in the *VvMYBA3* sequence (Fournier-Level *et al.*, 2009). Consequently, Fournier-Level *et al*. suggest that although *VvMYBA3* may not encode a functional protein, it could affect berry pigmentation by competing with other VvMYBA proteins.

Due to the highly conserved nature of the R2R3-MYB family, the number of copies of *MYBA* in the sweet cherry genome was unable to be accurately determined. Numerous oligonucleotide probes designed to different genomic regions were unable to specifically detect *MYBA* genes, despite high stringency Southern blotting conditions. The detection of three *PaMYBA* sequences is similar to the Arabidopsis and grape genomes, which both contain four anthocyanin R2R3-MYB factors (Stracke *et al.*, 2001; Walker *et al.*, 2007). However, the existence of additional *PaMYBA* genes cannot be ruled out.

3.2.2.1.2 PaMYBPA1 as a putative regulator of tannin biosynthesis

The 287 amino acid predicted protein encoded by PaMYBPA1 bears the greatest sequence resemblance to R2R3-MYB factors that have been confirmed to regulate tannin synthesis. In particular, PaMYBPA1 shares considerable identity with VvMYBPA1 (Bogs *et al.*, 2007), where 94% and 60% of the residues in the R2R3 domain and entire sequence, respectively, are identical (Table 3.3). To a lesser extent, PaMYBPA1 is also similar to other tannin regulators, such as VvMYBPA2 and AtTT2, with which it respectively shares 74% and 71% identities within the R2R3 domain. Recently, rapeseed (*Brassica napus*) (Wei *et al.*, 2007) and lotus (*Lotus japonicus*)(Yoshida *et al.*, 2008) have been identified as possessing three MYB transcription factors believed to regulate tannin synthesis, while grape has two MYBPA genes (Terrier *et al.*, 2009). Despite the isolation of this single MYBPA gene from sweet

Table 3.3 – Amino acid comparisons between sweet cherry transcription factors and related sequences from other species

Sequence	Best BLAST hit*			Arabidopsis homologue		
	Name	% ident / sim		Name	% ident / sim	
		R2R3	whole		R2R3	whole
PaMYBF1	VvMYBF1	89.3 / 95.1	32.5 / 41.5	AtMYB12^	83.7 / 95.2	23.2 / 30.4
PaMYBPA1	VvMYBPA1	94.2 / 98.1	60.1 / 70.5	AtTT2	71.2 / 84.6	29.8 / 41.2
PaMYBR	EgMYB1	98.1 / 100.0	68.2 / 76.5	AtMYB4	67.3 / 83.7	25.9 / 35.5
PaTT8	PhAN1	N / A	56.9 / 70.1[§]	AtTT8	N / A	41.1 / 53.3[§]
PaTT12	VvAM1	N / A	70.0 / 86.5[†]	AtTT12	N / A	48.2 / 69.4[†]

Amino acid identities and similarities based on global alignment using EMBOSS-Align (Needle program, BLOSUM62 matrix, gap open/gap extend penalties 25/0.5)

* Best hit with a characterised sequence

^ AtMYB12 sequence used as it was more similar to PaMYBF1 than AtMYB11 / AtMYB111

[§] Identities/similarities over 568 and 572 residues (local alignment with incomplete PaTT8 sequence)

[†] Identities/similarities over 223 and 222 residues (local alignment with incomplete PaTT12 sequence)

Accession numbers are provided in Appendix 6

cherry, and the absence of *Prunus* MYBPA2-like ESTs in the limited database, Southern blotting indicated there were most likely two related genes present in the genome (data not shown). A more extensive search of the EST databases revealed that within the genome of apple, a fruit closely related to sweet cherry, sequences exist that are more similar to either VvMYBPA1 (eg. GO519378), or VvMYBPA2 (eg. AF336284). Thus, it is possible that another sweet cherry *MYBPA* gene exists.

3.2.2.1.3 The candidate flavonol regulator PaMYBF1

The sweet cherry putative flavonol regulator proved more difficult to clone than the putative anthocyanin and tannin MYBs, mostly due to the lack of characterised sequences. At the time, only the Arabidopsis MYB11, MYB12 and MYB111 regulators of flavonol synthesis had been reported. Homologues have since been identified and characterised from tomato (SlMYB12) and grape (VvMYBF1) (Luo *et al.*, 2008; Czemmel *et al.*, 2009). Following numerous unsuccessful attempts to optimise PCR conditions with nested degenerate primers, a semi-degenerate approach was attempted. The conserved flavonol-specific motifs DNEIKNYNSHLSRK in the MYB domain, and the subgroup 7 motif GRTxRSxMK were used to blast the nucleotide database (tBlastn), yielding several good matches with cDNA and genomic DNA from a range of plants, including apple, lotus, gerbera (*Gerbera hybrida*), rice (*Oryza sativa*), rapeseed and cotton (*Gossypium hirsutum*). Based on these sequences, primers with a lower degree of degeneracy could be designed (Table 3.1), and proved successful in enabling the isolation of a sweet cherry flavonol-type MYB factor, PaMYBF1.

The *PaMYBF1* gene encodes a large 533 amino acid predicted protein. This represents a protein that is approximately 200 amino acids longer that other characterised flavonol regulators from Arabidopsis, grape, lotus and tomato, which range in size from 326-369 amino acids. The increased length of PaMYBF1 is due to a large number of tri-nucleotide repeats in the C-terminal domain that result in homopeptides, also called single amino acid repeats. One such example is the repeat of a codon $(act)_{6,}$ to encode a string of six threonine (T) residues within the PaMYBF1 sequence. Homopeptides are particularly common in eukaryotic proteins involved in transcription, and it has been suggested that they generate flexible regions of importance in mediating protein-protein interactions within large, multi-protein complexes (Faux *et al.*, 2005). Similar repeats can be found within the flavonol-associated regulators MdMYB22 and *Sorghum bicolor* yellow seed1 (Y1), the broad-acting phenylpropanoid regulators VvMYB5a and VvMYB5b, and the rice bHLH Rc that regulates tannin biosynthesis. Occasionally, homopeptides can also be detrimental to function, such as

the association of glutamine repeats (poly-Q) with protein misfolding and the development of degenerative diseases (Faux *et al.*, 2005).

Although the amino acid repeats make comparison of the full-length sequence of PaMYBF1 with other characterised flavonol-type MYBs difficult, strong similarities in the MYB domain suggest these proteins could be functionally related. Within the R2R3 domain, PaMYBF1 shares 89% and 84% amino acid identities with VvMYBF1 and AtMYB12, respectively, but has 95% of residues that are similar to these two sequences (Table 3.3). The copy number of *MYBF* in the sweet cherry genome was not determined. However, as three functionally conserved flavonol MYBs are present in Arabidopsis, it is possible that more than one gene is capable of regulating flavonol biosynthesis in sweet cherry.

3.2.2.1.4 Other regulators

In addition to the putative transcriptional activators isolated from sweet cherry, an R2R3-MYB was cloned that displays a striking degree of similarity to a number of known negative regulators. Within the MYB domain of the 256 amino acid deduced PaMYBR protein, 100% of amino acids were similar to those of EgMYB1, a repressor of lignin biosynthesis in Eucalyptus (Legay *et al.*, 2007). The extreme level of conservation within this domain extended to the uncharacterised MYB proteins hops (*Humulus lupulus*) MYB, grape MYB4 and cotton MYB9 (for accession numbers see Appendix 6). This conservation over a range of divergent species suggests that the structure of the MYB domain is critical to the function of these proteins. PaMYBR also had a large percentage of residues in common with AtMYB4 (Table 3.3), which has been shown to control the production of sinapate ester sunscreens through repression of the phenylpropanoid gene *cinnamate 4-hydoxylase* (*C4H*) (Jin *et al.*, 2000).

Besides the R2R3-MYB transcription factors mentioned, partial sequences for flavonoid-related genes of the bHLH and MATE transporter families were also isolated from sweet cherry. The bHLH gene was named *PaTT8* as the tannin regulator *AtTT8* (Nesi *et al.*, 2000) was the Arabidopsis gene generating the best local alignment with the partial sequence, with 41% amino acid identities and 53% similarities to the deduced protein (Table 3.3). However, the sequence of PaTT8 more closely resembles PhAN1 as the two sequences share 57% amino acid identity and 70% similarity. PhAN1 has been shown to regulate pigment synthesis, vacuolar pH and seedcoat development (Spelt *et al.*, 2002). Therefore, it seems

very likely that PaTT8 is involved in regulation of flavonoid biosynthesis, and its control may also extend to other cellular processes.

The sweet cherry putative homologue of the AtTT12 transporter, which is required for the vacuolar localisation of flavonoids (Debeaujon *et al.*, 2001; Baxter *et al.*, 2005) was designated PaTT12. The 3' region of the *PaTT12* gene was isolated using primers based on *TT12*-like Prunus_v2_contig1162 (Genome Database for Rosaceae). While the PaTT12 sequence was similar to AtTT12, it showed 86.5% amino acid similarities (Table 3.3) to the anthocyanin transporting MATE-type protein from grape, VvAM1 (Gomez *et al.*, 2009). The *PaTT8* and *PaTT12* sequences were not used in the characterisation of the flavonoid pathway as reported in this thesis. However, their future molecular characterisation would be a useful tool in further understanding flavonoid biosynthesis and transport.

3.2.2.2 Phylogenetic clustering of candidates with characterised regulators

Phylogenetic analysis of R2R3-MYB factors isolated from sweet cherry was performed in order to confirm their putative roles in flavonoid pathway regulation. As expected from the sequence homology described in Section 3.2.2.1, the sweet cherry MYBs fell into functional clades with characterised and putative branch-specific flavonoid pathway regulators from different plant species (Figure 3.3). Therefore, the three PaMYBA proteins, PaMYBPA1, and PaMYBF1 were confirmed as good candidates for the regulation of anthocyanin, tannin and flavonol biosynthesis, respectively. PaMYBR definitely clusters with other transcriptional repressors, but it is unclear from the dendogram whether or not it is related to other regulators of the flavonoid pathway.

The only apparent exception to the functional clustering of the MYB factors within these clades was the maize anthocyanin regulator C1, which the analysis revealed was most closely related to the group of proteins involved in regulating the tannin and general phenylpropanoid pathways. However, the ability of C1 to activate the Arabidopsis *ANR* promoter (Baudry *et al.*, 2004) provides a functional tie to this clade.

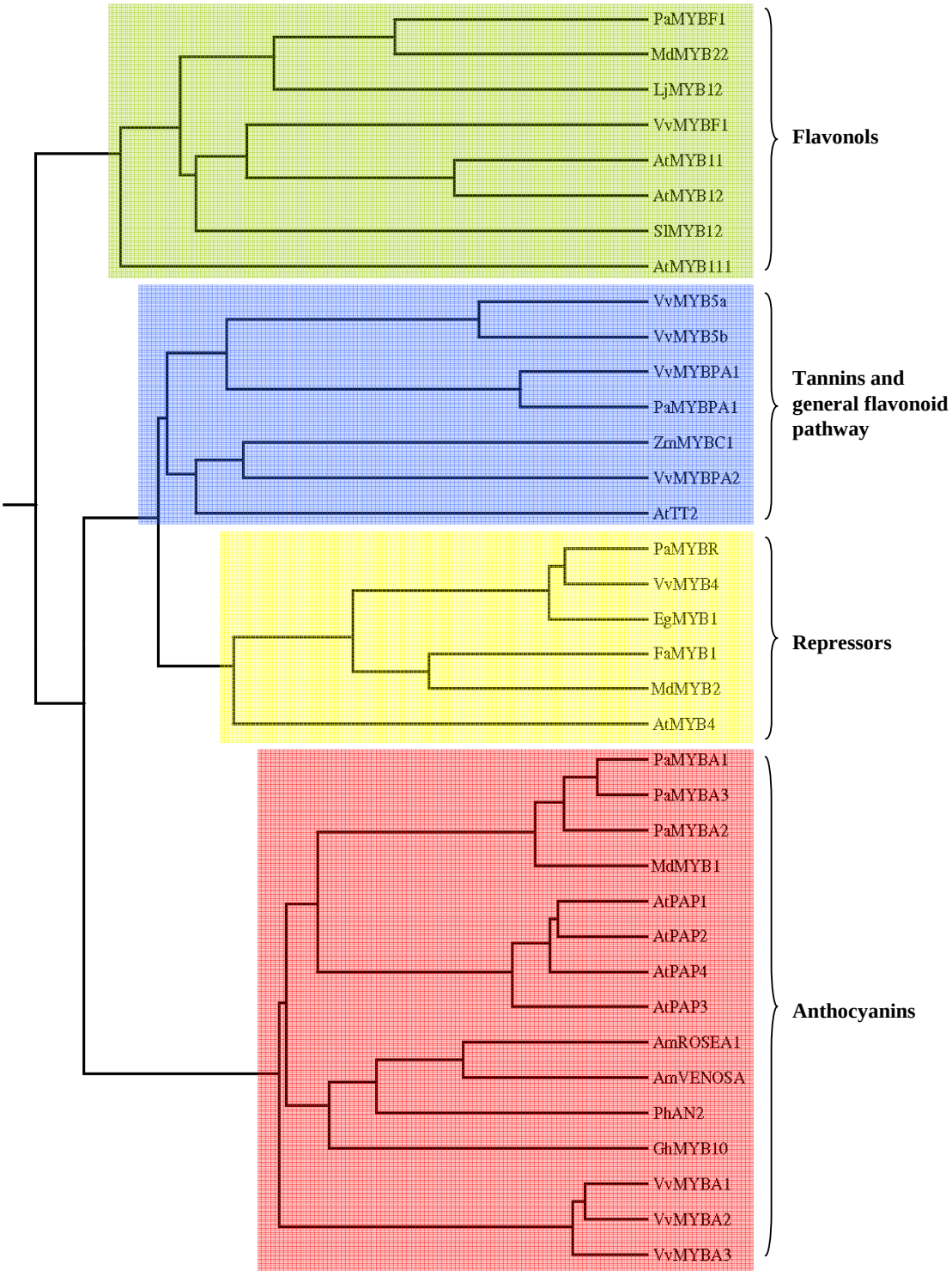


Figure 3.3 – Sweet cherry R2R3-MYBs cluster in flavonoid branch-specific nodes

Unrooted phylogenetic tree was constructed by Neighbour-Joining method and 1000 bootstraps using Phylogeny Inference Package (PHYLIP; Felsenstein J., 1989). Functional clades are highlighted in colour. Accessions of sequences used for analyses are listed in Appendix 6

3.2.2.3 Presence of conserved motifs C-terminal to the MYB domain

Besides sequence similarity leading to phylogenetic clustering, the presence of motifs supports the putative assignments of function to the sweet cherry MYB factors. Conserved motifs in the C-terminal region are used to classify MYB factors into subgroups (SGs), within which the TFs often share similar functions (Stracke *et al.*, 2001). In the years since these motifs were defined, primarily from Arabidopsis sequences, MYB factors from numerous other plant species have been identified. In some cases, this has resulted in the revision of the motifs to incorporate the new sequences; for example the SG5a and SG7 motifs were recently redefined upon isolation of the grape MYBPA2 (Terrier *et al.*, 2009) and MYBF1 sequences (Czemmel *et al.*, 2009), respectively}.

Within the sweet cherry R2R3-MYB factors isolated in this study, conserved amino acid sequences that closely resembled some previously described motifs were identified. These classified the PaMYBA sequences as belonging to SG6 which encompasses anthocyanin regulators, PaMYBF1 to SG7 wich includes flavonol regulators, and PaMYBR to the transcriptional repressors of SG4 (Table 3.4). PaMYBPA1, like VvMYBPA1, did not contain either SG5 or SG5a motifs characteristic of tannin regulators. However, these sequences had four motifs (SG5-2 to SG 5-5) in common with three other tannin-type MYB factors (Section 2.5.7 for motif identification), and these are described in Table 3.4. Another notable motif absent from the MYB domain of PaMYBF1 was [D/E]Lx_2[R/K]x_3Lx_6Lx_3R, required for interaction with bHLH factors (Zimmermann *et al.*, 2004). This motif is also absent from the flavonol regulators of Arabidopsis and grape (Stracke *et al.*, 2007; Czemmel *et al.*, 2009), which can reportedly cause transcriptional activation independently of a bHLH.

Table 3.4 – Identification and modification of R2R3-MYB motifs

Protein name	Motif Name	Motif Sequence	Reference
MYB	bHLH interaction	[DE]Lx$_2$[RK]x$_3$Lx$_6$Lx$_3$R	Grotewold *et al*., 2000
MYBA1	SG6	KPRPR[ST]F	Stracke *et al*., 2001
	SG6 (mod1)	[KR]P[QR]P[QR][ST]F	This work
	SG6-A1*	TS[PS]STKNGN	This work
MYBA2	SG6	As above	Stracke *et al*., 2001
	SG6-A2^	TSTSTRIGS	This work
MYBPA1	SG5	DExWRLxxT	Stracke *et al*., 2001
	SG5-2	TDPxTH[KR]K	This work
	SG5-3	KVHLPK	This work
	SG5-4	[ST]LExLYEEYLQ	This work
	SG5-5	LxSFAxSLL[IV]	This work
MYBPA2	SG5a	VxxIRTKA[IL]RC[SN]	Nesi *et al*., 2001
	SG5a (mod1)	V[IV]R[TP][KR]A[ILV][RK]C	Terrier *et al*., 2009
	SG5a (mod2)	V[ITV]x[RK]Ax[RK]CxK	This work
MYBF1	SG7	GRTxRSxMK	Stracke *et al*., 2001
	SG7 (mod1)	[KR][RX][RX]xGRT[SX][RG]xx[MX]K	Czemmel *et al*., 2009
	SG7-2	[WX][LX]LS	Czemmel *et al*., 2009
	SG7-2 (mod1)	[LM]xxWLLS	This work
MYBR	C2/SG4	pdLNL[ED]Lxi[GS]	Jin *et al*., 2000
	SG4	LNL[ED]L	Stracke *et al*., 2001
	SG4 (mod1)	PDLNL[ED]L	This work
	C1/SG4-2	LIsrGIDPx[TS]HRx[IL]	Aharoni *et al*., 2001
	C1/SG4-2 (mod1)	GIDPx[TN]H	This work
	SG4-3	R[SG]LEMK	This work
	Zn finger	Cx1-2Cx7-12Cx1-2C	Aharoni *et al*., 2001

Hyphenated (-) motif names indicate an additional motif within a subgroup
Mod# refers to modifed/redefined motifs
* Motif is specific to Rosaceae family members
^ Motif is specific to *Prunus* spp.
Motif variations in grey are not present in the *P. avium* sequence (see Figure 3.4)
Sequences used in motif identification are listed in Appendix 7

In light of the sweet cherry MYBs described here and other recently deposited MYB sequences, some of these motifs were tentatively modified using MEME (Bailey *et al.*, 2006) to more accurately reflect the variations amongst different species (Table 3.4). For example, the SG6 motif KPRPR[S/T]F characteristic of anthocyanin regulators from Arabidopsis and petunia becomes [K/R]P[Q/R]P[Q/R][S/T]F when the fruit-derived MdMYB1, VvMYBA1 and PaMYBA sequences are considered. Additionally, the putative motifs TS[P/S]STKNGN and TSTSTRIGS, designated SG6-A1 and SG6-A2, are only present within the uncharacterised sequences of Rosaceae species MYB10 factors, but may assist in the identification of anthocyanin regulators from this family. Figure 3.4 summarises the motifs present in the C-termini of the sweet cherry MYB factors, and their locations with respect to each other. However, modified motifs should not be used to infer function, as they have not been characterised and the role of the sweet cherry MYBs has not yet been confirmed.

3.2.3 Isolation and analysis of flavonoid biosynthetic genes from sweet cherry

Flavonoid biosynthetic genes were isolated from sweet cherry using the PCR-based approached described for the MYB factors. In this study, partial or full-length sequences were cloned for the shared pathway genes *PaCHS2*, *PaCHS3*, the flavonol gene *PaFLS*, and the tannin genes *PaLAR1*, *PaLAR2*, and *PaANR*. Additionally, 3' ends of the anthocyanin biosynthetic genes *PaCHS1*, *PaDFR*, *PaLDOX* (*PaANS*) and *PaUFGT* were obtained from the partial sequences provided by Dr Paul Wiersma, in order to more accurately determine the expression of closely-related genes. During February 2010, Dr Wiersma independently determined the full-length sequences of these anthocyanin genes, which are in agreement with the sequences used for the analysis described within this thesis. GenBank accession numbers for all of the sweet cherry flavonoid gene sequences are listed in Table 2.1.

The sequences of the flavonoid biosynthetic genes were used as molecular probes to determine the copy numbers of these genes in the *P. avium* genome by Southern blotting. Figure 3.5 provides an example of the *PaCHS* Southern blot, indicating the presence of between three and five *CHS* genes within the genomes of the 'Lapins' and 'Dame Nancy' genomes. Indeed, three *CHS* sequences have been isolated (*PaCHS1*, *PaCHS2* and *PaCHS3* share between 97-98% amino acid identities), and their potential roles in different aspects of flavonoid synthesis will be further discussed in Chapter 4.

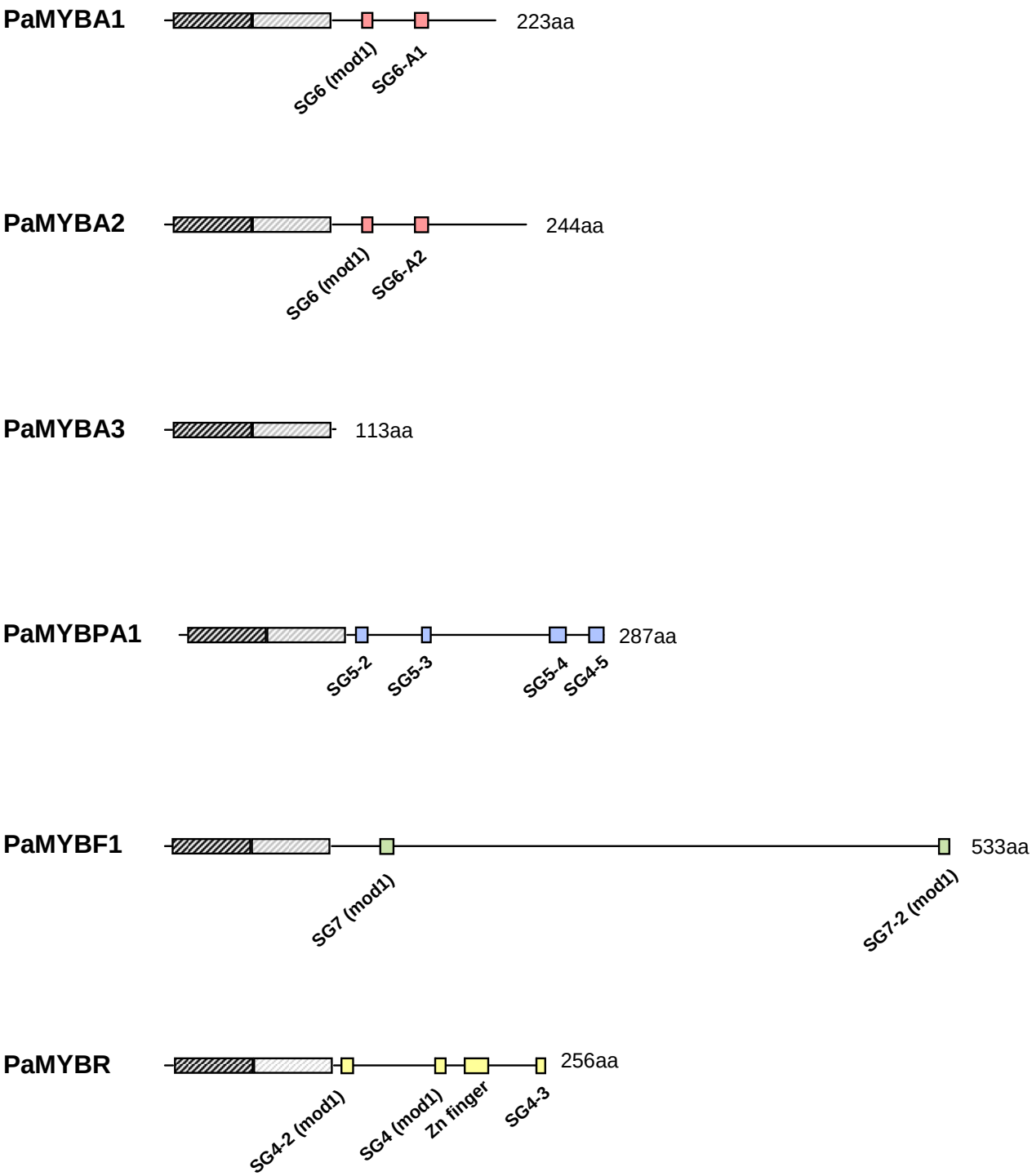


Figure 3.4 – Organisation of sweet cherry R2R3-MYB proteins

Diagrammatic representation of sweet cherry MYBs isolated in this study. Grey boxes depict R2 and R3 repeats. Small coloured boxes represent characteristic motifs further detailed in Table 3.4. Proportions are comparable as diagrams are drawn to scale.

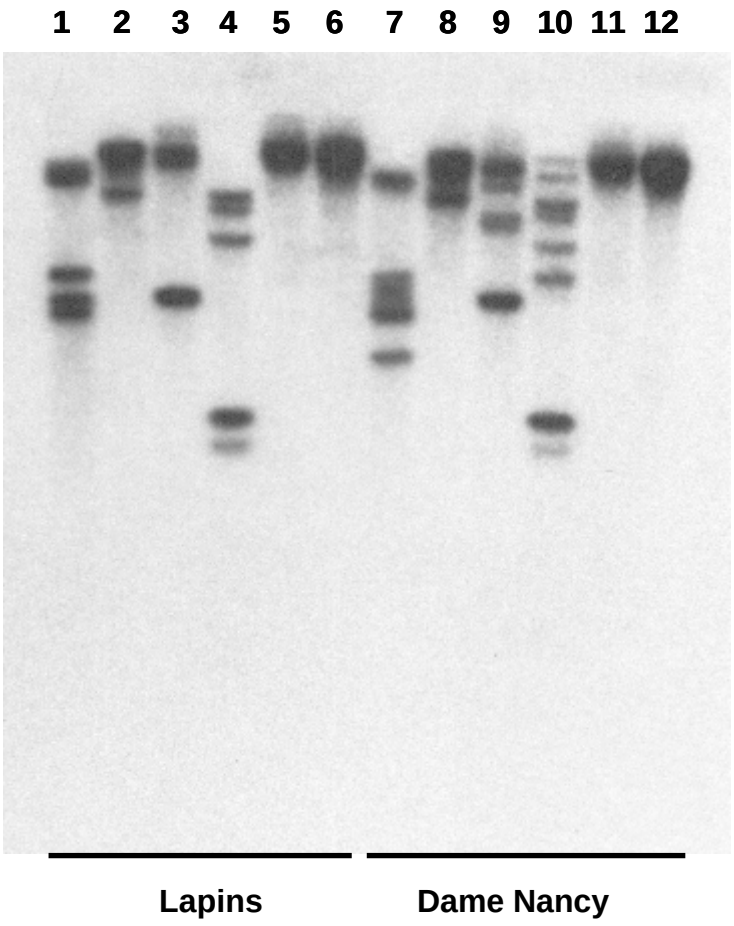


Figure 3.5 - Determination of *CHS* copy number in the *P. avium* genome

Genomic DNA from 'Lapins' (red fruit, lanes 1-6) and 'Dame Nancy' (blush fruit, lanes 7-12) sweet cherry cultivars was digested with one of six restriction enzymes, electrophoresed on an agarose gel and blotted to a GeneSreen Plus membrane. The Southern blot was probed with a [^{32}P]-labelled *PaCHS* PCR fragment (see Table 2.2), washed under high stringency (0.1x SSC) and exposed to Kodak BioMax MS film for 3h at -70°C. Lanes contain DNA digested with the following enzymes: Lanes 1 and 7 – *Eco*RV; lanes 2 and 8 – *Bam*HI; lanes 3 and 9 – *Bgl*II; lanes 4 and 10 – *Hind*III; lanes 5 and 11 – *Sma*I; lanes 6 and 12 – *Xho*I.

Besides *CHS*, single copies were detected for all of the other flavonoid genes analysed, similar to findings in Arabidopsis, but in contrast to the predominantly multiple copy genes of other fruit species (Table 3.5). In contradiction to the Southern blot result, two *PaLAR* genes were found in sweet cherry. The lack of detection of the second *PaLAR* gene may be due to the 70% nucleotide conservation between the two sequences, in combination with the high stringency conditions used to wash the probe from the membrane.

Given that the flavonoid biosynthetic genes have been previously studied in numerous plant species, and that their isolation is not the focus of this research, the organisation of the sweet cherry genes will not be discussed in detail. As was found for the *PaMYB* genes, the number and position of introns within the structural genes were well conserved with respect to previously described sequences. The considerable degree of sequence homology between these proven flavonoid genes and the sweet cherry genes, together with the single copy nature of most genes, indicated that the functional orthologues had been cloned. Deduced sweet cherry flavonoid proteins were generally more similar to those from the fruit crops apple, pear, raspberry, strawberry and grape, ranging in amino acid identities from 67% between PaUFGT and MdUFGT, to 96% between PaLDOX and LDOX from peach (data not shown). Even the more distantly related Arabidopsis flavonoid genes displayed between 70% and 94% similarity to the sweet cherry genes at the protein level.

3.2.4 Development of primers for analysis of gene expression

Using the sweet cherry flavonoid gene sequences, gene-specific primers were developed to assist in the confirmation, through molecular characterisation, of the MYB candidates for regulators of the flavonoid pathway. The primers were designed to amplify a small 150-300bp cDNA fragment, the abundance of which could be detected by real time PCR to calculate the level of gene expression in a biological sample. The primer design considerations and testing of the PCR product specificity were presented in Section 2.6.1. The successful primer pairs are listed in Table 3.6 for those based on regulatory genes, and Table 3.7 for structural genes, along with their primer efficiency values. These values enable differences in the rate of amplification to be accounted for (see Section 2.6.3), thus allowing comparisons between expression levels of different genes to be made, when analysed in a single experiment. Towards the final stages of the research encompassed in this thesis, the *PaMYBA3* gene, almost identical to *PaMYBA1*, was identified. Several sets of real time PCR primers were unable to distinguish between these two genes, and their polymorphisms were insufficient even to identify the sequences through a shift in the melt temperature of the PCR product.

Table 3.5 – Comparison of the copy numbers of sweet cherry flavonoid genes to those of other fruit species

Gene	Approximate copy number			
	Sweet cherry	Apple	Pear[3]	Other species
CHS	3-5	several[1]	4-5	Peach, 3[4]; Grape, 3[5]
FLS	1	3[1]	3-4	Grape, 5[6]; Strawberry, up to 8[7]; Mandarin, 2-5[8]
DFR	1	3-5[2]	2-3	Peach, 1[4]; Grape, 1[9]; Strawberry, 4-5[7]
LAR	[2]	3-4[1]	2	Strawberry, 4-5[7]
LDOX	n.d.	1[1]	1-2	Strawberry, 4-5[7]
ANR	1	3[1]	2-3	Strawberry, 1-2[7]; Grape, 1[10]
UFGT	n.d.	3[1]	2-3	Grape, 1[5]

n.d. - not determined

[1] Takos *et al*., 2006c, [2] Fischer *et al*., 2003, [3] Fischer *et al*., 2007, [4] Tsuda *et al*., 2004, [5] Sparvoli *et al*., 1994

[6] Fujita *et al*., 2006, [7] Almeida *et al*., 2007, [8] Moriguchi *et al*., 2002, [9] Gollop *et al*., 2002, [10] Fujita *et al*., 2005

[11] Winkel-Shirley, 2001, [12] Owens *et al*., 2008

Note; Arabidopsis contains single copies of all of the above genes[11], except for *FLS* (6 copies[12])

Table 3.6 – Real time PCR primers for expression analysis of sweet cherry flavonoid regulators

Gene	Real time PCR primers (5' to 3')				
	Forward	Reverse	Size of PCR product (bp)	Amplification efficiency	R^2 value
*PaMYBA1/A3**	TGGATGATATGCCACAATCG	gccatcaaacattacacacc	279	1.9267	0.999
PaMYBA2	GAACGTGCATCAATTTTTCTG	aatccagactaggtctcttcac	176	1.8758	0.9982
PaMYBPA1	CGATCAAGTTTTAGTTCATCCTTGG	GATGATGATCTGTCTTCAGCAGC	149	2.0665	0.9953
PaMYBF1	GATATGGAGGATGGTCAAGCTG	aacgggcggtacaatatataac	365	1.9713	0.9919
PaUBQ[#]	AGGAGTCTACCCTTCACCTTGTC	ccatcggccaagtacgag	143	2.0113	0.9947

Gene accession numbers are listed in Appendix 6

[#] *PaUBQ* primers were designed by Dr Paul Wiersma

* Primers could not distinguish between *PaMYBA1* and *PaMYBA3* sequences

Nucleotides in uppercase are located in coding region, lowercase located in untranslated region

Amplification efficiency and R^2 values for real time primer sets were determined as described in Appendix 5

Table 3.7 - Real time PCR primers for expression analysis of sweet cherry flavonoid structural genes

Gene	Real time PCR primers (5' to 3')				
	Forward	Reverse	Size of PCR product (bp)	Amplification efficiency	R^2 value
PaCHS1	GCTGTTTGGGTTTGGACCAGGA	caatgaagtgtaccccatgatgc	235	1.9014	0.9998
PaCHS2	GAAGCCTGAAAAATTAGAGCC	taggaacaagagcagacaaatc	277	1.9503	0.9971
PaCHS3	ATGTCGAGTGCTTGTGTGCTG	tacaccatgatgcatctggaag	285	1.9424	0.9997
PaF3'H	AGAAATTGAACATGGACGAAGC	tggaacagaaatagtaggagctagg	193	1.9345	0.9989
PaFLS	AAAATCCACCTAAATACAAGACC	ctcaccaacacatacaacttcc	209	1.9877	0.9966
PaDFR	CTTGGAGGACATGTTCGTAGG	aaagaacccaacagacactaatcc	189	2.0916	0.9716
PaLAR1	GCTGTCACTGCCACGTGTGC	cgaacaatcaaattagcatgtgtc	216	1.8648	0.983
PaLAR2	AGCAAAACTAGCTCAAAAGTCG	gggaagaaaagaagaaaaggg	238	2.0438	0.9961
PaLDOX[#]	CGAGTATGCTAAGGAACTGAGG	TGAGGGCAAACTGGGTAGTAG	156	2.0033	0.9973
PaANR	AGTTCCTCAACGAAAGATACCC	gctagataaacgccaatcacac	265	1.9872	0.9956
PaUFGT	TGTTGAGGATGGGGTTTTTAC	agtacagctcggttattcttatgc	243	2.0852	0.9793
PaUBQ[#]	AGGAGTCTACCCTTCACCTTGTC	ccatcggccaagtacgag	143	2.0113	0.9947

Gene accession numbers are listed in Appendix 6

[#] *PaUBQ* and *PaLDOX* primers were designed by Dr Paul Wiersma

Nucleotides in uppercase are located in coding region, lowercase located in untranslated region

Amplification efficiency and R^2 values for real time primer sets were determined as described in Appendix 5

The inability to discriminate between such related sequences is a limitation of this method of gene expression analysis. However, the real time PCR analysis had already been performed and the determination of protein levels, based on differences in size, was unfeasible at this late stage of candidature. Therefore, when *PaMYBA1* expression is referred to in this thesis, this value may also include any expression of *PaMYBA3*.

3.3 DISCUSSION

Within the flavonoid biosynthetic pathway, the key regulatory factors and their target genes can vary amongst different plant species. The research presented in this thesis will focus on the role of MYB TFs in flavonoid regulation, as this aspect of the regulatory complex displays the greatest functional specificity (Zhang *et al.*, 2003) and has been shown to govern pigmentation in apple and grape (Kobayashi *et al.*, 2004; Takos *et al.*, 2006a; Walker *et al.*, 2007). In this study, a PCR-based cloning approach has led to the discovery of genes encoding six R2R3-type MYB factors from sweet cherry. Strong amino acid similarities between the sweet cherry MYBs and characterised regulators from other species suggested putative roles as branch-specific regulators of flavonoid biosynthesis. Putative functions were also supported by the presence of C-terminal motifs, and the clustering with functionally related MYBs in a phylogenetic analysis. The putative negative regulator, PaMYBR, featured an EAR repression domain, but due to a strong similarity to the lignin repressor EgMYB1, has not been further characterised.

MYB factors with overlapping regulatory targets exist in a number of plant species. The presence of such regulators can reflect the need for tissue specific gene regulation. Examples of this are the Arabidopsis MYBs controlling trichome development, AtWER and AtGL1. Reciprocal complementation experiments have shown AtWER and AtGL1 to be functionally equivalent proteins; however *AtWER* is expressed in root epidermal cells, while *AtGL1* is expressed in the epidermal cells of developing shoot tissue (Lee and Schiefelbein, 2001). Therefore, the PaMYBA proteins could potentially regulate anthocyanin in a tissue-specific manner, a line of investigation that will be pursued in Chapter 6. Results from Southern blotting experiments indicated that there are also two related *PaMYBPA* genes in the sweet cherry genome. Although the second gene is yet to be identified, the future availability of sequences from *Prunus* ESTs and their genome should assist to this end.

In order to understand how MYB factors regulate flavonoid biosynthesis the effect that they have on their targets, the flavonoid pathway structural genes, must be studied. We have

cloned and determined the copy number for many of the genes encoding flavonoid pathway enzymes from sweet cherry. Of interest is the finding that many of these genes are present as single copies in the sweet cherry genome, which is similar to the grape genome where all enzymes except for CHS and PAL are encoded by a single gene (Sparvoli *et al.*, 1994). This is in contrast to the larger gene families seen within other fruits such as pear and apple, and in petunia. In sweet cherry, multiple gene copies were found for *CHS* and *LAR*. The apparent redundancy amongst the highly similar PaCHS1, PaCHS2 and PaCHS3 suggests the possibility that sweet cherry uses these multiple isoforms to mediate the production of different flavonoid molecules. This has been achieved by other plants through differential expression of *CHS* genes regulated spatially as for the maize seed-expressed *c2* (Dooner, 1983) and for *chsA* and *chsJ* expressed in petunia flower corolla and anthers (Koes *et al.*, 1989), temporally during grape berry development (Jeong *et al.*, 2008), and also in response to external cues such as light in soybean (*Glycine max*)(Shimizu *et al.*, 1999). The differential expression of the sweet cherry *CHS* and *LAR* isoforms will be investigated in Chapter 4. Additionally, multiple CHS enzymes may also be required due to differences in substrate specificities (Heller and Forkmann, 1993) or the association of particular CHS enzymes with multi-enzyme complexes, facilitating metabolic channeling as reviewed by (Winkel-Shirley, 1999), but these biochemical approaches were not explored in the work reported here.

3.4 CONCLUSION

In this work not only have many sweet cherry genes potentially involved in flavonoid synthesis been isolated, but their sequences have been used to design primers that will specifically detect the expression of each gene. These primers will be utilised extensively throughout the work described in this thesis, to analyse flavonoid structural gene expression and thereby characterise flavonoid pathway regulation in response to developmental, genetic and abiotic factors. Another outcome is the discovery of a number of new conserved motifs C-terminal to the MYB domain of the R2R3-type transcription factors. In particular, four putative motifs have been identified that describe a group of related sequences involved in tannin regulation, but lacking the SG5 and SG5a motifs generally used to classify tannin MYBs. The newly defined motifs may be applied to identify currently available sequences related to this group of proteins, or used for a degenerate cloning approach, with the aim of further understanding the function of this second tannin regulator. Additional investigations are required to determine the extent of any conservation of these new motifs within the plant kingdom. Mutational analysis would also prove useful in determining whether the motifs are required for the function of the protein.

Chapter Four: Regulation of flavonoid synthesis in developing sweet cherry fruit

4. REGULATION OF FLAVONOID BIOSYNTHESIS IN DEVELOPING SWEET CHERRY FRUIT

4.1 INTRODUCTION

The temporal accumulation of flavonoids in developing flowers and fruit is dictated by the needs of the plant at particular stages. Anthocyanins function to attract pollinating insects to flowers at early developmental stages, and attract animals to ripe fruit for seed dispersal (Whitney and Lister, 2004). Astringent tannins are required early in fruit development, as they deter animals from feeding on growing fruit (Feeny, 1970; Forkner *et al.,* 2004). Flavonols present in developing flowers have been shown to be necessary for pollen tube growth in maize and petunia, while in developing fruits they most likely confer UV protection (Lois and Buchanan, 1994; Deboo *et al.*, 1995; Ryan *et al.*, 2002).

These flavonoid compounds have been shown to accumulate at developmental stages that correspond to these functional requirements in the flowers and fruits of grape (Downey *et al.*, 2003b, a) and a number of Rosaceae fruits, including apple (Takos *et al.*, 2006c), and strawberry (Cheng and Breen, 1991). In many of these instances, there is a temporal separation between the synthesis of flavonols and tannins, and that of anthocyanins. Therefore, it seems that at a predetermined stage of fruit development, the metabolic flux of the flavonoid pathway switches to divert intermediates towards the production of anthocyanins. Additionally, this indicates suggests that in many plant species, due to substrate competition, anthocyanin synthesis cannot occur at the same time as the synthesis of flavonols and tannins. Vvedenskaya and Vorsa (2004) suggest from their studies in American cranberry (*Vaccinium macrocarpon* Ait) that partitioning of the precursor pool in the flavonoid pathway is influenced by both variety and phenological stage.

In contrast to a few fruit species such as apple and grape, relatively little is known about flavonoid biosynthesis during sweet cherry development. The composition and concentrations of flavonoid compounds in the mature fruits of a number of sweet cherry varieties have been reported, as reviewed in Section 1.2.3. Also presented in Chapter 1 were the somewhat conflicting regression models that have been used to describe anthocyanin accumulation in ripening sweet cherry fruit (Poll *et al.*, 2003; Mozetic *et al.*, 2004; Remon *et al.*, 2006). These differences could probably be explained by cultivar and maturity stage variations (Vangdal *et al.*, 2006), and could also be influenced by environmental conditions. Upon dissection of sweet

cherry fruit, skin has been found to synthesise anthocyanins up to two weeks prior to flesh (Watanabe and Taira, 1986). As the focus of flavonoid research in this species has clearly been on anthocyanins, details of the temporal and spatial accumulation of flavonols and tannins in sweet cherry fruit have not been reported.

The expression of specific groups of flavonoid structural genes shows tight correlation with the accumulation of corresponding flavonoid compounds during fruit development. In a number of fruit species the shared pathway genes, including *CHS*, *CHI*, *F3H*, *DFR*, and *LDOX*, show a biphasic pattern of expression that mirrors the accumulation of flavonols and tannins, followed by anthocyanins (Cheng and Breen, 1991; Kumar and Ellis, 2001; Jaakola *et al.*, 2002; Tsuda *et al.*, 2004; Takos *et al.*, 2006c; Palapol *et al.*, 2009). Transcript levels of flavonol-, tannin-, and anthocyanin-specific genes *FLS*, *LAR* and *ANR*, and *UFGT* respectively are only detectable when that particular compound is being actively synthesised in apple (Takos *et al.*, 2006c) and grape (Downey *et al.*, 2003b; Bogs *et al.*, 2005; Fujita *et al.*, 2005). It appears that sweet cherry is no exception, with the anthocyanin gene expression profile matching the accumulation of anthocyanins in 'Lapins' fruit (P. Wiersma (AAFC), personal communication). The developmental expression of flavonoid biosynthetic genes is usually controlled by R2R3-MYB transcription factors that share the same expression profile. The MYB factors that are characterised regulators of the flavonoid pathway in fruit species were presented in Section 1.4.1.

Functional characterisation of transcription factors is often performed to confirm the role of putative regulatory proteins. This can involve molecular profiling and analysis of transformed plants in which a gene/s have been silenced or over-expressed. However, stable plant transformation and the subsequent screening process can be complex and lengthy (Berger *et al.*, 2007). Consequently, a variety of heterologous systems have been developed in which transcription factors can be transiently expressed and tested for their ability to bind to the target promoter, causing the gene to be transcribed. These include Arabidopsis, tobacco, petunia floral tissues, and grape suspension cells. Within these systems, trans-activation is measured with a reporter gene, such as ß-glucuronidase (*uidA*, GUS) or LUC, fused to the target promoter. Heterologous systems have been used to confirm the role of a number of candidate regulators of flavonoid biosynthesis (Baudry *et al.*, 2004; Takos *et al.*, 2006a; Bogs *et al.*, 2007; Czemmel *et al.*, 2009). A limitation of these methods, however, is that the degree of trans-activation achieved does not strictly translate to what occurs *in planta*. There may be differences in binding affinities, transcriptional repressors present, or even co-factors absent compared with

the endogenous environment. The co-transformation of the MYB factor with a bHLH partner has been shown to partially circumvent this problem (Takos *et al.*, 2006a; Bogs *et al.*, 2007; Espley *et al.*, 2007).

Utilising the sequences from Chapter 3, in this study flavonoid production was analysed during fruit development in the red (skin and flesh) sweet cherry variety 'Lapins' as a result of increased expression of flavonoid pathway genes. Furthermore, functionality of the putative flavonoid pathway regulators isolated in Chapter 3 was tested in the grape heterologous expression system. The aim of this study was to understand the biosynthesis of flavonoids in developing sweet cherries, and to support the homology-based assignment of function given to the candidate MYB proteins regulating this pathway.

4.2 RESULTS

4.2.1 Developmental observations in 'Lapins' sweet cherries

To enable investigation of the flavonoid biosynthetic pathway, flowers and fruit of the sweet cherry cultivar 'Lapins' were sampled at weekly intervals during the 2008 season. Early developmental stages were defined by a growth reference chart (Figure 2.1 from Michigan State University Extension) and fruit defined by days after full bloom (DAFB). Figure 4.1 depicts the developmental stages analysed in this investigation, and displays the development of fruit colour. Approximately two weeks prior to the detection of the first red skin pigmentation, the green fruit became more straw coloured, possibly due to the breakdown of chlorophyll. At 65 DAFB, skin began to exhibit red colouration but flesh remained yellow (Figure 4.2). After this point, colour deepened and became more evenly distributed in both skin and flesh as fruit developed, reaching maximum intensity at 107 DAFB (Figure 4.1).

4.2.2 Temporal expression of flavonoid pathway genes correlates with flavonoid levels in fruit

Whole fruit (without seed) was used to determine flavonoid concentrations and expression levels of flavonoid structural and regulatory genes (described in Chapter 3) throughout sweet cherry development. The composition of each of the three main flavonoid classes was also determined, and will be discussed in Chapter 5. Expression patterns, together with knowledge of the gene function in other species, allowed genes to be classified as involved mainly in anthocyanin, flavonol or tannin biosynthesis.

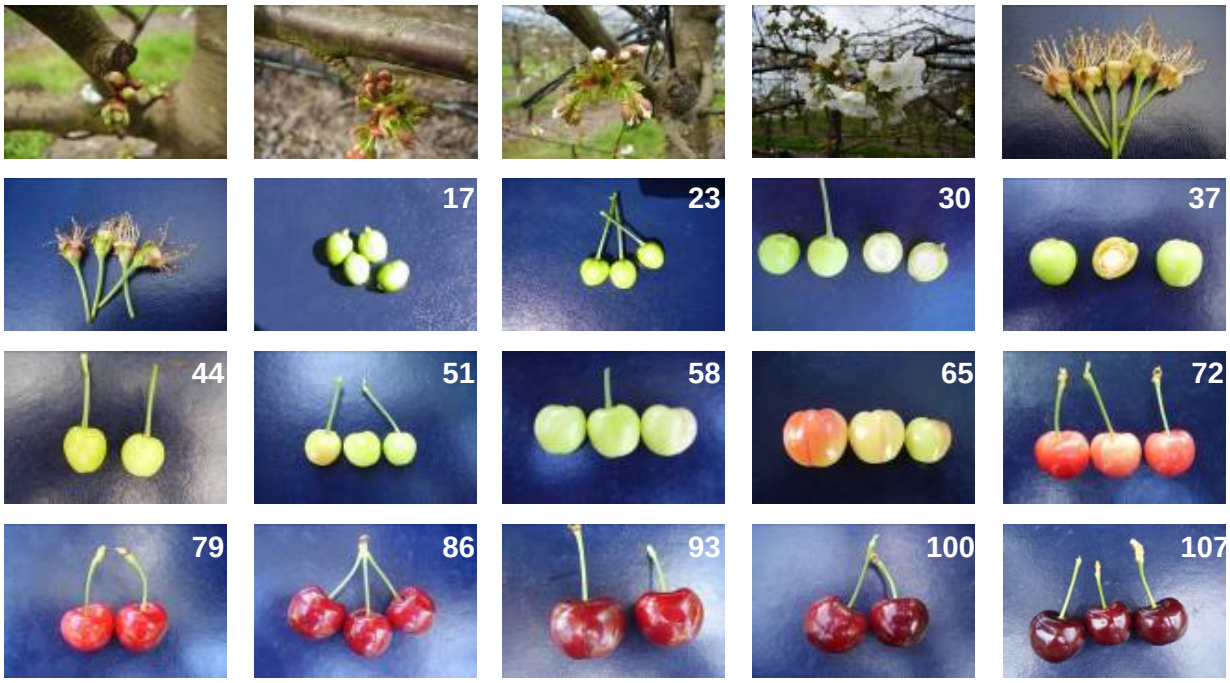


Figure 4.1 – Photographs of ‘Lapins’ sweet cherry developing flowers and fruit

Photographs represent samples taken at weekly intervals during the 2006 season in Lenswood, South Australia. Developmental stages in the first six photographs (left to right) are ‘tight cluster’, ‘open cluster’, ‘first white’, ‘first bloom’, ‘in the shuck’ and ‘shuck split’. Number in the top right corner indicates days after full bloom (September 11). Photographs are not to scale.

Figure 4.2 – Skin of ‘Lapins’ sweet cherry fruit colours before flesh does

Fruit was sampled 65 DAFB, halved and photographed. Not actual size.

4.2.2.1 Anthocyanin biosynthesis

A small amount of anthocyanin was detected in the reproductive tissues of sweet cherry. However, no anthocyanin was measured in developing 'Lapins' fruit until 65 DAFB, corresponding to the first visible evidence of skin pigmentation (Figure 4.3a). Anthocyanin accumulation was responsible for the observed deepening of colour during fruit development, with the concentration of total anthocyanins rising gradually to around 800µg g^{-1} of tissue.

Expression of a number of flavonoid pathway genes closely mirrored the developmental accumulation of anthocyanin. Transcript levels of *PaUFGT*, encoding the glycosyl transferase that produces stable anthocyanins, increased from 65 DAFB, peaking at 93 DAFB with over 30,000-fold higher than abundance during early developmental stages (Figure 4.3b). The expression of *PaF3'H* and *PaLDOX* during 'Lapins' development showed almost identical profiles to *PaUFGT*, with 34-fold and 150-fold increases in transcript abundance respectively (Appendix 8). These results are consistent with the 'Lapins' gene expression profiles obtained by Dr Paul Wiersma (personal communication).

Anthocyanin pathway-specific expression was demonstrated by two of the three *CHS* genes isolated from sweet cherry. The expression *PaCHS1* increased 66-fold between 58 and 107 DAFB, correlating with anthocyanin accumulation (Figure 4.3c). Additionally, *PaCHS1* was expressed early in development, with mean normalised gene expression 16-fold higher than at 51 DAFB, preceding the late developmental peak. A small early peak and a larger 120-fold late peak in gene expression were also observed for *PaCHS3*, although this transcript was less abundant than that of *PaCHS1* (Appendix 8).

The putative anthocyanin regulator, *PaMYBA1*, also displayed a temporal expression profile that correlates with anthocyanin accumulation (Figure 4.3d). The *PaMYBA1* transcript was barely detectable in buds, flowers and green fruit. Expression of *PaMYBA1* rose sharply between 51 and 58 DAFB and continued to increase, with a 12,000-fold peak in gene expression observed 93 DAFB. Consequently, PaMYBA1 is an excellent candidate to control the transcription of the genes *PaCHS1*, *PaCHS3, PaF3'H, PaLDOX* and *PaUFGT*, whose expression at the late stages of fruit development leads to the production of anthocyanin.

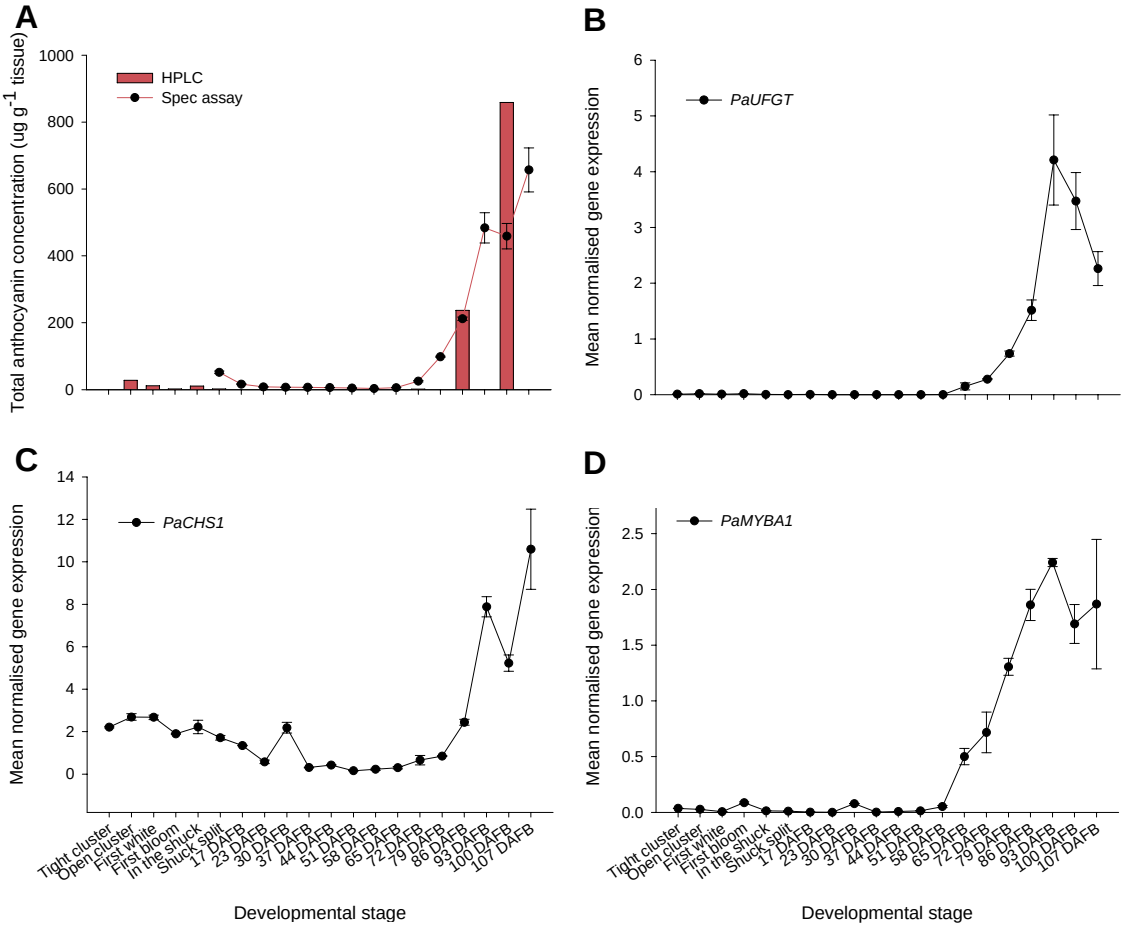


Figure 4.3 – Anthocyanin accumulation correlates with the expression pattern of anthocyanin-related genes

A) Total anthocyanin concentrations were determined for all fruit samples by absorbance at 530nm (red line with error bars showing ± 1SD, n=3). Total anthocyanin concentrations of selected samples were also analysed by HPLC (red columns). Gene expression was determined by real time PCR for B) the anthocyanin-specific gene *PaUFGT*, C) the core pathway gene *PaCHS1*, and D) the putative anthocyanin regulator *PaMYBA1*. Mean gene expression of biological triplicates (± SEM) was normalised with *ubiquitin*.

4.2.2.2 Tannin biosynthesis

Similar to flavonols, the concentration of total tannins (adducts, terminal units and free monomers) in sweet cherry was highest early in development. 'Open clusters' contained 9.1 mg g^{-1} of total tannins, after which time the concentration declined to 4.4mg g^{-1} by 'first bloom' (Figure 4.4a). As fruit emerged from 'in the shuck', tannin concentration again rose to 7.5mg g^{-1} before steadily decreasing throughout the remainder of development to just 400µg g^{-1} at 100 DAFB.

The structural genes of the tannin branch of the flavonoid pathway correlated with tannin concentration in the pattern of their developmental expression in sweet cherry. The expression of *PaLAR1*, involved in the production of catechin in other plant species, peaked at 'first bloom' to a level 144-fold greater than in ripe fruit (Figure 4.4b). After this peak, expression generally decreased steadily and levels of the *PaLAR1* transcript were minimal by 44 DAFB. *PaLAR2* expression was also analysed (Appendix 8). Early developmental stages displayed a generally increasing trend of *PaLAR2* expression, with maximum expression reached at 30 DAFB and little expression detected after 37 DAFB. The pattern of expression of the tannin biosynthetic gene that most closely correlates with tannin accumulation is *PaANR*, required for epicatechin production. Like total tannin concentration, *PaANR* was already expressed at a high level in 'tight cluster' tissue, 275-fold higher than in ripe fruit (Figure 4.4c). With only relatively small fluctuations, the expression of *PaANR* remained high until the 'shuck split' stage, followed by a decline in transcript abundance to minimal levels after 37 DAFB. All of the tannin genes mentioned displayed slightly elevated transcript abundance at 30 DAFB.

The early to mid developmental synthesis of tannins in sweet cherry, as indicated by tannin accumulation and structural gene expression, is supported by expression of the putative tannin regulator, *PaMYBPA1*. Transcript abundance of *PaMYBPA1* followed the pattern of expression described above; high initial expression, then a slight drop followed by a quick rise to peak around shuck stage, with minimal expression in the later stages of sweet cherry development (Figure 4.4d). Unlike the other tannin genes, the second peak in *PaMYBPA1* expression at 'in the shuck' stage was higher than initial levels, 841-fold and 670-fold higher than in ripe fruit, respectively. The elevation in transcript abundance seen in *PaANR* and the *PaLAR* genes occurred seven days earlier, at 23 DAFB for *PaMYBPA1*. Finally, *PaMYBPA1* transcripts remained present in the fruit until 51 DAFB, slightly longer than *PaLAR1* and *PaANR*. The level of expression of *PaMYBPA1* was lower that than of both *PaLAR1* and *PaANR* by one order of magnitude.

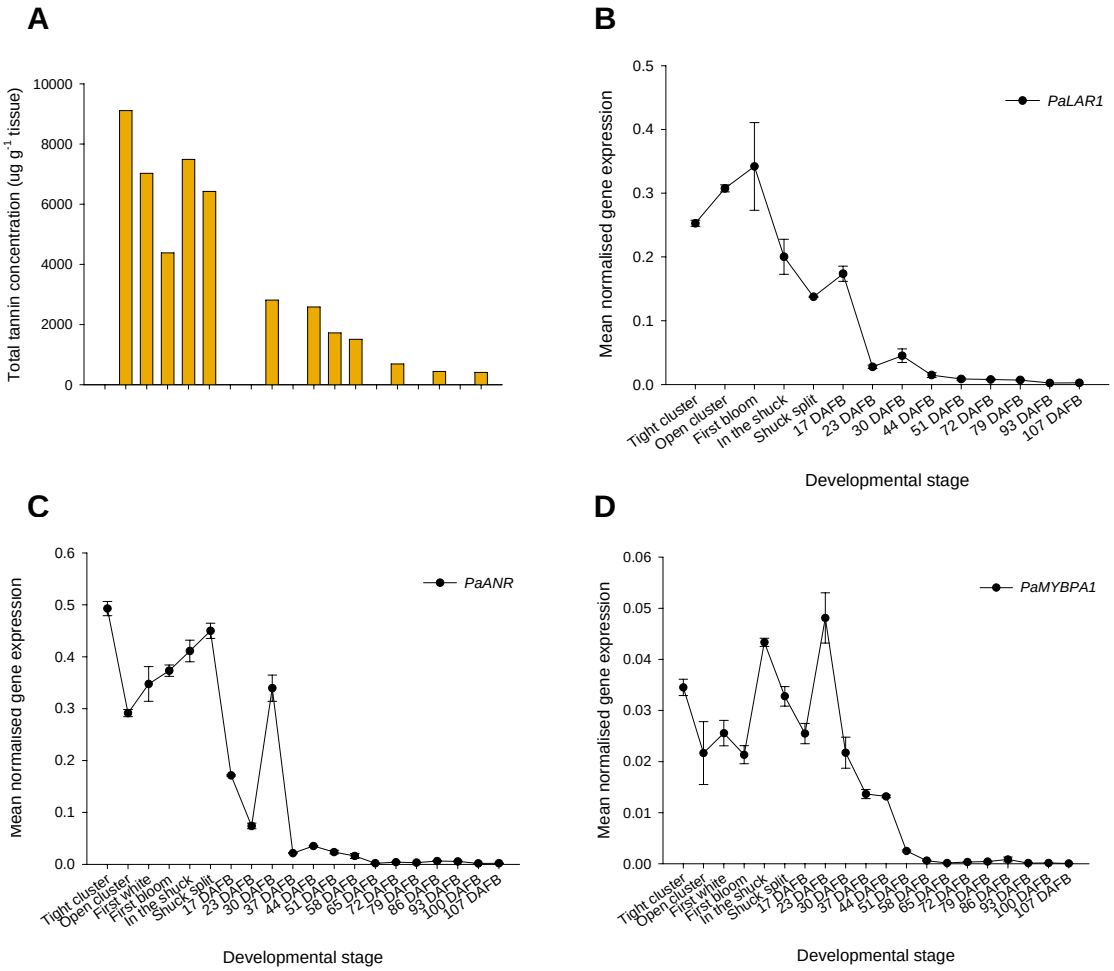


Figure 4.4 – Tannin accumulation correlates with the timing of expression of tannin-related genes

A) Total tannin concentrations were determined for selected samples by HPLC. Gene expression was determined by real time PCR for the tannin-specific genes B) *PaLAR1* and C) *PaANR*, and D) the putative flavonol regulator *PaMYBPA1*. Mean gene expression of biological triplicates (± SEM) was normalised with *ubiquitin*. Some time points in B) were excluded as expression was too low to reliably detect a single, specific *PaLAR1* sequence.

4.2.2.3 Flavonol biosynthesis

During the development of 'Lapins' cherries, flavonol concentrations were highest in budding tissues, reaching 2.5mg g^{-1} (Figure 4.5a). At flowering flavonol concentration had dropped significantly to below 1mg g^{-1}, continuing to fall to just 4µg g^{-1} of tissue by 72 DAFB. A slight increase in flavonol levels was detected in the later stages of fruit development, with 46µg g^{-1} measured 100 DAFB.

Expression of two of the flavonoid structural genes that were studied showed a correlation with the accumulation of flavonol compounds in developing 'Lapins' fruit. Abundance of the *PaFLS* transcript, required for the synthesis of flavonols, was over 3,000-fold higher in the early stages of sweet cherry development than in later stages (Figure 4.5b). *PaFLS* expression was barely detectable after petal fall. The developmental expression profile of *PaCHS2* matched that of *PaFLS*, with almost 1,300-fold higher expression pre-petal fall (Figure 4.5c). However, neither *PaFLS* nor *PaCHS2* displayed an increase in expression in ripening fruit, when a slight rise in total flavonols was noted.

The temporal expression pattern of the putative flavonol regulator *PaMYBF1* failed to confirm its role in flavonol synthesis. The pattern did not coincide with the expression of the flavonol biosynthetic genes and timing of flavonol accumulation. Similar to *PaFLS* and *PaCHS2*, *PaMYBF1* expression in 'tight cluster' tissue was relatively high, dropping approximately 4-fold by the shuck stage (Figure 4.5d). Nonetheless, between 44 and 65 DAFB *PaMYBF1* expression varied significantly whilst the other measures of flavonol synthesis remained steadily low. Transcript abundance of *PaMYBF1* was an order of magnitude lower than that of *PaFLS*. Although the developmental expression of *PaMYBF1* was inconsistent with flavonol synthesis, it displayed a high degree of similarity to characterised flavonol regulators, and encoded a putative protein containing motifs common to subgroup 7, as described by Stracke *et al*. (2001). Therefore, this candidate regulator was still subjected to functional characterisation.

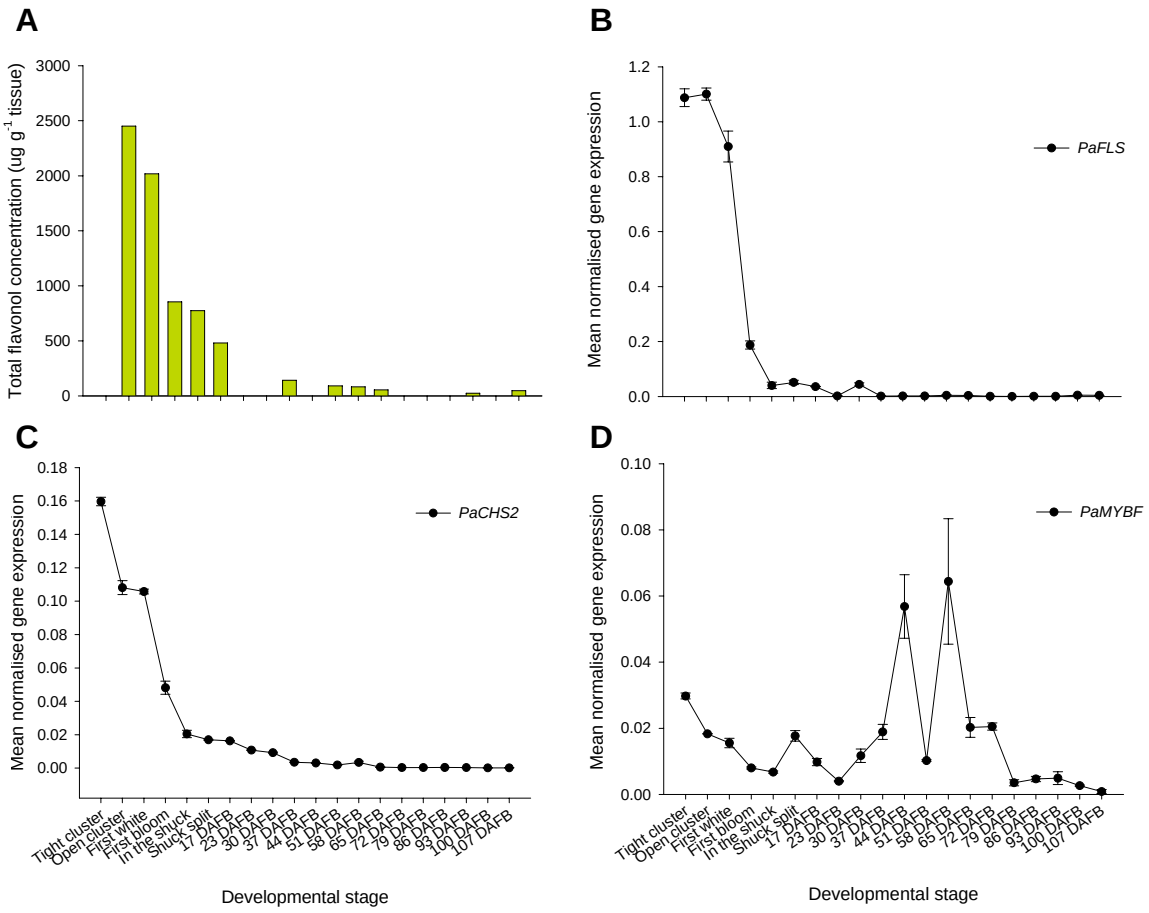


Figure 4.5 – Flavonol accumulation correlates with the expression of some flavonol-related genes

A) Total flavonol concentrations were determined for selected samples by HPLC. Gene expression was determined by real rime PCR for B) the flavonol-specific gene *PaFLS*, C) the core pathway gene *PaCHS2*, and D) the putative flavonol regulator *PaMYBF1*. Mean gene expression of biological triplicates (± SEM) was normalised with *ubiquitin.*

4.2.3 Functional characterisation of putative regulators of flavonoid synthesis

Gene expression analysis confirmed that *PaMYBA1* is a very good candidate for regulation of anthocyanin synthesis in sweet cherry. Developmental expression data were less convincing for control of flavonol and tannin synthesis by PaMYBF1 and PaMYBPA1, respectively. Thus, a demonstration of the functionality of the MYB proteins was required.

4.2.3.1 Validation of grape system for characterisation of sweet cherry MYB proteins

Characterisation of MYB transcription factors by transient trans-activation assays can be performed using various systems (as reviewed in Section 4.1.). MYB transcription factors from apple have been successfully characterised in a system utilising grape (cv. Chardonnay) petiole callus cells (Takos *et al.*, 2006a). In this system the ability of MYB proteins to activate target promoters from another species has also been demonstrated (Deluc *et al.*, 2008; Czemmel *et al.*, 2009). Nonetheless, before proceeding with functional characterisation, we first tested that the sweet cherry MYB proteins were compatible with the grape system, and that they were able to able to activate the apple and grape target promoters we had available.

Compatibility of sweet cherry MYBs with this heterologous system was confirmed by co-bombardment of *PaMYBA1* and the *bHLH*, *AtEGL3* using grape cells. Although fewer red grape cells were observed compared with using VvMYBA1, the presence of this pigmentation demonstrates the ability of PaMYBA1 to bind to the promoter of the endogenous grape *UFGT*, activating gene transcription and resulting in anthocyanin production.

The trans-activation of the *DFR* promoter from apple and grape by sweet cherry MYB transcription factors was compared. Strength of promoter activation was measured by the amount of luminescence, produced as a result of the promoter driving expression of the firefly luciferase gene. The putative flavonoid pathway transcription factors PaMYBA1, PaMYBF1 and PaMYBPA1 were tested. Transient transformation with PaMYBF1 was performed without the bHLH co-factor because its presence reduced activation by up to 55% (data not shown), a finding consistent with others using grape and Arabidopsis flavonol regulators (Stracke *et al.*, 2007; Czemmel *et al.*, 2009), and with the absence of a bHLH interaction domain in PaMYBF1 (Chapter 3). No significant differences were measured between activation of apple and grape *DFR* from any of the MYBs tested (Figure 4.6). These data indicate compatibility of sweet cherry MYB proteins with apple and grape flavonoid gene promoters, with comparable activation from promoters of both species. This result eliminates

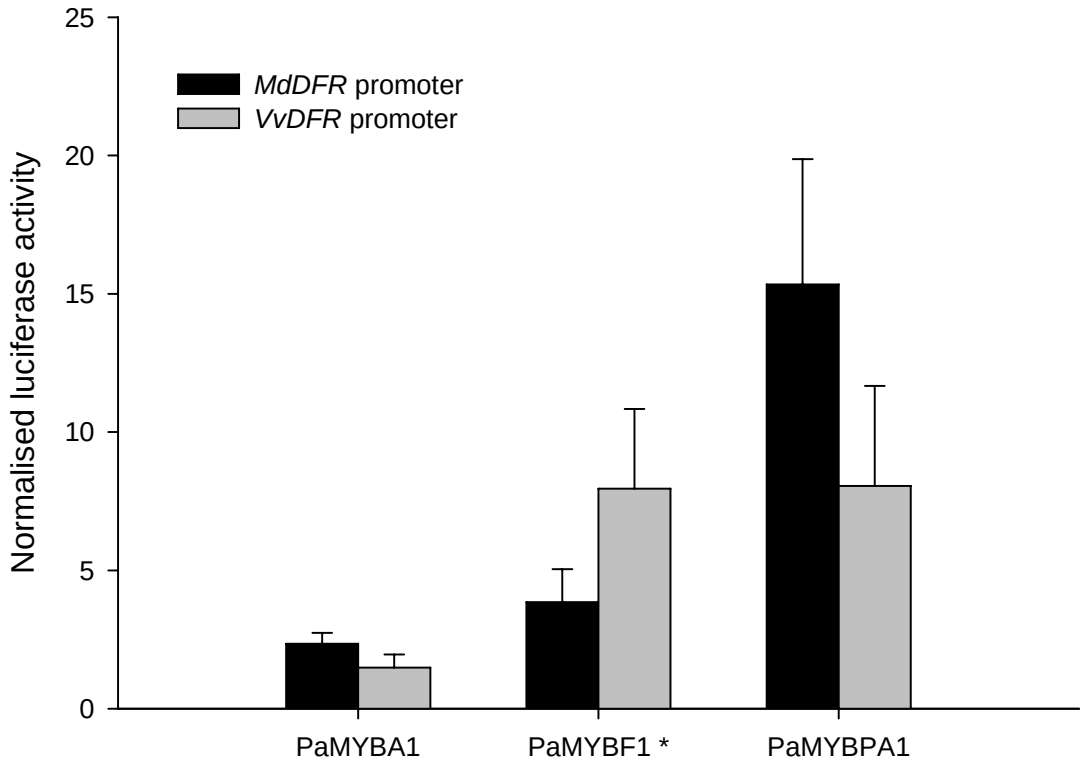


Figure 4.6 – *DFR* promoters from apple and grape can be comparably activated by sweet cherry MYBs transiently expressed in Chardonnay cells

Stimulation of LUC activity driven by either an apple or grape *DFR* promoter fused to the firefly *LUC* gene and co-bombarded into grape cells with a sweet cherry *MYB* cDNA. * All bombardments were performed with the bHLH co-factor *AtEGL3*, with the exception of PaMYBF1. Data is the mean ratio of LUC activity (see methods) normalised to a separate bombardment with the promoter only. Error bars are ± 1SD of triplicate bombardments.

the requirement for the creation of promoter constructs from sweet cherry in order to achieve functional characterisation of the cherry MYBs.

4.2.3.2 Transient trans-activation assays

To confirm the roles of the MYB transcription factors in the synthesis of specific flavonoid compounds, and to identify their target genes, trans-activation assays were performed with various flavonoid gene promoters. The luciferase activity ratio obtained from co-bombardment of a promoter and MYB factor was normalised to that of bombardment with the promoter LUC construct (plus *AtEGL3*) only. For some promoters, including those from *MdCHS*, *MdDFR* and *VvFLS*, a high basal level of activity was observed in the absence of effectors, suggesting that endogenous transcription factors capable of trans-activation might be present in the grape cells.

PaMYBA1 was able to strongly activate (37-fold) luciferase expression under the control of the *MdUFGT* promoter, confirming its role as a regulator of anthocyanin synthesis (Figure 4.7a). Furthermore, the activation of *MdCHS* and *MdDFR* promoters (~10-fold and 3.6-fold respectively) suggests PaMYBA1 is involved in regulation of other flavonoid pathway genes to provide substrates for anthocyanin synthesis. It seems likely that PaMYBA1 does not regulate *LDOX* in sweet cherry, as it did not significantly activate the *VvLDOX* promoter in the assay.

The transient trans-activation assay showed the ability of PaMYBF1 to activate the promoters of *MdCHS* about 15-fold and *MdANR* 4.5-fold (Figure 4.7b). However, *VvFLS* and *MdUFGT* promoters were only marginally activated by PaMYBF1, which increased luciferase activity by approximately 2 fold in both cases. These results suggest a role for PaMYBF1 in the regulation of the flavonoid pathway, but it may not be a functional regulator of flavonol synthesis.

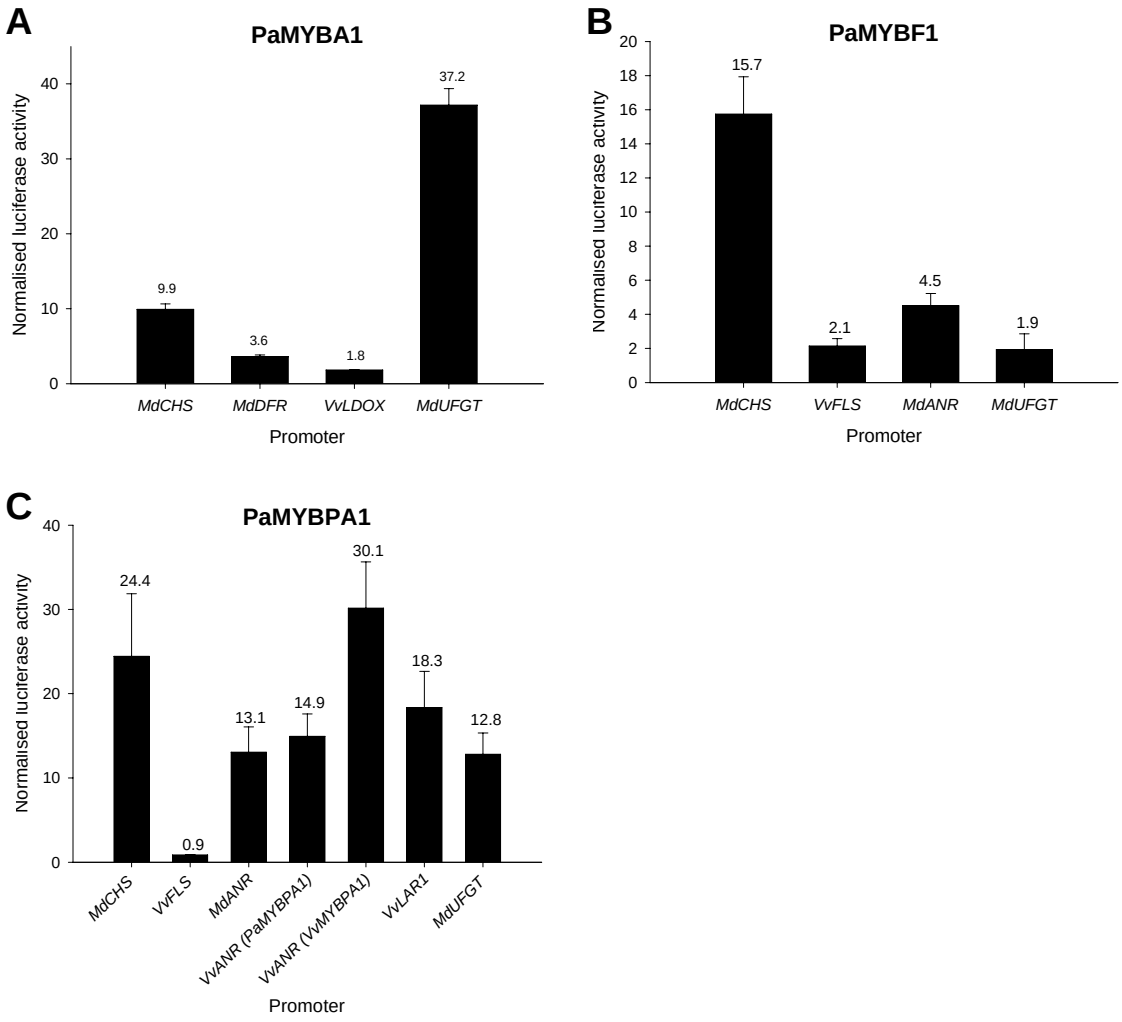


Figure 4.7 – Functional testing of sweet cherry MYBs transiently expressed in a heterologous system

Stimulation of LUC activity driven by either an apple or grape promoter fused to the firefly *LUC* gene and co-bombarded into grape cells with A) *PaMYBA1* cDNA, B) *PaMYBF1* cDNA or C) *PaMYBPA1* cDNA (Note: *VvLAR* was tested with both PaMYBPA1 and VvMYBPA1, all other promoters in C) were tested with PaMYBPA1 only). All bombardments were performed with the bHLH co-factor *AtEGL3*, with the exception of PaMYBF1. Data is the mean ratio of LUC activity (see methods) normalised to a separate bombardment with the promoter only (quantified above the error bar). Error bars are ± 1SD of triplicate bombardments.

Functional characterisation of PaMYBPA1 confirmed it is able to control tannin synthesis via transcriptional regulation of the tannin-related genes *LAR1* and *ANR.* Transiently transformed grape cells exhibited ~18-fold and 13-15-fold increases in luciferase activity, driven by the promoters of *VvLAR1* and both *MdANR* and *VvANR* promoters respectively. Activation of *VvANR* by PaMYBPA1 was approximately half that by VvMYBPA1, similar to the difference seen in activation of *VvFLS* by AtMYB12 compared with VvMYBF1 (Czemmel *et al.*, 2009). In addition to regulating *LAR* and *ANR,* PaMYBPA1 also activated the promoters of the flavonoid biosynthetic genes *MdCHS* and *MdUFGT* (~25-fold and ~13-fold), but was unable to induce to the *VvFLS* promoter.

4.3 DISCUSSION

4.3.1 Synthesis of anthocyanins is temporally separated from synthesis of other flavonoid classes in sweet cherry fruit

To investigate flavonoid synthesis in sweet cherry fruit, we analysed the concentrations of flavonols, tannins and anthocyanins in 'Lapins' buds, flowers and fruit at regular intervals throughout a growing season. We found that flavonol and tannin concentrations were highest in the earliest bud tissues sampled, declining during development on a per gram basis to relatively low and steady levels post 58 DAFB. In contrast, anthocyanin only began to accumulate in fruit post 58 DAFB. The anthocyanin content of 'Lapins' fruit increased exponentially, as previously observed for the 'Burlat' sweet cherry (Remon *et al.*, 2006). These two distinct phases of flavonoid production are in agreement with findings in other fleshy fruits like apple (Takos *et al.*, 2006c), bilberry (Jaakola *et al.*, 2002), strawberry (Cheng and Breen, 1991), raspberry (Kumar and Ellis, 2001) and peach (Tsuda *et al.*, 2004). Similar to observations in other Rosaceae family fruits we also noted a transient accumulation of anthocyanin early in development, which presumably became diluted as fruit began to expand (Saure, 1990; Tsuda *et al.*, 2004). The concentration and composition of flavonoids in mature 'Lapins' fruit was comparable to other reports for sweet cherry (Gao and Mazza, 1995; Kim *et al.*, 2005; Harnly *et al.*, 2006; Serrano *et al.*, 2009). The temporal separation of metabolite production from different flavonoid branches during fruit development suggests regulatory control of the flavonoid pathway in sweet cherry fruit.

4.3.2 Anthocyanin accumulation in 'Lapins' fruit is controlled by PaMYBA1

Using real time PCR we determined the abundance of transcripts for a number of flavonoid genes during 'Lapins' fruit development. As expected, the expression of the documented anthocyanin pathway genes *PaLDOX* and *PaUFGT* coincided with the timing of anthocyanin

accumulation. Although transcript levels declined post 93 DAFB, they still remained relatively high, and presumably the substrates and enzymes required for synthesis remained present, enabling subsequent anthocyanin accumulation. Two *CHS* variants *PaCHS1* and *PaCHS3*, but not *PaCHS2*, also displayed a developmental expression pattern indicative of a role in anthocyanin synthesis. In peach (Tsuda *et al.*, 2004) and grape (Ageorges *et al.*, 2006) one of three isolated *CHS* fragments were detected in fruit tissues that were accumulating anthocyanin. The data indicate that co-ordinate regulation of a set of anthocyanin-related genes is responsible for the initiation of anthocyanin synthesis at a specific stage of fruit development, as has previously been shown for apple (Takos *et al.*, 2006c), peach (Tsuda *et al.*, 2004) and mangosteen (Palapol *et al.*, 2009).

Co-expression of the putative anthocyanin regulator *PaMYBA1* with the anthocyanin-related genes suggests that it is the transcription factor controlling their expression during development. Increased *PaMYBA1* expression slightly preceded the increase in structural gene expression, as seen for the expression of the gentian (*Gentiana triflora*) anthocyanin MYB factor *GtMYB3* in flower petals (Nakatsuka *et al.*, 2005a). This lag is consistent with the requirement for translation of *MYBA1*, and trans-activation and transcription of target mRNA.

Further characterisation of PaMYBA1 was used to confirm it is a functional regulator of anthocyanin synthesis. When transiently expressed in cultured grape cells, PaMYBA1 was able to drive anthocyanin production via trans-activation of endogenous *UFGT*. Compared to bombardment with VvMYBA1, PaMYBA1 produced fewer red cells that took longer to develop. This is similar to bombardment of Chardonnay cells with *MdMYBA1* (M. Walker, personal communication); however, differences are anticipated when using a heterologous system. Confirmation of functionality was also achieved by the ability of PaMYBA1 to drive transcription from promoters of the apple *CHS*, *DFR* and *UFGT* genes required for anthocyanin synthesis. Therefore, unlike VvMYBA1 which controls only *VvUFGT* transcription (Kobayashi *et al.*, 2002; Kobayashi *et al.*, 2004; Walker *et al.*, 2007), PaMYBA1 is able to control anthocyanin production by transcriptional activation of multiple flavonoid pathway genes similar to the findings for apple MdMYB1 (Takos *et al.*, 2006a) and for Arabidopsis AtPAP1, AtPAP2, AtMYB113 and AtMYB114 (Borevitz *et al.*, 2000; Tohge *et al.*, 2005; Gonzalez *et al.*, 2008).

4.3.3 PaMYBPA1 regulates shared pathway and tannin-specific flavonoid genes

During sweet cherry development, we found tannin accumulation to closely follow flavonol accumulation. Previous studies have shown expression of the flavonoid pathway genes *ANR* (Xie *et al.*, 2003) and *LAR* (Tanner *et al.*, 2003) is necessary for the production of tannins around flowering in legumes. Expression of these genes at a comparable time has also been shown to correlate with tannin accumulation in grape (Bogs *et al.*, 2005) and apple (Takos *et al.*, 2006c). The presence of both catechin and epicatechin subunits units suggested that expression of *PaLAR* and *PaANR*, respectively, would also be required for tannin synthesis in sweet cherry. *PaANR* and *PaLAR1* genes were expressed only until 44 DAFB, coinciding with the accumulation of tannins within the fruit and consistent with a role in protecting very young fruit from being eaten by animals. It is interesting to note that there was no overlap in the temporal expression of tannin- and flavonol-synthesis genes with anthocyanin-synthesis genes, suggesting a switch in the metabolic flux of the flavonoid pathway. This is different from grape, in which flavonols accumulate again in the last weeks of berry ripening (Downey *et al.*, 2003b).

The *PaANR* expression pattern fits best with tannin accumulation during fruit development, which was anticipated given that throughout fruit growth ~90% of the terminal subunits present were epicatechin. Although *PaLAR2* was also expressed during early development, its abundance did not seem tightly linked with the concentration of fruit tannins. This is in agreement with the *VvLAR2* (Bogs *et al.*, 2005) and *MdLAR2* genes of grape and apple, respectively, which have not yet been assigned functions. Based on their expression profiles *VvLAR2* and *MdLAR2* are thought to be involved in tannin synthesis in seed rather than fruit skin or flesh. Similarly, *PaLAR2* expression could be seed-specific, as expression in this tissue was not determined. The seed could not be removed from the skin and flesh of the immature fruit prior to lignification of the endocarp ("pit hardening") that encases the seed. A spike in the expression of all of the tannin genes analysed was observed at around 30 DAFB and did not correlate with an increase in tannin accumulation. Due to the relatively low levels of expression of these tannin biosynthetic genes it is possible that increased transcription was the result of changes in environmental conditions, such as increases in maximum air temperature and average solar radiation, experienced at the field site at this time in early October 2006.

PaMYBPA1 was generally co-expressed with *PaANR* and *PaLAR1* during sweet cherry development. Abundance of the *PaMYBPA1* transcript displayed seemingly large

fluctuations; however, considering the low steady state level, consistent with that of *VvMYBPA1* (Bogs *et al.*, 2007), these fluctuations are largely due to biological noise and minor experimental errors that are magnified by the small scale of gene expression. In grape a second regulator VvMYBPA2 has been found to regulate tannin synthesis in fruit exocarp and leaves, rather than in seed like VvMYBPA1 (Terrier *et al.*, 2009). In Chapter 3 we concluded it is likely that a second *MYBPA* gene also exists in the sweet cherry genome. The expression of such a gene, when identified, may display closer correlation with the accumulation of tannins in fruit tissue.

Functional characterisation of PaMYBPA1 revealed its ability to drive transcription from the promoters of a large set of genes, including those required for both tannin and anthocyanin but not flavonol synthesis. This is similar to the trans-activation of the grape promoters of *CHI*, *F3'5'H1* and *LDOX* in addition to *ANR* and *LAR1* by VvMYBPA1 (Bogs *et al.*, 2007). The activation of *UFGT* by a characterised tannin regulator has not been previously reported. However, the grape flavonoid pathway regulator VvMYB5b is able to drive transcription from *LDOX*, *CHI* and *F3'5'H1* as well as the tannin genes *LAR1* and *ANR* (Deluc *et al.*, 2008). The PaMYBPA1 sequence displays greater similarity with VvMYBPA1 than with VvMYB5b, but it could play a broader role in flavonoid pathway regulation in a similar way to VvMYB5b. Given that *PaUFGT* is not expressed when the *PaMYBPA1* transcript is more abundant, we suggest that PaMYBPA1 might have a low binding affinity for the sweet cherry *UFGT* promoter. Additional regulatory control, for example the presence of transcriptional repressors or the absence of PaMYBPA1 co-factors, could also explain this lack of *PaUFGT* transcription early in fruit formation despite its promoter being responsive to PaMYBPA1. In our experiments we observed only about half as much luciferase activity driven by the *VvANR* promoter when PaMYBPA1 was bombarded compared to VvMYBPA1. This disparity could be due to sequence differences within the MYB and/or promoter binding sites between grape and sweet cherry. The lower degree of activation could also be influenced by the ability of endogenous co-factors and/or the co-bombarded AtEGL3 to interact with PaMYBPA1. Nonetheless, the apple and grape flavonoid gene promoters were sufficient to demonstrate the functionality of PaMYBA1 and PaMYBPA1.

4.3.4 PaMYBF1 contributes to flavonoid pathway regulation

In grape, VvMYBF1 controls flavonol synthesis by modulating the abundance of *FLS* transcripts. FLS catalyses the synthesis of flavonols from dihydroflavonols, and its expression correlates with the accumulation of flavonols at early stages of apple and grape development

(Downey *et al.*, 2003b; Takos *et al.*, 2006c). Similarly, this study found that sweet cherry flavonol concentration and *PaFLS* expression were highest in the earliest tissue samples, and displayed a parallel decline during fruit development. The similar expression profiles of *PaCHS2* and *PaFLS* implicates the *CHS2* gene as being important for flavonol synthesis, although with its early developmental expression *PaCHS2* could also play a role in tannin synthesis. The involvement of a particular isoform of a *CHS* multigene family in the production of flavonols has also been described for petunia (Koes *et al.*, 1989), grape (Jeong *et al.*, 2008), gerbera (Helariutta *et al.*, 1995) and morning glory (*Ipomoea purpurea* and *Ipomoea nil,* Johzuka-Hisatomi *et al.*, 1999). Further investigation of the sweet cherry *CHS* gene family, such as determining substrate preference and physical interaction with flavonoid enzymes for substrate channeling, may help to clarify the roles of these different genes.

The putative flavonol regulator, *PaMYBF1*, did not appear to be co-expressed with *PaFLS* and *PaCHS2*, nor did its expression coincide with flavonol accumulation during sweet cherry development. In contrast, when the flavonol regulator *AtMYB12* is over-expressed or silenced in Arabidopsis seedlings, the abundance of the *AtFLS* transcript abundance follows the *AtMYB12* expression levels (Mehrtens *et al.*, 2005). Similarly, *VvMYBF1* reportedly displays the same light-induced expression pattern as *VvFLS1* in grape (Czemmel *et al.*, 2009). The lack of correlation of *PaMYBF1* expression with that of *PaFLS* and *PaCHS2* was for the most part due to large variations in *PaMYBF1* transcript abundance. Such variations could possibly be attributed to a combination of the very low level of expression of *PaMYBF1* and the influence of abiotic factors such as environmental factors. It is also possible that co-expression was not observed because another unidentified MYB may control flavonol synthesis in sweet cherry instead of PaMYBF1. With the presence of three characterised flavonol regulators, AtMYB11, AtMYB12 and AtMYB111 in Arabidopsis (Stracke *et al.*, 2007), sweet cherry may also possess multiple MYB transcription factors belonging to subgroup 7.

Transient trans-activation assays were performed with PaMYBF1, to help clarify its role in flavonoid pathway regulation. The up-regulation of luciferase from the *MdCHS* and *MdANR* promoters indicates PaMYBF1 is able to exert transcriptional control over flavonoid synthesis. However, as the *MdCHS* promoter utilised in the assay was one of several related apple *CHS* sequences (Takos *et al.*, 2006c), it is unclear whether PaMYBF1 regulates only flavonol-related *CHS* variants such as *PaCHS2*, or if it can control all *CHS* family members. To determine this it would be necessary to isolate and test the responsiveness of the promoters

of *PaCHS1*, *PaCHS2* and *PaCHS3*. The conventional target of flavonol regulation in grape, *VvFLS*, can be trans-activated by both VvMYBF1 and AtMYB12 in vitro (Mehrtens *et al.*, 2005; Czemmel *et al.*, 2009), whereas PaMYBF1 was unable to drive gene expression from this promoter. Considering that flavonol synthesis is light-induced in grape (Downey *et al.*, 2004), endogenous production of flavonols could be enhanced by the experimental conditions, resulting in underestimation of the level of activation from the *VvFLS* promoter. Performing the transformations in the dark may be necessary to obtain a true indication of promoter responsiveness to PaMYBF1. The possibility also exists that the PaMYBF1 protein is unable to activate to the *VvFLS* promoter but would be able to induce expression from the *PaFLS* promoter. However, considering the temporal expression of PaMYBF1, it is likely that it is not a functional regulator of flavonol synthesis during sweet cherry development. The expression of the genes required for flavonol biosynthesis must be governed by another MYB that is co-expressed with *PaFLS* during fruit development.

4.4 CONCLUSION

Knowledge of the flavonoid composition of developing fruit is important, as it enables the harvesting of fruit at a time which will maximise the health benefits that flavonoids provide (Serrano *et al.*, 2005). In this study, we have investigated the production of flavonoid compounds in sweet cherry during fruit development. Consistent with published results for other fruit species, flavonols and tannins were produced prior to flowering. Anthocyanin accumulated towards harvest, when flavonol and tannin synthesis had ceased. Molecular and functional characterisation putative flavonoid regulators have shown that the expression of *PaMYBPA1* early in development is likely to produce tannins by controlling the transcription of a broad set of shared pathway and tannin-specific flavonoid biosynthetic genes. Similarly, we have demonstrated that PaMYBA1 is a functional regulator of anthocyanin synthesis during ‘Lapins’ sweet cherry development, controlling the transcription of another set of genes. Further characterisation of PaMYBF1 is required to resolve its potential role in flavonoid regulation; however co-expression analysis and trans-activation suggest it is not the transcription factor controlling flavonol synthesis in sweet cherry.

Chapter Five: The role of PaMYBA1 in varietal colour differences

5. INVESTIGATION OF THE ROLE OF PaMYBA1 IN FRUIT OF DIFFERENT COLOURS

5.1 INTRODUCTION

The red, blue and purple colours that colour plant tissues are primarily due to the presence of anthocyanins. Within a particular plant species, there can be a great deal of naturally occurring colour variation. Ornamental flowers like petunia can be range from pale pink, through to purple or blue depending on the type of anthocyanin produced, the presence of co-pigments and vacuolar pH (Section 1.2.2.3, Mol *et al.*, 1998). Fruits and vegetable varieties are often highly coloured yet a number of varieties exist that contain little or no anthocyanin, such as those yielding green cabbage (*Brassica oleracea*), white grapes, yellow corn and green/yellow apples. Molecular comparisons between diversely coloured varieties have assisted in isolation of key flavonoid pathway genes, as well as understanding how flavonoid biosynthesis is regulated and how genes determining colour are inherited (Davies *et al.*, 2009). In addition to the characterisation of naturally occurring flavonoid mutants, further insight has been gained through characterisation of induced mutations, and transformed plant lines in which structural and/or regulatory genes have been silenced or over-expressed, as mentioned in Section 1.3.5.

Although not as easily detected as colour variations, flavonol and tannin levels also vary amongst cultivars. Flavonoid concentrations are determined by transcript abundance of genes required for flavonoid biosynthesis. For instance, almost undetectable levels of expression of all anthocyanin biosynthetic genes for green apple (Honda *et al.*, 2002), and *CHS* and *DFR* for white peach (Tsuda *et al.*, 2004), mean that these fruits generally lack colour. Conversely, the intense colourations of red cabbage and red-fleshed apple are attributable to very high transcript levels of all anthocyanin genes (Espley *et al.*, 2007; Yuan *et al.*, 2009). Spontaneous somatic mutations have also been reported to produce bud sports which either reduce or increase the degree of colouration via altered gene expression for a number of fruits, including apple (Dickinson and White, 1986), pear (Pierantoni *et al.*, 2009), and grape (Walker *et al.*, 2006; Ramazzotti *et al.*, 2008).

In some instances, the lack of gene expression is the result of a mutation of that gene. Nucleotide base substitutions, as well as insertions and deletions of bases, regions of genes or whole genes can alter the ability of a gene to be transcribed. A naturally occurring, cultivated

white gentian flower colour mutant reportedly lacks blue pigmentation due to an *anthocyanidin synthase* (*ANS*, commonly referred to as *LDOX*) structural gene mutation (Nakatsuka *et al.*, 2005b). Similarly, mutated *DFR-A* alleles are responsible for the colour difference between yellow and red onions (*Allium cepa*, Kim *et al.*, 2009). Transposon-related mutations in genes like *CHS* and *DFR* have also been found to produce white flowers in mutants of morning glory cultivars (Inagaki *et al.*, 1994; Habu *et al.*, 1998).

When the altered flavonoid levels of a cultivar result from diminished or enhanced expression of one of more genes, transcriptional regulators of flavonoid biosynthesis are often involved. Mutations in genes that encode proteins required for transcriptional activation of flavonoid biosynthetic genes have been described for a number of flavonoid-deficient mutants. Green apples and white grapes have both been shown to lack pigmentation because they contain non-functional anthocyanin *MYB* genes (Kobayashi *et al.*, 2004; Takos *et al.*, 2006a; Walker *et al.*, 2007). While the wild ancestors of rice display a ubiquitous red pericarp, the white pericarp of domesticated cultivars is thought to have arisen from mutation of the *bHLH* gene, *Rc* (Sweeney *et al.*, 2006). The *bHLH* gene *IpbHLH2* from morning glory, and *PhAN1* from petunia, are also mutated in their respective white flower cultivars (Gerats *et al.*, 1985; Park *et al.*, 2007). Due to the regulatory promiscuity of bHLH and WD40 proteins, mutations in these genes often affects not only flavonoid production, but other traits such as trichome formation and cell morphology (Walker *et al.*, 1999; Spelt *et al.*, 2002; Zhang *et al.*, 2003). Finally, variable flavonoid levels between cultivars might be attributed to the presence of repressors of flavonoid biosynthesis (Aharoni *et al.*, 2001; Park *et al.*, 2008), or competitive inhibitors of proteins regulating transcription (Matsui *et al.*, 2008; Zhu *et al.*, 2009).

Amongst sweet cherry cultivars, flesh can be yellow, red/mahogany or purple/black and skin can be yellow, blush, red/mahogany or purple/black. Through studying the segregation of colour traits in sweet cherry breeding populations, it has been determined that dark fruit colour results from incompletely dominant flesh and skin colour traits, which were introduced in Section 1.2.2 and will be studied in greater detail in Chapter 6. Although incomplete dominance explains the intermediate red fruit colour phenotype of, for example, ‘Lapins’, the genes/s responsible for the difference between so-called “light” (yellow or blush) and “dark” (red, purple or black) sweet cherries have not yet been determined.

Characterisation of *PaMYBA1* has revealed it is able to control colour in developing sweet cherry fruit by regulating the transcription of genes required for anthocyanin synthesis

(Chapter 4). Furthermore, it is similar in sequence to the anthocyanin MYB regulators in apple (MdMYB1) and grape (VvMYBA1 and VvMYBA2) that determine whether fruits are pigmented. To test the hypothesis that this gene is responsible for different colour intensities amongst sweet cherry cultivars, *PaMYBA1* gene sequence was isolated from leaves of different-coloured cultivars and the expression of the gene was analysed in fruit displaying a variety of colours. Varietal variations in the flavonol and tannin pathways and their putative regulators in sweet cherry were also investigated.

5.2 RESULTS

The sweet cherry cultivars utilised in this study were 'Yellow Glass' ("yellow fruit", yellow skin and flesh), 'Ranier' ("blush fruit", red blush on yellow skin with yellow flesh), 'Lapins' ("red fruit", red skin and flesh), and 'Sam' ("black fruit", dark red skin and flesh) (Figure 5.1). No sequence variations within the flavonoid biosynthetic genes *PaF3'H*, *PaDFR*, *PaLDOX* or *PaUFGT* were detected within the genomic DNA of any of the varieties that would apparently render them non-functional and explain any lack of pigmentation. Therefore, our attention was focused to the *PaMYBA1* gene.

5.2.1 Analysis of *PaMYBA1* in sweet cherry varieties

The abundance of the *PaMYBA1* transcript in the ripening skin of fruit from four sweet cherry cultivars was determined by real time PCR. Samples used for analysis were all collected on a single day; therefore fruit were not necessarily at the same maturity stage, ranging from 67-78 DAFB. This approximate developmental stage in 'Lapins' fruit was found to correspond with the 2-4 weeks following the induction of *PaCHS1*, *PaUFGT* and *PaMYBA1* gene expression and the onset of anthocyanin accumulation (Chapter 4). With that considered, the expression of *PaMYBA1* was comparable in the anthocyanin producing varieties 'Ranier', 'Lapins' and 'Sam', despite colour variations between their fruits (Figure 5.1). Consistent with its role in anthocyanin regulation, the *PaMYBA1* transcript was not detected in cDNA from 'Yellow Glass' fruit that lacked anthocyanin.

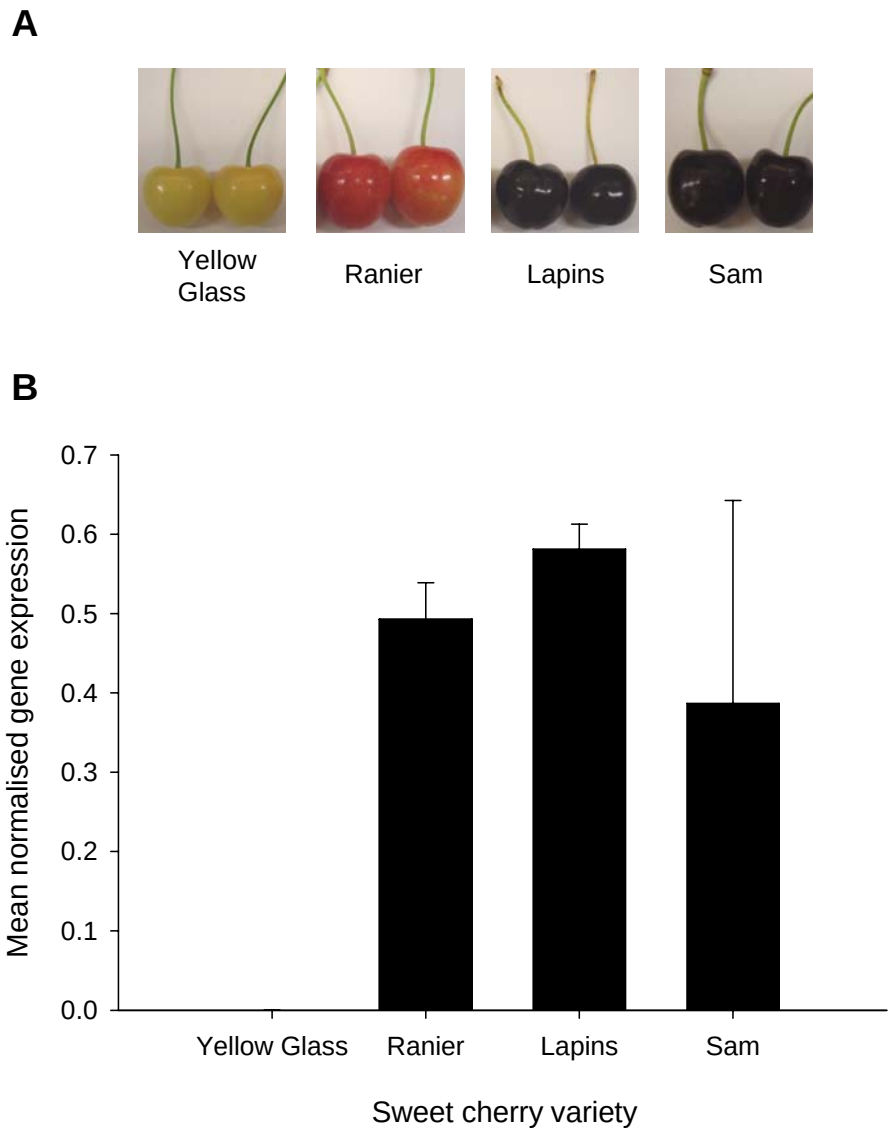


Figure 5.1 – Levels of the *PaMYBA1* transcript in four sweet cherry varieties

A) Photographs of the four sweet cherry varieties tested. Photographs are not to scale but demonstrate fruit colour. B) Expression of the *PaMYBA1* gene in the skin of ripening fruit (67-78 DAFB) was determined by real time PCR. Samples from all four varieties were collected on the same day. Mean gene expression of biological triplicates (± SEM) was normalised with *ubiquitin*.

To further investigate the genetic differences between the sweet cherry cultivars with respect to colour, the genomic sequences for *PaMYBA1* were analysed in the four cultivars. Within the coding regions of the *PaMYBA1* sequences from the four cultivars very few differences were found (data not shown). The very few nucleotide substitutions that were identified either did not translate to altered residues or only encoded conserved amino acid changes. The high degree of conservation extended to the untranslated regions of the *PaMYBA1* gene. However, the first intron in 'Yellow Glass' *PaMYBA1* contained an insertion of the nucleotides GCCAAT when compared to the sequence of the other varieties (Figure 5.2). This is a binding site for transcription factors belonging to the CCAAT/enhancer binding protein (C/EBP) family, which, if recruited to the 5' untranslated region, can result in over-expression of the human osteopontin gene (Giacopelli *et al.*, 2003). However, a DNA-bound protein obstacle, such as a C/EBP within the gene, may block the progression of the RNA polymerase during transcriptional elongation and result in premature termination of transcription (Rasmussen and Lis, 1995). Such a block in transcriptional elongation has been shown to down-regulate the human c-*myc* gene (Eick and Bornkamm, 1986). This could be a possible explanation for the lack of detection of *PaMYBA1* transcripts in cDNA from 'Yellow Glass' fruit.

Figure 5.2 also shows a single nucleotide deletion in the region -18 to -40 nucleotides from the 3' splice site of the first intron of 'Yellow Glass' *PaMYBA1*. This region often contains a branch point sequence for lariat RNA formation, and subsequent intron splicing (Padgett *et al.*, 1984; Reed and Maniatis, 1985; Brown, 1986). Mutations in this sequence could lead to reduced transcript abundance through inefficient gene splicing (Simpson *et al.*, 1996), although cryptic branch points may also be utilised (Ruskin *et al.*, 1985). An altered lariat branch point sequence is not likely to prevent expression of *PaMYBA1* in the 'Yellow Glass' cultivar, as if this were the case, some gene expression would still be detected. Additionally, the position of the nucleotide deletion in 'Yellow Glass' DNA does not appear to fall within the plant consensus for the branch point site, CTPuAPy that is found in the majority of plant introns (Brown, 1986).

Figure 5.2 – Alignment of the nucleotide region spanning the first intron of *PaMYBA1*

The *PaMYBA1* gene was amplified from 'Lapins' and 'Yellow Glass' genomic DNA (see Chapter 2). Products were cloned, sequenced, aligned using ClustalW software (Thompson *et al.*, 1994) and presented with GeneDoc Version 2.7.000 (Nicholas and Nicholas, 1997). Only a section of the gene containing the first intron of *PaMYBA1* is presented. Nucleotides within an exon are presented as capital letters, while those within introns are in lower case. Nucleotides highlighted in blue are not identical in the two sequences. The C/EBP binding site insertion in the 'Yellow Glass' sequence is shown in red. The boxed nucleotides represent the putative region in which a branch point site would be expected (Brown, 1986).

5.2.2 Comparison of flavonoid accumulation in sweet cherry varieties during fruit development

Analysis of *PaMYBA1* did not reveal differential expression of the gene between blush, red and black cherry fruit collected on the same day, corresponding to 78 DAFB for 'Lapins'. Therefore, flavonoid accumulation was studied in green, colouring and ripe fruit from blush, red and black fruited sweet cherry varieties to obtain a picture of flavonoid pathway activity and metabolic flux. The average TSS of ripe fruit samples were 20.2°Brix for 'Ranier', 17.6°Brix for 'Lapins' and 25.6°Brix for 'Sam' (data not shown). In the sweet cherry industry these TSS values are considered to be within the range of commercial ripeness (San Martino *et al.*, 2008), which can vary between cultivars. Flavonoid concentrations of fruit from the three different stages were measured by HPLC, quantified against standard curves as described in Chapter 2 and identified based on both retention time and anticipated abundance in sweet cherry. Flavonoid concentrations in the ripe fruit of all three sweet cherry varieties tested were within the range of previous studies (Gao and Mazza, 1995; Kim *et al.*, 2005; Harnly *et al.*, 2006; Serrano *et al.*, 2009). The quantity per fruit of the individual flavonoid classes are presented here to account for the expansion of fruit during development.

The accumulation of anthocyanins in 'Ranier', 'Lapins' and 'Sam' fruit is shown in Figure 5.3a. No anthocyanins were detected in the green fruit of any of the three sweet cherry varieties. Anthocyanins were also barely detectable in 'Ranier' and 'Lapins' fruit that was beginning to display red skin pigmentation (colouring fruit). However, anthocyanins were present in ripe fruit (86-93 DAFB) of 'Ranier' and 'Lapins', with 61.5µg and almost 4mg of anthocyanin per fruit, respectively. In comparison, the level of anthocyanins in 'Sam' ripe fruit reached 18mg per fruit, over four times more than in 'Lapins', explaining the intensely coloured skin and flesh of this variety. Cyanidin 3-rutinoside was the predominant anthocyanin in all three varieties, accounting for ~85% of the total anthocyanins in 'Lapins' and 'Sam', and 100% in 'Ranier' (Figure 5.4). Interestingly, 'Lapins' fruit seemed to preferentially make peonidin rutinoside, accounting for 10% of anthocyanins while 'Sam' fruit contained 10% cyanidin glucoside. These findings are in agreement with Mozetic *et al.* (2004), who reported that 85-92% and 5-16% of total cherry anthocyanins are cyanidin 3-rutinoside and cyanidin 3-glucoside, respectively. A small amount (less than 2%) of peonidin glucoside was also found in 'Lapins' and 'Sam' fruit.

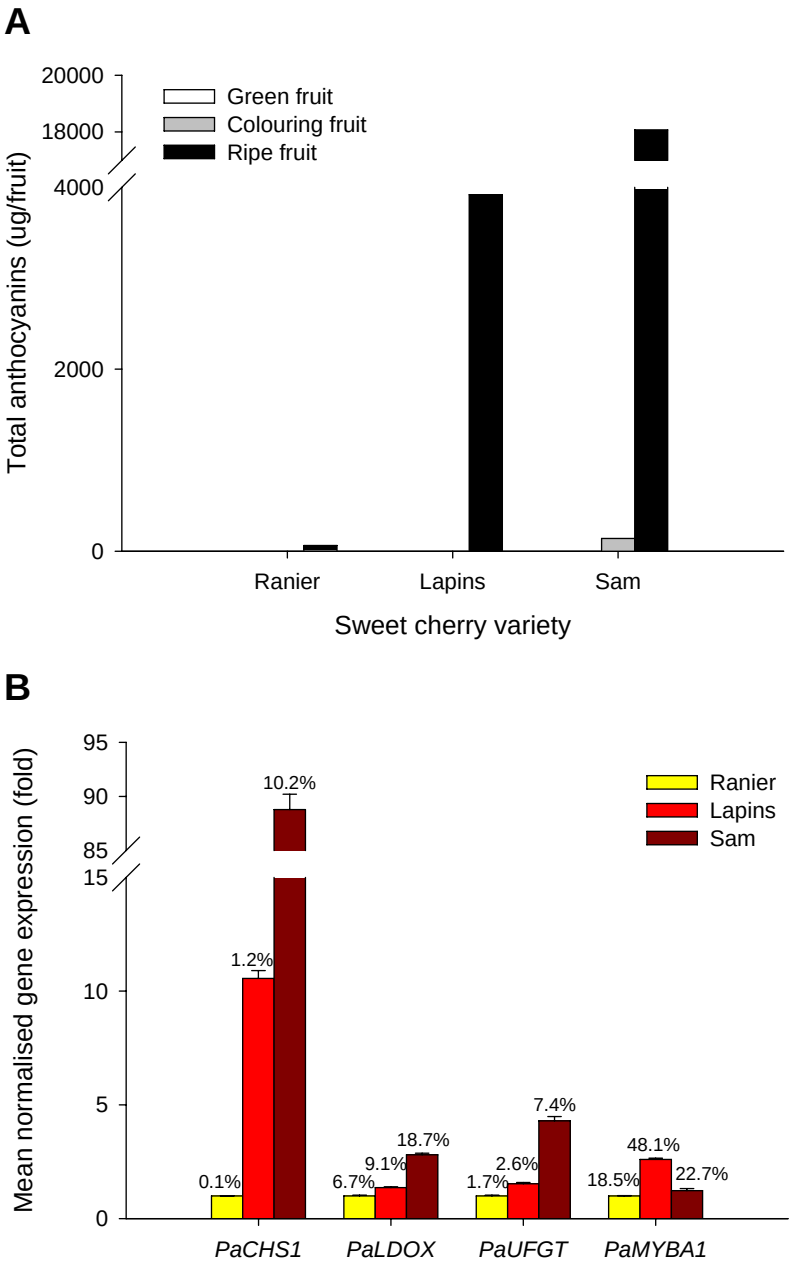


Figure 5.3 – Comparison of anthocyanin accumulation and gene expression in sweet cherry varieties

A) Concentrations per fruit of total anthocyanins in the cultivars ‘Ranier’, ‘Lapins’ and ‘Sam’ at three developmental stages, determined by HPLC on a single, homogenous sample. Within each broad developmental stage, fruit collected from each variety were not necessarily of equal maturity. B) Expression of anthocyanin-related genes in colouring whole fruit minus seed (56-65 DAFB) was determined by real time PCR. Mean gene expression of technical triplicates (± SEM) was normalised with *ubiquitin*, and is plotted relative to the expression in ‘Ranier’ for each gene. Expression of the gene compared with its maximum expression during ‘Lapins’ development is shown as a percentage above the columns.

Cultivar-specific differences in flavonol concentration, like anthocyanins, only became apparent in ripe fruit. 'Ranier', 'Lapins' and 'Sam' fruits possessed similar levels of total flavonols in green and colouring fruit (Figure 5.5a). 'Ranier' and 'Lapins' fruits produced a relatively small amount of flavonols between the onset of fruit colouring and ripening, representing 48% and 15% increases, respectively. On the other hand, 'Sam' fruit tripled the amount of total flavonols present in this time, with ripe fruit containing 712µg per fruit, compared to 286µg and 304µg per fruit in 'Ranier' and 'Lapins' respectively. Total flavonols found within all of the sweet cherry varieties included rutinosides of quercetin and kaempferol, as well as quercetin glucoside (data not shown).

Similar to the differences seen for anthocyanins and flavonols, the level of tannin in the ripe fruit of 'Sam' was 3-4 times higher than that of 'Ranier' and 'Lapins'. Between the green and ripe stages of fruit development, tannin levels declined in 'Ranier' and 'Lapins' from 6.5 to 3.6mg per fruit and from 4.5 to 2.9mg per fruit, respectively (Figure 5.6a). This is consistent with the decreasing tannin concentration measured in 'Lapins' cherries during fruit development (Chapter 4), which could be due in part to reduced extractability of complexed tannins (Downey *et al.*, 2003a). In 'Sam' fruit on the other hand, tannin synthesis continued during development, as indicated by an increase from 10 to 12mg of total tannins per fruit. Within all three of the varieties analysed, over 85% of the terminal subunits of the tannin polymers were epicatechin. The mean degree of polymerisation (mDP) was very low, with an average of only 1.5 subunits per tannin polymer in all fruit throughout development, with the exception of 'Sam' which displayed a mDP of 2 in ripe fruit (data not shown).

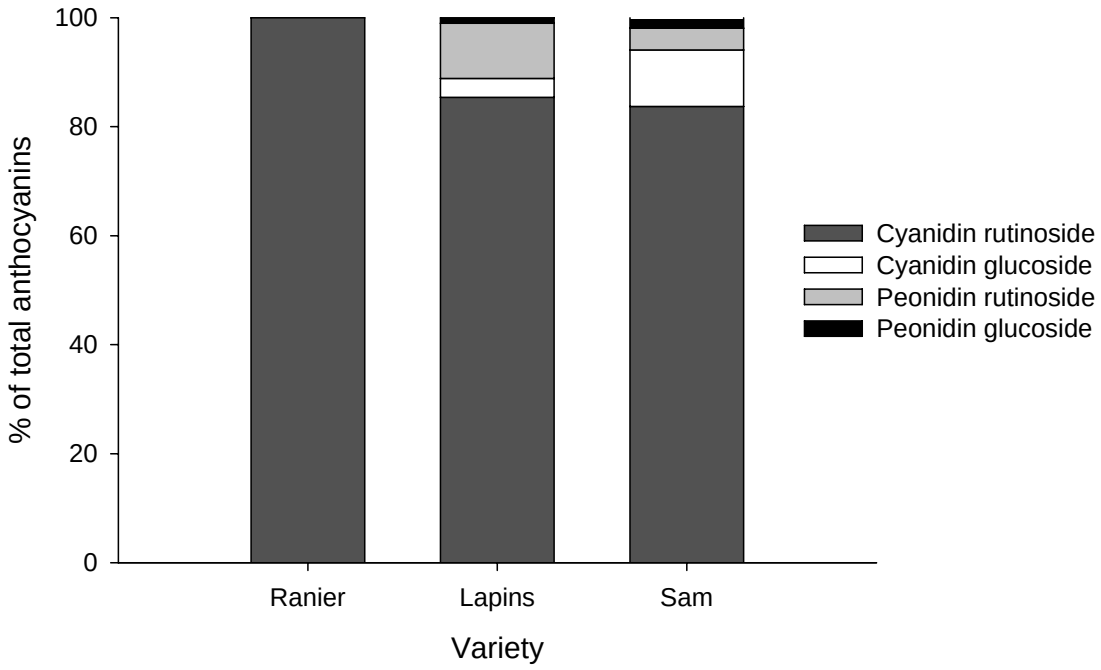


Figure 5.4 – Anthocyanin composition in ripe sweet cherry fruits

Concentrations of the major anthocyanins present in the ripe fruit of ‘Ranier’, ‘Lapins’, and ‘Sam’ sweet cherry fruit were determined by HPLC on a single, homogenous sample. Anthocyanins were identified by comparison with known standards. Anthocyanins are shown as a percentage of the total anthocyanins present within the sample.

Figure 5.5 – Comparison of flavonol accumulation and gene expression in sweet cherry varieties

A) Concentrations per fruit of total flavonols in the cultivars ‘Ranier’, ‘Lapins’ and ‘Sam’ at three developmental stages, determined by HPLC on a single, homogenous sample. Within each broad developmental stage, fruit collected from each variety were not necessarily of equal maturity. B) Expression of flavonol-related genes in colouring whole fruit minus seed (56-65 DAFB) was determined by real time PCR. Mean gene expression of technical triplicates (± SEM) was normalised with *ubiquitin*, and is plotted relative to the expression in ‘Ranier’ for each gene. Expression of the gene compared with its maximum expression during ‘Lapins’ development is shown as a percentage above the columns.

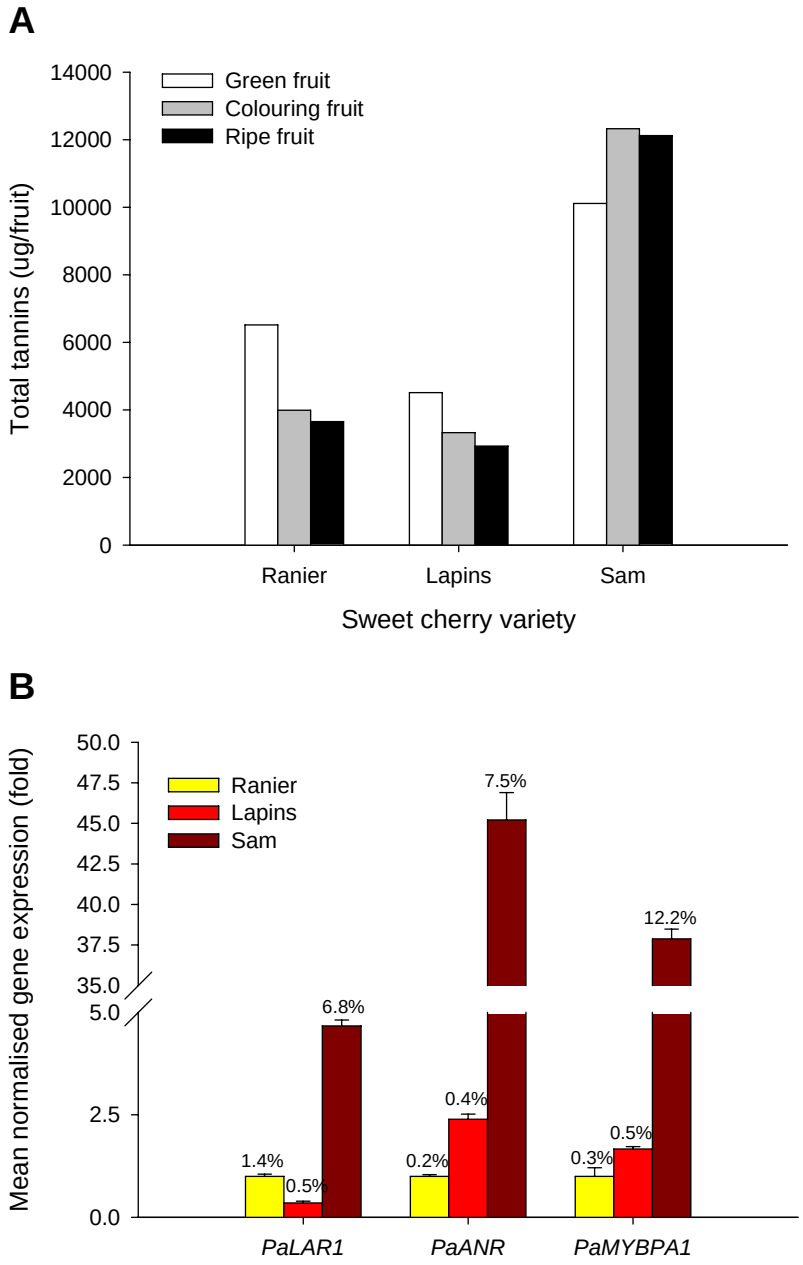


Figure 5.6 – Comparison of tannin accumulation and gene expression in sweet cherry varieties

A) Concentrations per fruit of total flavonols in the cultivars ‘Ranier’, ‘Lapins’ and ‘Sam’ at three developmental stages, determined by HPLC on a single, homogenous sample. Within each broad developmental stage, fruit collected from each variety were not necessarily of equal maturity. B) Expression of tannin-related genes in colouring whole fruit minus seed (56-65 DAFB) was determined by real time PCR. Mean gene expression of technical triplicates (± SEM) was normalised with *ubiquitin*, and is plotted relative to the expression in ‘Ranier’ for each gene. Expression of the gene compared with its maximum expression during ‘Lapins’ development is shown as a percentage above the columns.

5.2.3 Expression levels of some flavonoid genes are cultivar dependent

Expression of the genes responsible for flavonoid synthesis was studied to further understand the variable flavonoid levels and skin/flesh colours displayed by different sweet cherry cultivars. The abundance of flavonoid gene transcripts was determined by real time PCR for colouring fruit of 'Ranier', 'Lapins' and 'Sam' cultivars (same sample as in Figures 5.3a, 5.5a, 5.6a, grey bars). In our analysis of this gene expression data, we have related each value to the maximum expression of that gene during 'Lapins' fruit development (Chapter 4, referred to as 'Lapins' maximum developmental expression or LDE_{max}), shown as a percentage above the columns in Figures 5.3b, 5.5b and 5.6b. As discussed in Chapter 4, the expression of the flavonoid biosynthetic genes is quite low at this developmental stage (56-65 DAFB). Consequently, notable differences in gene expression at this time might suggest significant differences in transcript levels at earlier or later stages of development, depending on the flavonoid being produced. Once again, absolute comparisons between the three cultivars cannot be made due to their different flowering and harvest dates. The expression levels of *PaCHS2* and *PaLAR2* are not presented here as transcript abundance was below the detection threshold.

'Sam' colouring fruit was found to express a number of flavonoid pathway genes at a higher level than 'Ranier' and 'Lapins', consistent with the greater production anthocyanins, flavonols and tannins in this cultivar as fruit continue to develop. The transcripts for *PaCHS1*, which correlates with anthocyanin synthesis, and *PaFLS*, and *PaANR*, representing the flavonol and tannin branches of the flavonoid pathway respectively, were between 45- and 90-fold more abundant in 'Sam' than in 'Ranier' fruit, and over 20-fold higher in 'Sam' when compared to 'Lapins' fruit. In contrast, the varietal differences in the expression of *PaLDOX* and *PaUFGT* do not seem as pronounced, with their transcripts only 2-3 fold more prevalent in 'Sam' than in 'Lapins' colouring fruit (Figure 5.3b). However, these small differences become more relevant when taken in the context of developmental stage. At this point in fruit development, the co-ordinate induction of anthocyanin-related gene expression is just beginning (Figure 4.3). Therefore, in 'Lapins' colouring fruit, *PaLDOX* and *PaUFGT* transcript levels are just 9.1% and 2.6%, respectively, of the level they will reach in ripe fruit (LDE_{max}). In contrast, the expression of these genes in 'Sam' colouring fruit is already 18.2% and 7.4% of LDE_{max}. If transcript abundance remains consistently higher, these small increases in *PaLDOX* and *PaUFGT* expression at the onset of fruit colouring could translate into large varietal differences later in fruit development. Such differential gene expression may be amplified by the enhanced availability of substrate for these anthocyanin biosynthetic

enzymes due to the abundance of *PaCHS1* in colouring 'Sam' fruit. Conversely, expression of the tannin biosynthetic gene *PaLAR1* in colouring 'Sam' fruit remained at 6.8% of LDE_{max} whilst in 'Lapins' it had declined to just 0.5%, suggesting an increased ability of 'Sam' fruit to synthesise tannins late in fruit development.

The expression levels of the *PaMYBA1* gene within 'Ranier', 'Lapins' and 'Sam' colouring fruit did not display the same flavonoid-linked variation seen for *PaCHS1*, *PaFLS* and *PaANR*. Minimal variation between cultivars was detected for the expression of *PaMYBA1* and another flavonoid regulatory gene *PaMYBF1*, with between 2- and 3-fold differences in transcript abundance (Figures 5.3b and 5.5b). On the other hand, the abundance of the transcript for the tannin regulator *PaMYBPA1* was considerably higher in colouring 'Sam' fruit, similar to the pattern displayed by *PaCHS1*, *PaFLS* and *PaANR*. At this point in development, *PaMYBPA1* expression was almost 40-fold and 20-fold higher in 'Sam' fruit than in 'Ranier' and 'Lapins' respectively (Figure 5.6b). It appears that some genes, for example *PaLAR1* and *PaMYBF1*, are expressed at a higher level in 'Ranier' fruit than in 'Lapins' fruit. However, these are small differences in the abundance of genes that are expressed at low levels throughout development. Considering that developmental stage cannot be synchronised to compare fruit at exactly the same maturity level, these discrepancies may not be significant.

5.3 DISCUSSION

It is well documented that flavonoid biosynthesis is transcriptionally regulated (Mol *et al.*, 1998; Winkel-Shirley, 2002b). A number of defects within genes encoding regulatory proteins have been reported to produce plant products with altered anthocyanin levels, as we will discuss. Therefore, it seems possible that the colour variations seen amongst sweet cherry cultivars are attributable to the anthocyanin regulator *PaMYBA1*. We have investigated this possibility by studying *PaMYBA1* and the flavonoid pathway in sweet cherry fruits with yellow, blush, red and black skin. Whilst variation in maturity stage between cultivars is difficult to account for, care has been taken to draw general trends rather than conclude that small differences in gene expression and flavonoid accumulation are significant.

5.3.1 *PaMYBA1* expression is necessary for anthocyanin production

We have shown that *PaMYBA1* is a gene that controls colour in sweet cherry fruit. The 'Yellow Glass' *PaMYBA1* gene isolated from genomic DNA encodes a protein that is almost identical to the PaMYBA1 predicted protein of the anthocyanin-producing varieties 'Ranier',

'Lapins' and 'Sam'. However, this gene was not expressed in the skin and flesh of the sweet cherry cultivar 'Yellow Glass', corresponding with the absence of anthocyanin. Similarly, apple alleles have also been described that encode functional MYB transcription factors but are deficient in regulating anthocyanin synthesis (Takos *et al.*, 2006a; Espley *et al.*, 2007).

Investigation of the lack of *PaMYBA1* expression in 'Yellow Glass' fruit revealed a transcription factor binding site in the first intron of the *PaMYBA1* from this variety that is not present in this position for the other varieties. We suggest that the binding of this C/EBP could interfere with elongation of *PaMYBA1* transcripts, thus preventing initiation of anthocyanin biosynthesis through trans-activation of flavonoid genes. In the future, this could be tested by the removal of the inserted binding site to see if the gene could then be transcribed, using transgenic apples to test this hypothesis. Alternatively, the absence of the 'Yellow Glass' *PaMYBA1* transcript may be due to structural abnormalities in the promoter of *PaMYBA1* that prevent assembly of the transcriptional apparatus. In grapes, a retrotransposon insertion in the *VvMYBA1* promoter inhibits its transcription (Kobayashi *et al.*, 2004), and, together with mutations in *MYBA2*, contributes to the inability of white grapes to produce anthocyanin (Walker *et al.*, 2007). Another possibility for why *PaMYBA1* is not expressed in 'Yellow Glass' fruit could be a non-functional mutation in a positive regulator of *PaMYBA1*. Transcription factors that regulate the expression of *MYB* genes have been described for maize (Suzuki *et al.*, 1997) and petunia (de Vetten *et al.*, 1997), as will be discussed in Section 6.3.7.

5.3.2 More intense colouration in cherries correlates with higher flavonoid pathway activity

The flavonoid pathway generally functions at a much higher level in the intensely pigmented 'Sam' sweet cherry fruit, compared with red and blush-skinned cherries. Whilst flavonol levels remained relatively steady and tannin levels declined in developing 'Ranier' and 'Lapins' fruit, the synthesis of these compounds continued right throughout the development of 'Sam' fruit. In apples and grapes, flavonol and tannin accumulation in fruit is generally completed when ripening starts (Bogs *et al.*, 2005; Takos *et al.*, 2006c), although 'Shiraz' grapes reportedly continue to accumulate flavonols throughout the later stages of fruit development (berry ripening) (Downey *et al.*, 2003b). However, like 'Sam' fruit, the American cranberry with its very high phenolic content displays increases in flavonol and tannin accumulation during fruit ripening (Vvedenskaya and Vorsa, 2004). In ripe fruit, flavonoids accumulated to higher levels in 'Sam' than in 'Ranier' or 'Lapins'. 'Sam' fruit

contained at least four times more anthocyanins than in the other varieties, consistent with its deeper skin and flesh colour. In addition, 'Sam' ripe fruit contained double the amount of total flavonols and at least triple the amount of tannins than were found in the ripe fruit of blush or red varieties. Most of the tannin subunits present were epicatechin rather than catechin, which is consistent with the elevated levels of *PaANR* but not *PaLAR1* in 'Sam' fruit. The surprisingly low degree of polymerisation (1.5-2 subunits per molecule) is in contrast to the 10-49mers found in grapes and the 7.5-16mers in apple, depending on developmental stage (Bogs *et al.*, 2005; Takos *et al.*, 2006c), and suggests that activity of the enzyme required for transport of tannin precursors to the vacuole, or the enzyme/s that catalyses the polymerisation reaction in sweet cherry fruit is insufficient for the production of longer tannin molecules.

Consistent with the increased accumulation of flavonoids, the transcripts of the majority of flavonoid biosynthetic genes were also more abundant in colouring 'Sam' fruit than that of the other varieties tested. The co-ordinated up-regulation of each branch of the flavonoid pathway, particularly *PaCHS1, PaFLS* and *PaANR*, indicates that the high levels of flavonol and tannin accumulation are not merely the result of increased flavonoid pathway flux, as is the case for the Arabidopsis *banyuls* mutants lacking ANR activity that accumulate greater levels of anthocyanins than wildtype plants (Devic *et al.*, 1999). This co-ordinate expression also suggests that the genes are under transcriptional control. However, neither *PaMYBA1* nor *PaMYBF1* seem to be involved in the up-regulation as their expression was not increased in colouring 'Sam' fruit relative to 'Ranier' or 'Lapins'. Although expression at this single time point alone is insufficient to draw a firm conclusion, the comparable expression of *PaMYBA1* across the three varieties later in fruit development (Figure 5.1) supports this hypothesis.

The higher expression of multiple flavonoid pathway genes detected in 'Sam' fruit could be co-ordinated in part by *PaMYBPA1*, as the 20- and 40-fold abundance of the gene in 'Sam' over 'Lapins' and 'Ranier', respectively, reflects the varietal expression differences of *PaCHS1, PaFLS* and *PaANR*. An additional factor, presumably the as yet unidentified sweet cherry flavonol MYB transcription factor/s, must regulate the increased transcription of *PaFLS* in 'Sam', as PaMYBPA1 was unable to activate the *VvFLS* promoter in the transient assay (Chapter 4). We also found that the promoter of the anthocyanin gene *MdUFGT* was trans-activated by PaMYBPA1, which also regulates a broad set of flavonoid genes in the assay. It is therefore possible that the highly expressed *PaMYBPA1* is also responsible for the modest comparative increases in *PaLDOX* and *PaUFGT* expression seen in 'Sam' fruit versus

other cultivars, although perhaps PaMYBPA1 has a lower binding affinity for these promoters than for those that are highly activated, like *PaCHS1* and *PaANR*. Slightly enhanced expression of the anthocyanin genes, combined with greater substrate availability from the highly expressed early biosynthetic genes, could subsequently result in greater accumulation of anthocyanin in ripe 'Sam' fruit compared with that of 'Lapins'.

There are a number of factors that could contribute to the greater transcript abundance of *PaMYBPA1*, and potentially other regulators, in dark sweet cherry fruit. Steady state abundance of an mRNA is determined by the balance between transcription and decay. In terms of transcription, the promoter of 'Sam' *PaMYBPA1* might be stronger, containing more *cis*-acting elements than the promoters of 'Ranier' and 'Lapins'. Another possibility is that PaMYBPA1 is capable of auto-regulation, like the apple MYB10 which is responsible for the increased anthocyanin content of cultivars of *Malus* x *domestica*, *Malus* x *purpurea*, *Malus pumila*, *Malus sieversii* and *Malus marjorensis* displaying red flesh and foliage (Espley *et al.*, 2009). This apple MYB10 has been shown to transcriptionally activate its own promoter by binding to minisatellite repeat motifs in the upstream regulatory region, in a dose-dependent manner. Promoter studies could therefore help to elucidate the mechanism behind the enhanced flavonoid pathway activity seen in black sweet cherry fruit. On the other hand, mRNA decay could play a role in the differential transcript abundance displayed by the sweet cherry cultivars analysed here. The *PaMYBPA1* gene transcribed from the 'Sam' genome could be more stable and therefore less readily degraded than the 'Ranier' and 'Lapins' transcripts. Such differential decay has previously been shown for wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*), in which the effect of light on *phytochrome A* mRNA stability was found to be cultivar-specific (Plotnikov *et al.*, 2000)

5.3.3 Anthocyanin-specific genes are not responsible for reduced anthocyanin accumulation in blush fruit

The accumulation of flavonoids in mutants that are defective in the next step of flavonoid synthesis has helped to define the flavonoid pathway and its regulation as reviewed by Dooner *et al.* (1991), Shirley (1996) and Mol *et al.* (1998). In a similar way, silencing of flavonoid genes in transgenic plants can induce a shift towards the accumulation of flavonoid precursors (Griesser *et al.*, 2008; Rommens *et al.*, 2008; Szankowski *et al.*, 2009). Analysis of flavonoid concentrations in developing 'Ranier' and 'Lapins' fruit revealed similar levels of total tannins and flavonols, with little variation in their composition. The comparable flavonol and tannin levels correspond to similar levels of *PaFLS*, *PaANR* and *PaMYBPA1* expression

in 'Ranier' and 'Lapins' fruit. This indicates that the reduced pigmentation of the blush fruit is not due to a blockage in the pathway caused by a mutation in a structural gene, and is more likely to be the result of differences in transcriptional regulation.

As predicted by the colour of the fruit, ripe 'Ranier' cherries were found to contain much less anthocyanin than those of 'Lapins'. Whilst the red-fleshed cherry varieties produced cyanidin and peonidin derivatives, only cyanidin rutinoside was detected in 'Ranier' cherries. The presence of the flavonols kaempferol and quercetin shows that 'Ranier' must possess functional *F3H* and *F3'H* genes, but the conversion of the dihydroflavonol substrates to anthocyanins appears to be impaired. It is, however, possible that peonidin derivative are present in 'Ranier' fruit, but in a concentration below the limits detectable in this analysis. Despite the variation in anthocyanin levels between 'Ranier' and 'Lapins', both varieties displayed similar expression of *PaLDOX*, *PaUFGT* and *PaMYBA1* in whole fruit. Thus colour in blush sweet cherries is regulated quite differently to white grapes, where the lack of anthocyanin is the result of very low expression levels of a functional *UFGT* in comparison to that in red grape berries (Boss *et al.*, 1996b; Kobayashi *et al.*, 2001). The blush skin colour of cherry is more similar to that described for the skin of the closely related white peach (Tsuda *et al.*, 2004). Both of these colour mutants have the capacity for anthocyanin production, with functional *LDOX* and *UFGT* genes expressed at similar levels to darker skinned fruit. The prevention of anthocyanin synthesis in blush sweet cherry, and similarly in white peach, could be caused by a number of factors, including the presence of a negative regulator of anthocyanin synthesis, or the absence of a positive regulatory co-factor, such as a bHLH or WD40 repeat protein.

5.3.4 The reaction catalysed by PaCHS1 is a key regulatory step in anthocyanin synthesis

Flavonoid pathway studies in a number of plant species have revealed that different genes can be crucial to colour production in the various species. As previously mentioned, *UFGT* is not expressed in white grapes (Boss *et al.*, 1996b; Kobayashi *et al.*, 2001). In non-red apples, *UFGT* is hardly detectable in ripe fruit (Honda *et al.*, 2002), except in skin sections of sunlight exposed bagged fruit that accumulate anthocyanin (Kondo *et al.*, 2002b). For strawberry, *DFR* is the important gene controlling colour, displaying a tight correlation with anthocyanin accumulation in developing fruit (Moyano *et al.*, 1998; Li *et al.*, 2001). *DFR* also determines anthocyanin synthesis in white peach, along with *CHS*, as expression of both genes is only detectable in the occasional patches of red skin (Tsuda *et al.*, 2004). In sweet

cherry fruit, we have discovered that the major molecular difference between the skin of red and blush fruit is the expression of *PaCHS1*. The *PaCHS1* transcript, which was shown to correlate with anthocyanin accumulation in Chapter 4, is over 10-fold more abundant in 'Lapins' than in 'Ranier' colouring fruit. This difference in *PaCHS1* expression could result from either differential expression of a regulator of *PaCHS1*, or from the differential ability of a regulator to bind to the *PaCHS1* promoter, due to differences in sequence. The equivalent amounts of total flavonols and tannins in 'Ranier' and 'Lapins' fruit indicate these compounds are synthesised via a different *CHS* gene, probably by *PaCHS2* as its expression correlates with flavonol and tannin accumulation in developing 'Lapins' flowers and very early fruit development (Chapter 4).

The sweet cherry varieties analysed in this chapter did not display differential *PaMYBA1* expression. The lack of correlation of *PaMYBA1* expression with tightly linked skin colour and *PaCHS1* abundance suggests that *PaMYBA1* is not responsible for the phenotypic colour differences between blush, red and dark sweet cherry fruit. It is possible that *PaMYBA1* does give rise to variable *PaCHS1* levels as a result of cultivar-specific post-transcriptional or post-translational modification. Comparison of PaMYBA1 protein levels between cultivars would confirm whether or not modifications occur. Phosphorylation of the C-terminus of MYB proteins can result in altered binding and transcriptional activation of target DNA (Moyano *et al.*, 1996; Morse *et al.*, 2009). The phosphorylation can affect activation of some target genes and not others, resulting in differential regulation. In theory, this might explain why the differently pigmented cherries express *PaCHS1* at different levels but expression of *PaUFGT*, which we have also shown to be activated by PaMYBA1, is comparable. However, a more likely scenario is that another gene besides *PaMYBA1* is controlling the blush phenotype, and this will be discussed in further detail in the next chapter.

5.4 CONCLUSION

Yellow fruit have long been seen as distinctly different to anthocyanin-containing fruit and, as such, have generally been excluded from breeding programs and, as a result, from genetic studies. Until now, the genetic reason for this distinction has not been known. This research has uncovered that *PaMYBA1* expression is the molecular difference between yellow sweet cherries and those displaying all other colour variations. The lack of anthocyanin in the pale yellow cherry 'Yellow Glass' is explained by the absence of the *PaMYBA1* transcript in the fruit of this cultivar. However, expression of this gene does not account for the varying anthocyanin levels displayed by blush, red and black sweet cherry fruit. We have

demonstrated that a marked up-regulation of most of the flavonoid pathway in ‘Sam’ fruit, independent of *PaMYBA1* and could be mediated by enhanced *PaMYBPA1* expression, is the reason for the more intense colouration of this cultivar. Due to the abundance of flavonoid compounds, ‘Sam’ fruit would therefore presumably be a greater source of antioxidants than ‘Ranier’ or ‘Lapins’, although this would depend on their bioavailability. Very few differences in flavonoid biosynthesis exist between blush ‘Ranier’ and red ‘Lapins’ fruit, besides differing *PaCHS1* expression that correlates with anthocyanin accumulation. Therefore, the blush characteristic of sweet cherry fruit does not appear to be controlled by PaMYBA1, and is probably the result of another factor that controls anthocyanin synthesis through regulation of *PaCHS1* transcription.

Chapter Six: Effect of light and genetic factors on synthesis of flavonoids in sweet cherry fruit

6. EFFECT OF LIGHT AND GENETIC FACTORS ON SYNTHESIS OF FLAVONOIDS IN SWEET CHERRY FRUIT

6.1 INTRODUCTION

In fruit, skin colour is an important trait as it can be used as an indicator of fruit maturity and quality. Fruit colour can be significantly affected by light. For instance, stimulation of colour in apple skin upon exposure of dark-grown fruit to sunlight, a practice known as 'fruit bagging', is a tool that can produce apples with even and intense skin colouration (Ju, 1998). In the same way, apple skin colour is often enhanced by the used of reflecting films on the orchard floor, which increase light intensity within the tree canopy (Ju *et al.*, 1999). Sunlight can affect the degree of colouration or blush seen in some sweet cherry cultivars, evident by a reduction or lack of colour in fruit shaded by tree limbs or canopies (Patten and Proebsting, 1986). Consequently, sweet cherry fruit may be amenable to enhancement of skin colour through orchard management practices or postharvest processing. However, published research on the effect of light on sweet cherry colour is very limited, and the molecular mechanism responsible for colour synthesis in different light conditions has not been investigated. Additionally, the genes encoding the heritable factors that determine skin and flesh colour of sweet cherry fruit have not been identified. A greater understanding of the influence of abiotic and varietal factors (as discussed in Section 5.1) on flavonoid synthesis may help to determine the genetic basis of fruit colour, and assist in the enhancement of fruit quality.

Through centuries of traditional breeding a number of sweet cherry varieties exist, displaying vast differences in skin and flesh colour. The colours of fruit skin and flesh and are closely linked (Schmidt, 1998). Dark varieties can range from red to mahogany to purple to almost black. Light cherries are often referred to as yellow or white cherries and usually exhibit a red blush on their skin, although their juice is colourless. Generally, a deep red coloured fruit is preferred by North American consumers, regardless of their ethnicity (Crisosto *et al.*, 2002). In contrast, Japan commonly cultivates blush varieties of sweet cherry such as 'Napoleon' and 'Sato Nishiki' (McCracken *et al.*, 1989), with Asian breeders favouring yellow-fleshed fruit displaying a bright pinkish-orange blush (Sansavini and Lugli, 2008). From breeding population studies, flesh colour has been found to be determined by a single gene (Section 1.2.2.1). Meanwhile, skin colour is influenced by two incomplete dominant genes that act epistatically (Section 1.2.2.2); a major factor (*A*) confers dark skin colour, and a minor factor

(*B*) can manifest itself as a blush in the absence of the major factor. Through segregation of colour traits in the progeny of various testcrosses, the genotypes of a number of popular sweet cherry cultivars have been described (see Section 1.2.2). Amongst those cultivars genotyped for skin colour are two varieties studied in this thesis; 'Sam' has been described as homozygous dominant for the *A* gene (*AA*), whilst 'Lapins' is a heterozygote (*Aa*, Schmidt, 1998).

Light is essential for numerous plant growth and development processes, including photosynthesis, determination of flowering time and germination. Light also plays an important role in the accumulation of flavonoids in plants. Transcriptional regulation of flavonoid biosynthetic genes in response to light has been observed in both model plants and fruit crops. Dark-adaptation of Arabidopsis plants strongly reduced the expression of four flavonoid structural genes (Hartmann *et al.*, 2005). Exposure of bagged apple fruit to sunlight initiated anthocyanin accumulation in skin by inducing transcription of flavonoid structural genes, with the exception of those genes required for tannin synthesis (Takos *et al.*, 2006b). During grape berry development, exclusion of light resulted in altered flavonol levels in the fruit that correlated with changes in *VvFLS* gene expression, but did not affect anthocyanin accumulation (Downey *et al.*, 2004).

Early investigations into the effect of light on sweet cherry fruit have focused on the impact on fruit size and sugar content (Ryugo and Intrieri, 1972; Patten and Proebsting, 1986). However, the role of light in the development of skin colour has since been an important subject of investigation. Fruit colour seems to be influenced by light to differing degrees in a cultivar-specific manner for both sweet cherry and plum (Kataoka *et al.*, 2005; Murray *et al.*, 2005). The anthocyanin levels in sweet cherry skin have been demonstrated to be enhanced post-harvest in UV irradiated light-skinned fruit, such as the cultivar 'Satohnishiki' (Arakawa, 1993; Taira *et al.*, 1995; Kataoka *et al.*, 1996). Taira *et al*. (1995) also demonstrated that UV light can stimulate colour in blush sweet cherry fruit that is still attached to the tree. In a recent study, Mulubagal *et al*. (2009) showed that blush-skinned 'Ranier' fruit failed to produce anthocyanin when grown on trees protected from radiation outside of the visible light spectrum by a plastic tunnel structure. On the other hand, red- and black-fruited sweet cherries are able to develop colour independently of sunlight (Marshall, 1954). Sweet cherry fruit also appears more light sensitive in the later rather than earlier stages of development (Ryugo and Intrieri, 1972; Kataoka *et al.*, 2005). Nonetheless, there remains a lack of understanding about the effect of light on the molecular regulation of flavonoid synthesis.

To further understand the genetic difference in blush-skinned sweet cherries and the effect that sunlight has on the transcription of flavonoid pathway genes, we applied the practice of 'fruit bagging' to sweet cherry cultivars exhibiting a range of skin colours. The elimination of sunlight during fruit development and subsequent re-exposure at fruit ripening was found to initiate light-dependent skin colouration in only the blush cultivar 'Ranier'. As anthocyanin is produced solely in the skin of this cultivar, the abundance of flavonoid gene transcripts was compared in skin and flesh tissue of blush- and red-fruited cultivars.

6.2 RESULTS

6.2.1 Preliminary studies to exclude sunlight from sweet cherry fruit using "fruit bagging"

Blush-skinned 'Ranier' and red-skinned 'Lapins' sweet cherry fruit were subjected to a 'fruit bagging' experiment, conducted in at Lenswood, South Australia, to examine the regulation of the flavonoid pathway by sunlight. Bags placed on the fruit early in development excluded sunlight from the fruit but allowed air flow and drainage. Bags were removed from the fruit just before commercial harvest, and dark-grown ('unbagged') and naturally-grown ('control') fruit were sampled 0, 1, 2, 4 and 7 days after bag removal (DABR). Warm, moist weather conditions during the harvest period somewhat hindered the success of the experiment. Nonetheless, some physiological measurements and flavonoid concentrations were able to be reliably determined from fruit sampled before this inclement weather occurred. In this experiment, fruit bagging appeared to cause a mild elevation in total soluble sugar (TSS) content. The average TSS (° Brix) of 'Lapins' and 'Ranier' unbagged fruit at 1 DABR was 5.9 ± 2.0 (1SD) % and 22.5 ± 0.5% lower than that of control fruit, respectively. After seven days of light exposure, 'Lapins' and 'Ranier' unbagged fruit had a 9.5 ± 2.0% and 14.0 ± 6.9% higher average TSS than control fruit. Dark-treatment of sweet cherry fruit by fruit bagging did not seem to notably alter the size or firmness of the fruit in comparison with control fruit grown under normal light conditions (data not shown). This observation is consistent with findings in grape that shading of bunches does not alter berry weight (Downey *et al.*, 2004; Matus *et al.*, 2009).

6.2.2 Effect of light treatment on sweet cherry flavonoid accumulation

The effect of growth in the absence of light on sweet cherry flavonoid composition was quantified by HPLC. Table 6.1 shows flavonoid concentrations in control and bagged fruit on the day of bag removal (0 DABR) for 'Lapins' and 'Ranier'. In the red-skinned variety

'Lapins' the absence of light results in a doubling of the total anthocyanins and flavonols, from 225 and 21.8µg g^{-1} of tissue respectively, in comparison to control fruit. On the other hand, the blush-skinned variety 'Ranier' produced less total flavonols per gram of tissue when grown in the dark, with no anthocyanin detected in the bagged fruit. The main anthocyanin detected was cyanidin rutinoside, accounting for approximately 90% of total anthocyanins in both 'Lapins' and 'Ranier', with the exception of 'Ranier' bagged fruit in which anthocyanins were not detected. Other anthocyanins present included glucosides of pelargonidin, cyanidin and peonidin. The total hydroxycinnamate concentrations of both varieties were lower in bagged fruit than in control fruit. Both catechin (2,3-trans-flavan-3-ol) and epicatechin (2,3-cis-flavan-3-ol) tannin initiating units were present in all samples. Whilst total tannin concentration was not significantly different ($P < 0.05$) between 'Ranier' treatments, fruit bagging increased the tannin concentration of 'Lapins' fruit by over 50% (604.5µg g^{-1} tissue compared to 391.3µg g^{-1} in control fruit).

6.2.3 Effect of light treatment on flavonoid gene expression

A more extensive 'fruit bagging' experiment was conducted in Summerland, British Columbia, to further examine the regulation of the flavonoid pathway by sunlight. 'Sam', 'Lapins', 'Ranier' and 'Yellow Glass' fruit display black, red, blush and yellow skin, respectively. Photographs of these sweet cherry cultivars, demonstrating skin colour, can be found in Figure 5.1 (Chapter 5). Bags were applied to and removed from the fruit of these cultivars as described for preliminary studies. During the course of the experiment, weather conditions were generally fine, with daily maximum temperatures around 30°C. However, 6 mm of rain and a maximum temperature of 22°C were recorded on 2 DABR (Figure 6.2).

Variety	Sample	Flavonoid concentrations (µg/g tissue)				
		Total anthocyanins	Total flavonols	Total hydroxycinnamates	Total tannins	MDP
Lapins	Control	225.0	21.8	536.3	391.3	2.1
	Bagged	525.0	40.6	303.6	604.5	2.0
Ranier	Control	6.1	26.5	673.8	585.0	2.5
	Bagged	0.0	15.3	566.5	571.9	2.2

Table 6.1 – Concentrations of flavonoid metabolites in control fruit and bagged fruit at 0 DABR[§]

Concentrations were determined by HPLC from skin + flesh. Fruit samples were from the preliminary fruit bagging experiment conducted at Lenswood, South Australia. [§] DABR, days after bag removal. MDP, mean degree of polymerisation.

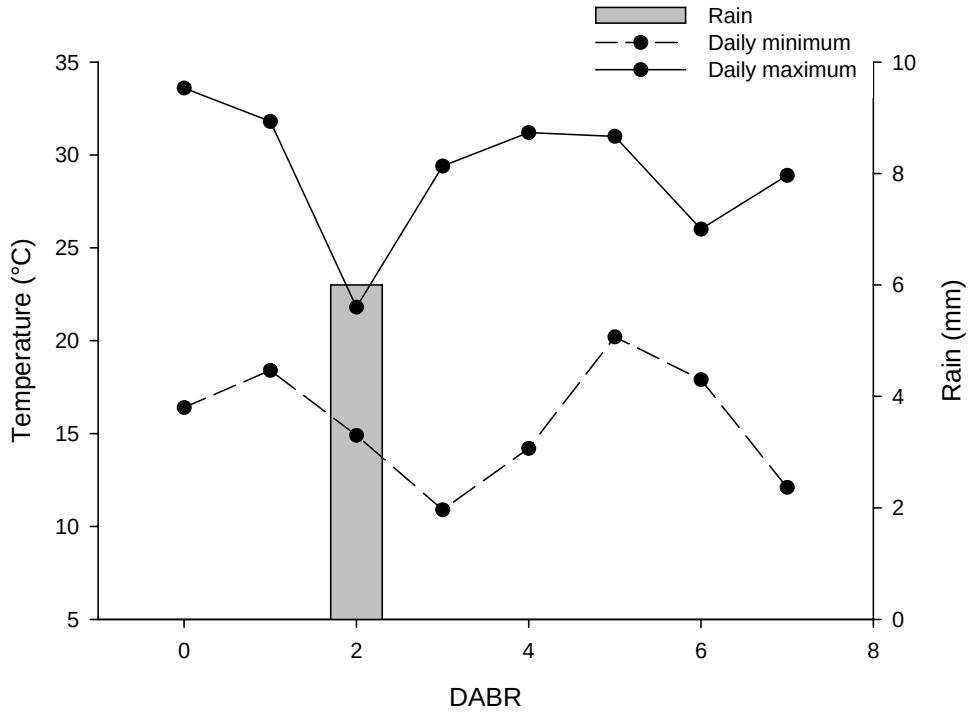


Figure 6.2 – Weather observations for Summerland, BC, during the fruit bagging experiment.

Sample collection began on July 21, 2008 (0 days after bag removal (DABR)) and continued for 7 days. Maximum (solid line) and minimum (dashed line) daily temperatures (degrees Celsius) were those reported for Summerland, and rainfall (mm) for nearby Penticton as no data was available for Summerland (National Climate Data and Information Archive, www.climate.weatheroffice.ec.gc.ca).

Light treatment of the sweet cherry varieties 'Lapins', 'Sam' and 'Yellow Glass' did not elicit a flavonoid response. Upon removal of bags from dark-grown sweet cherry fruit, there were no obvious skin colour differences from that of control fruit in these cultivars. Consistent with this observation, anthocyanin levels assayed in the three cultivars above were comparable in unbagged and control fruit throughout the experiment (data not shown). The expression of *PaCHS1*, a putative anthocyanin-related isoform (P. Wiersma (AAFC), personal communication) encoding the first step of the flavonoid pathway, was analysed in unbagged and control sweet cherry fruit by real time PCR. Figure 6.3 shows the abundance of the *PaCHS1* transcript relative to the 0 DABR treatments for each variety. Following four days of light exposure the expression of *PaCHS1* did not alter substantially in 'Yellow Glass', 'Lapins' or 'Sam' fruit (Figure 6.3a,b,d).

In contrast, the blush-skinned cultivar 'Ranier' displayed differences between light treatments. Dark-grown 'Ranier' fruit had yellow skin, whilst the skin of control fruit showed a red blush (Figure 6.4). Exposure of the unbagged 'Ranier' fruit to sunlight caused a gradual increase in red pigmentation in the fruit skin, which became evident 2 DABR. After seven days of light exposure, 'Ranier' skin displayed an even red colour although slightly less intense than that of control fruit. Total anthocyanin levels were assayed in the sweet cherry skin throughout the experiment to quantify the visible differences in pigmentation. 'Ranier' control fruit contained 100µg anthocyanin g^{-1} fresh tissue (Figure 6.4), falling within the reported range of 20 to 410µg g^{-1} fresh weight for light-coloured varieties (Gao and Mazza, 1995). 'Ranier' dark-grown fruit contained only 3.4µg anthocyanin g^{-1} of skin tissue at both the beginning and the end of the 7-day experiment. In agreement with the visual colour development, anthocyanin accumulated steadily in 'Ranier' unbagged fruit in response to light exposure, rising almost 10-fold to 29.6µg g^{-1} tissue. 'Ranier' was also the only cultivar tested that produced a notable light-induced change in gene expression, with a more than 40-fold increase in *PaCHS1* transcript abundance detected in fruit following 4 days of light exposure (Figure 6.3c).

6.2.3.1 Further investigation of the flavonoid pathway in light-treated blush fruit

In order to determine which branches of the flavonoid pathway might be stimulated in a light-dependent manner, 'Ranier' fruit was subjected to expression analysis of selected flavonoid genes (Figure 6.5). The expression of *PaCHS1*, a core pathway gene, was induced approximately 120-fold by 7 days of light exposure in the unbagged fruit (Figure 6.5a).

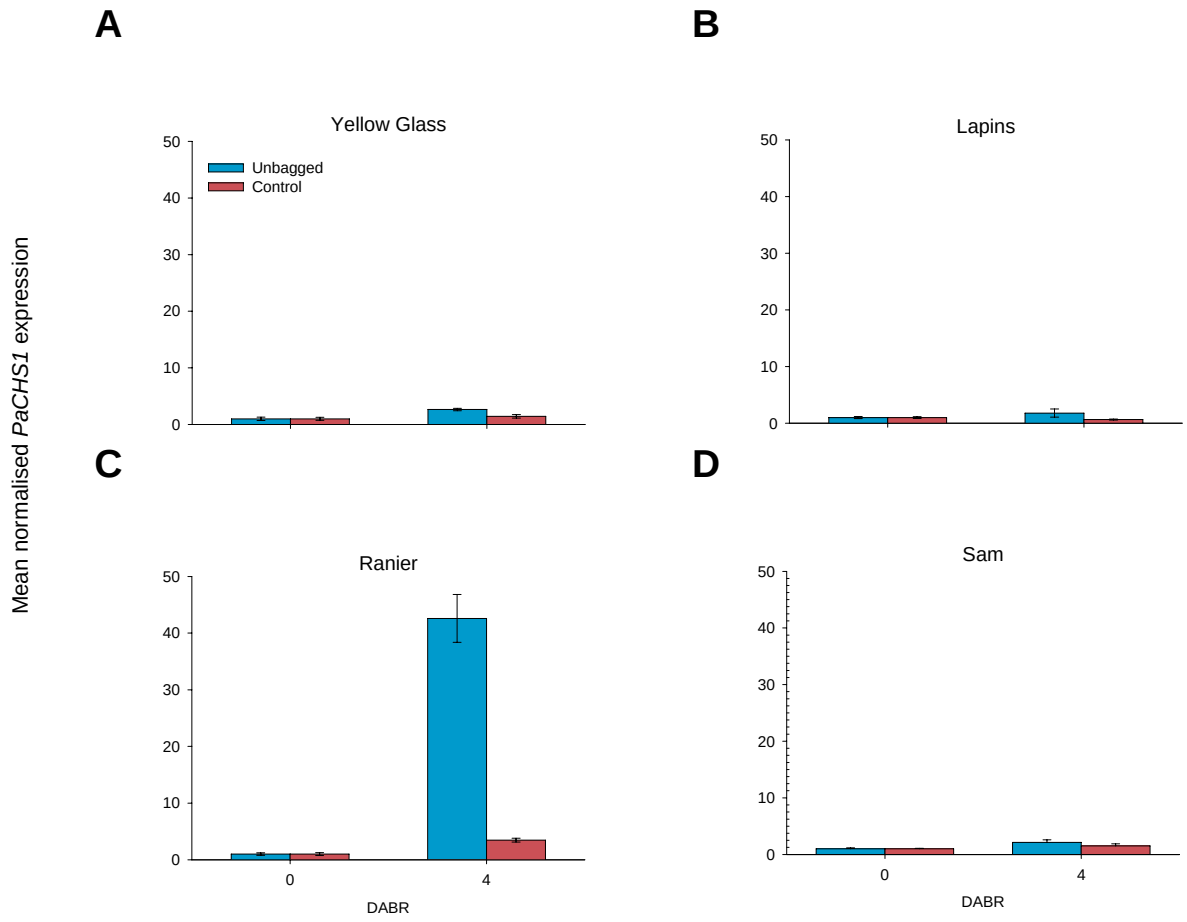


Figure 6.3 – Light exposure induces *PaCHS1* expression only in 'Ranier' fruit.

Mature sweet cherry fruit grown in the absence (unbagged) or presence (control) of light were harvested 0 and 4 days after bag removal (DABR). Transcript levels were determined by real time analysis and normalised with the *ubiquitin* housekeeping gene. Mean gene expression of biological triplicates (± 1SD) is plotted as a fold increase relative to the 0 DABR sample for each treatment. Gene expression was determined for four sweet cherry varieties, a) 'Yellow Glass', b) 'Lapins', c) 'Ranier' and d) 'Sam'.

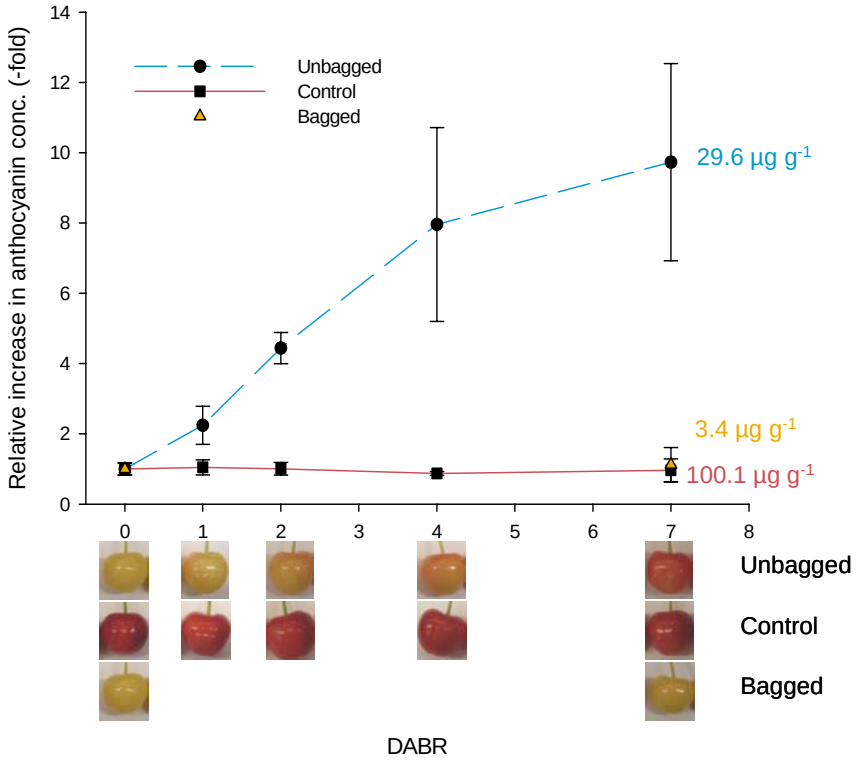


Figure 6.4 – Changes in anthocyanin concentration correlate with observations in 'Ranier' skin colour.

Foil-lined bags were placed on fruit of the blush variety 'Ranier' at 40 days after full bloom (DAFB) to exclude sunlight and (most) removed 78 DAFB (0 days after bag removal (DABR)). Unbagged fruit (dashed line), together with control fruit (grown under normal light conditions, solid line) from the same branch, were sampled on 0, 1, 2, 4, and 7 DABR. Bagged fruit (triangle) remained protected from light throughout the experiment and was sampled in triplicate 7 DABR as a baseline control. Anthocyanin concentration is expressed as a fold increase relative to the 0 DABR mean of the three replicates (3x n=3 ± 1SD) for each treatment, with final concentrations (µg g^{-1}) shown. Photographs of sweet cherry fruit representing each treatment group are shown below the graph, beneath the day on which they were sampled. Note photographs are not to scale.

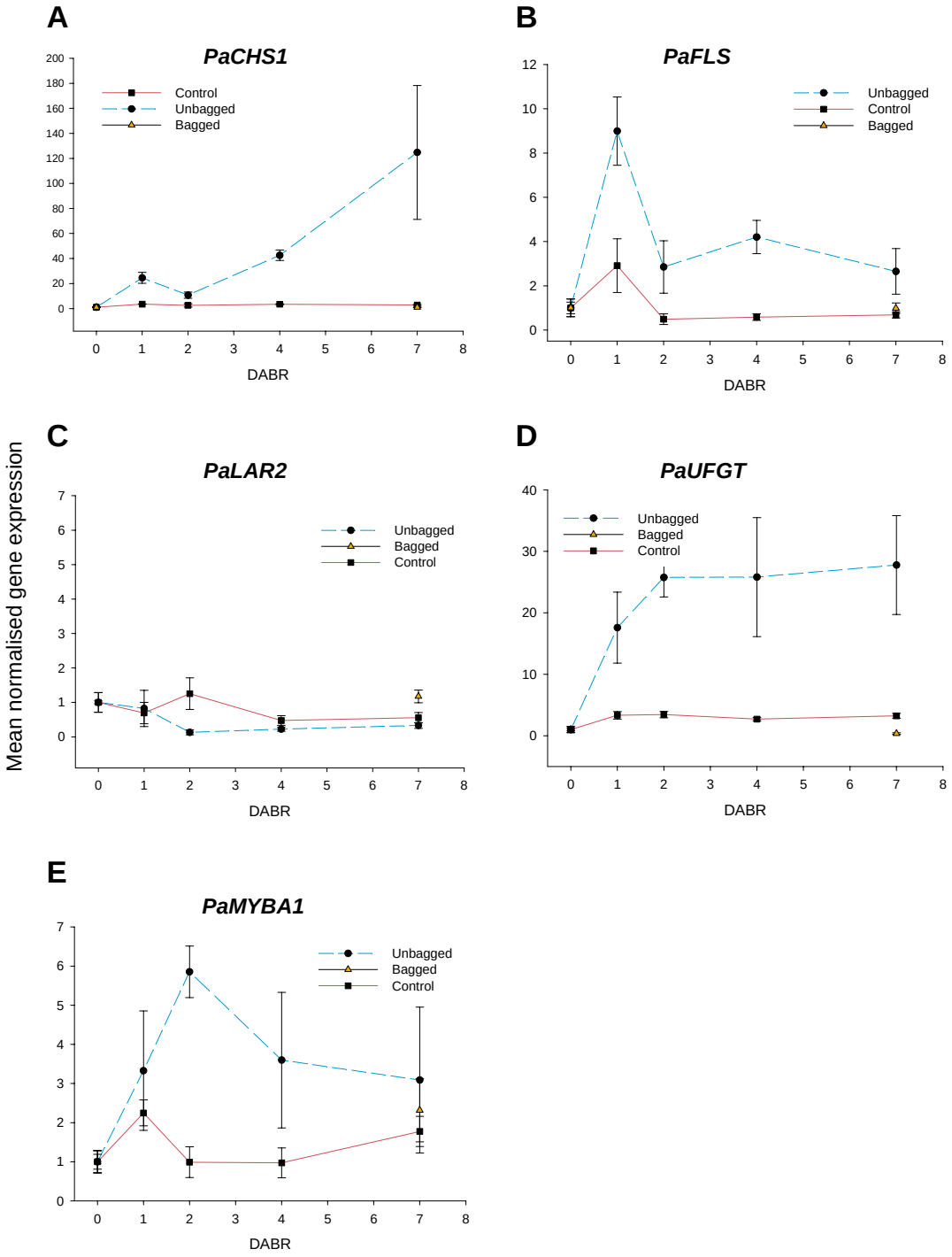


Figure 6.5 – Expression of flavonoid biosynthetic genes in 'Ranier' fruit in response to light exposure

Mature 'Ranier' sweet cherry fruit grown in the absence (unbagged) or presence (control) of light were harvested 0, 1, 2, 4 and 7 days after bag removal (DABR). Gene expression was determined by real time PCR for four genes representing different branches of the flavonoid pathway, a) the core pathway gene *PaCHS1*, b) *PaFLS* for flavonol synthesis, c) *PaLAR2* for tannin synthesis, d) *PaUFGT* for anthocyanin synthesis and e) the anthocyanin regulator *PaMYBA1*. Mean gene expression of biological triplicates (± 1SD) is plotted as a fold increase relative to the 0 DABR sample for each treatment, normalised with *ubiquitin*.

Expression of *PaCHS1* in control and bagged fruit remained constant during the experiment. The flavonol branch-specific gene *PaFLS* was up-regulated in unbagged fruit, with transcript levels 9-fold higher at 1 DABR than initial levels and then dropping to approximately 3-fold (Figure 6.5b). At this peak in gene expression, the *PaFLS* transcript was 4-fold more abundant in unbagged fruit than in control fruit. Expression levels of both *PaCHS1* and *PaFLS* dip at 2 DABR, corresponding to the cooler, wetter weather experienced that day. The expression of *PaLAR2* remained constant during the experiment and did not respond to light treatment (Figure 6.5c). In contrast, the anthocyanin synthesis gene *PaUFGT* displayed a strong light induction of transcript levels, rising approximately 25-fold by 2 DABR after which levels remained similar (Figure 6.5d).

6.2.4 Searching for the "blush gene"

The anthocyanin regulator *PaMYBA1* appears to play roles in the fruit colouration, or lack thereof, displayed by yellow-, red- and black-skinned sweet cherry varieties (see Chapters 4 and 5). However, the comparable expression of *PaMYBA1* in 'Ranier' and 'Lapins' fruit suggests that it is not the gene responsible for the reduced pigmentation in the skin of blush sweet cherry varieties. In an attempt to understand the genetic basis behind blush fruit, and identify the *B* gene (the epistatic skin colour factor that can result in a blush), the abundance of flavonoid gene transcripts were compared in skin (exocarp) and flesh (mesocarp) tissue of the blush cultivar 'Ranier' and the red cultivar 'Lapins' (Figure 6.6a,b). At this stage of development, fruit were beginning to ripen and colour development was not yet complete, as evident from the pink flesh of the 'Lapins' fruit (Figure 6.6b). As was the case in whole fruit (Figure 5.2), 'Ranier' and 'Lapins' displayed no notable differences in the expression of *PaUFGT* or *PaMYBA1* in skin tissue (Figure 6.6c), despite 'Lapins' containing a much higher concentration of anthocyanins. Within 'Ranier' flesh, the lack of *PaUFGT* expression was consistent with the absence of anthocyanins in this tissue. However, in the same tissue, the expression of *PaMYBA1* was still 48% of that in 'Ranier' skin. These results confirm that an additional factor must control production of anthocyanins in blush sweet cherry fruit.

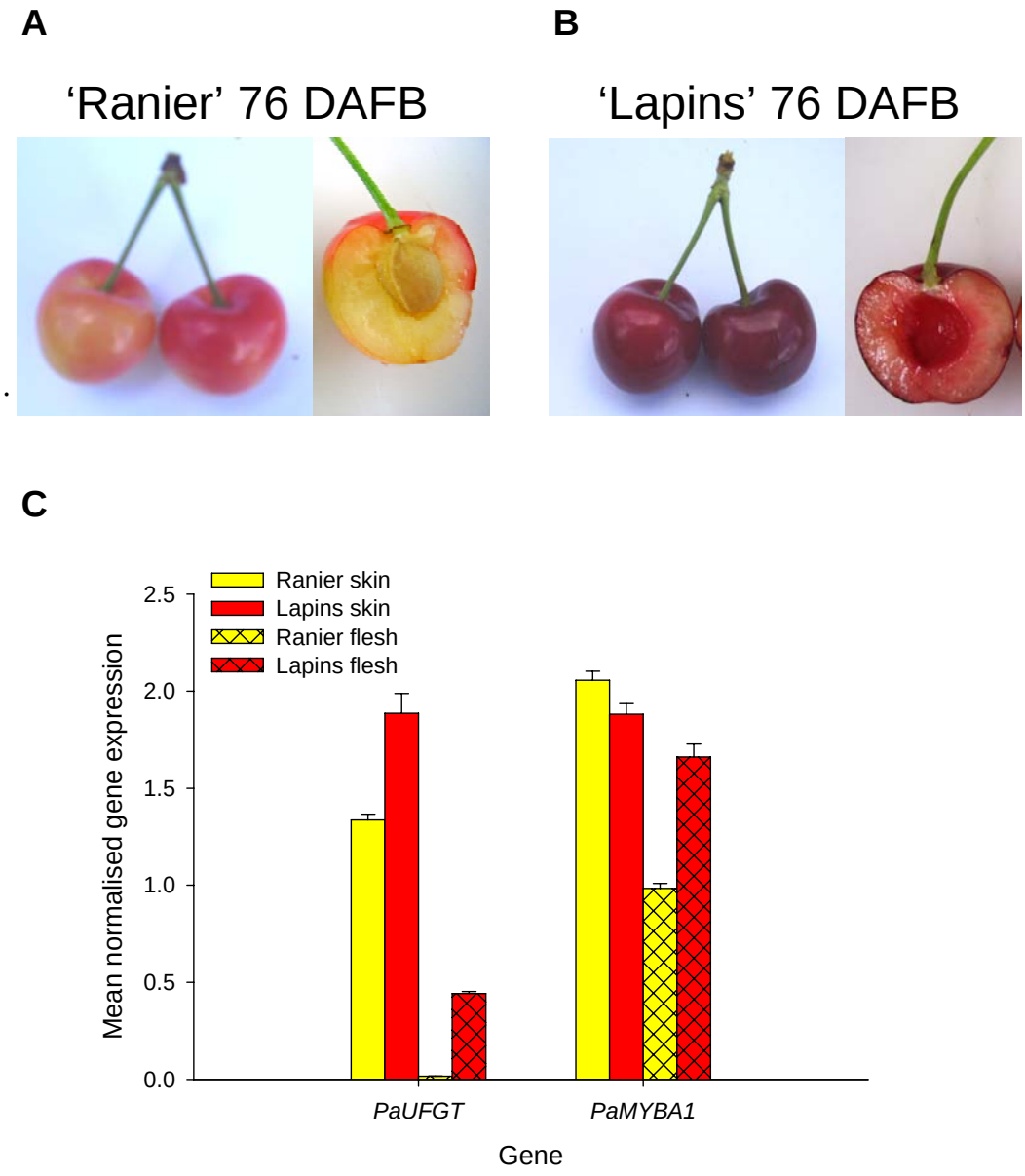


Figure 6.6 – Levels of *PaUFGT* and *PaMYBA1* transcripts in the skin of 'Ranier' and 'Lapins' sweet cherry fruit do not explain colour variations between these varieties

Photographs of A) 'Ranier' and B) 'Lapins' ripening (76 DAFB) fruit. Whole and halved fruit are shown to display skin and flesh colour differences. Photographs are not to scale. C) Expression of the *PaUFGT* and *PaMYBA1* genes in the skin or flesh of ripening fruit (76 DAFB) was determined by real time PCR. 'Ranier' fruit displays blush skin whilst 'Lapins' fruit is red-skinned. Mean gene expression of technical triplicates (± SEM) was normalised with *ubiquitin*.

A number of anthocyanin-related flavonoid biosynthetic genes were found to be expressed at significantly lower levels in 'Ranier' skin compared with 'Lapins' skin. The transcripts of two anthocyanin-associated *chalcone synthase* genes, *PaCHS1* and *PaCHS3*, as well as *PaDFR*, *PaF3'H* and *PaLDOX* were between 4- and over 20-fold more abundant in the colouring fruit skin of 'Lapins' than of 'Ranier' (Figure 6.7). No significant differences in expression of flavonol and tannin branch genes were detected between the two varieties. The co-ordinated expression of the anthocyanin pathway genes points to their transcriptional regulation, possibly by the *B* gene, or by another factor that influences either the expression of the *B* gene or the action of protein that it encodes.

One candidate for the blush gene is another putative anthocyanin regulator we isolated, *PaMYBA2*, which shares 91% amino acid identities with PaMYBA1 (see Chapter 3 for features of this sequence). The *PaMYBA2* transcript was barely detectable in 'Lapins' fruit during development, resulting in non-specific amplification with *PaMYBA2* primers. This was determined as the PCR product failed to melt at a single, discreet temperature (melt curve) and contained multiple DNA fragments when separated on an agarose gel. Expression of *PaMYBA2* was not induced by exposure of dark-grown fruit to light (data not shown). However, the *PaMYBA2* transcript was about 10-fold and 5-fold more abundant in skin and flesh from the colouring fruit of 'Ranier' than 'Lapins', respectively (Figure 6.8). Taken together, the diminished accumulation of anthocyanin, low expression of anthocyanin genes and abundance of *PaMYBA2* in 'Ranier' fruit indicate that PaMYBA2 might act as a negative regulator of anthocyanin synthesis.

Another putative anthocyanin MYB transcription factor, *PaMYBA3*, could also be the blush gene. The sequence of *PaMYBA3* is almost identical to *PaMYBA1*, with the exception of two nucleotide differences just before the end of the MYB DNA-binding domain, one of which is a deletion that shifts the reading frame and results in a truncated PaMYBA3 protein (Chapter 3). Thus PaMYBA3 should be able to bind to the same targets as PaMYBA1 as it contains almost the entire DNA-binding domain, but may not be able to initiate their transcription as the activation domain is absent. Both the *PaMYBA1* and *PaMYBA3* sequences were able to be amplified from genomic DNA and cDNA from all four sweet cherry cultivars utilised in this study, discounting the possibility that *PaMYBA3* is a pseudogene. However, the proportion of *PaMYBA1:PaMYBA3* transcripts differed amongst the cultivars, as depicted by the chromotograms of the cDNA products in Figure 6.9.

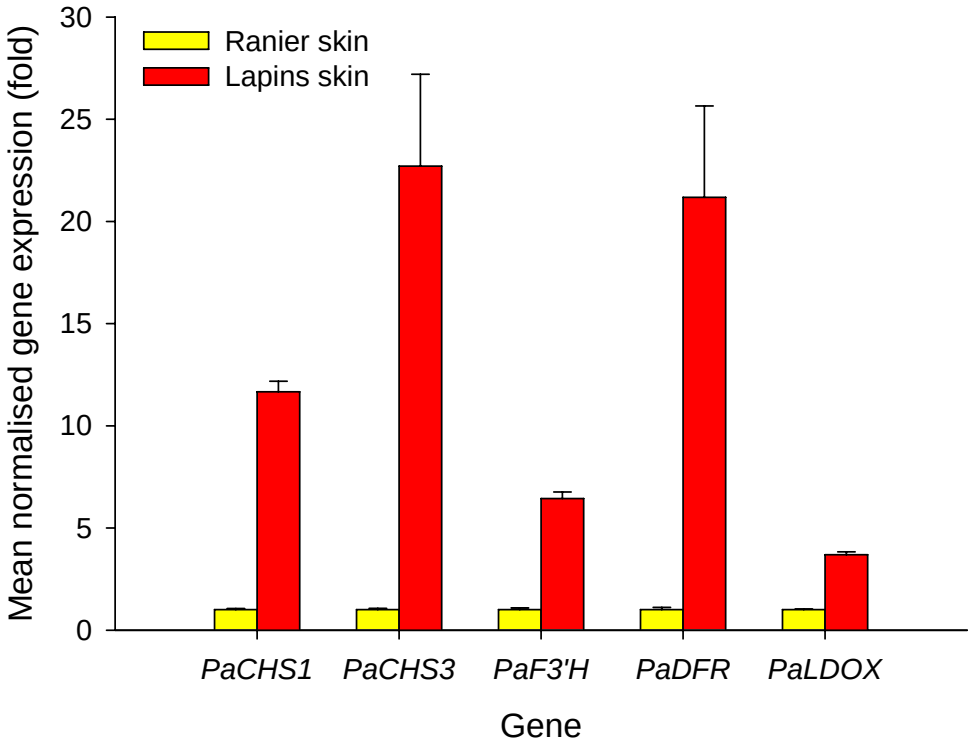


Figure 6.7 – Anthocyanin-related flavonoid genes are expressed at a lower level in the skin of blush fruit compared with red sweet cherry fruit

Expression of the *PaCHS1*, *PaCHS3*, *PaF3'H*, *PaDFR* and *PaLDOX* genes in the skin of ripening fruit (76 DAFB) was determined by real time PCR. 'Ranier' fruit displays blush skin whilst 'Lapins' fruit is red-skinned. Mean gene expression of technical triplicates (± SEM) was normalised with *ubiquitin*, and is plotted relative to the expression in 'Ranier' for each gene.

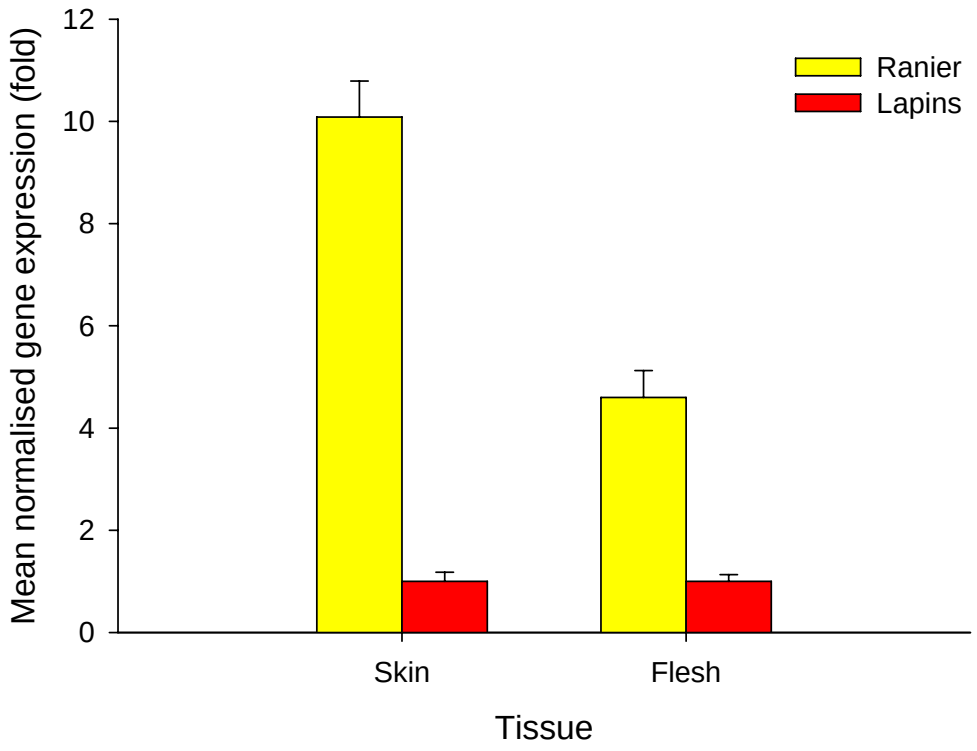


Figure 6.8 – The R2R3-MYB transcription factor *PaMYBA2* is preferentially expressed in the skin of blush sweet cherry fruit

Expression of the *PaMYBA2* gene in the skin and flesh of ripening fruit (76 DAFB) was determined by real time PCR. 'Ranier' fruit displays blush skin whilst 'Lapins' fruit is red-skinned. Mean gene expression of technical triplicates (± SEM) was normalised with *ubiquitin*, and is plotted relative to the expression in 'Lapins' for each gene.

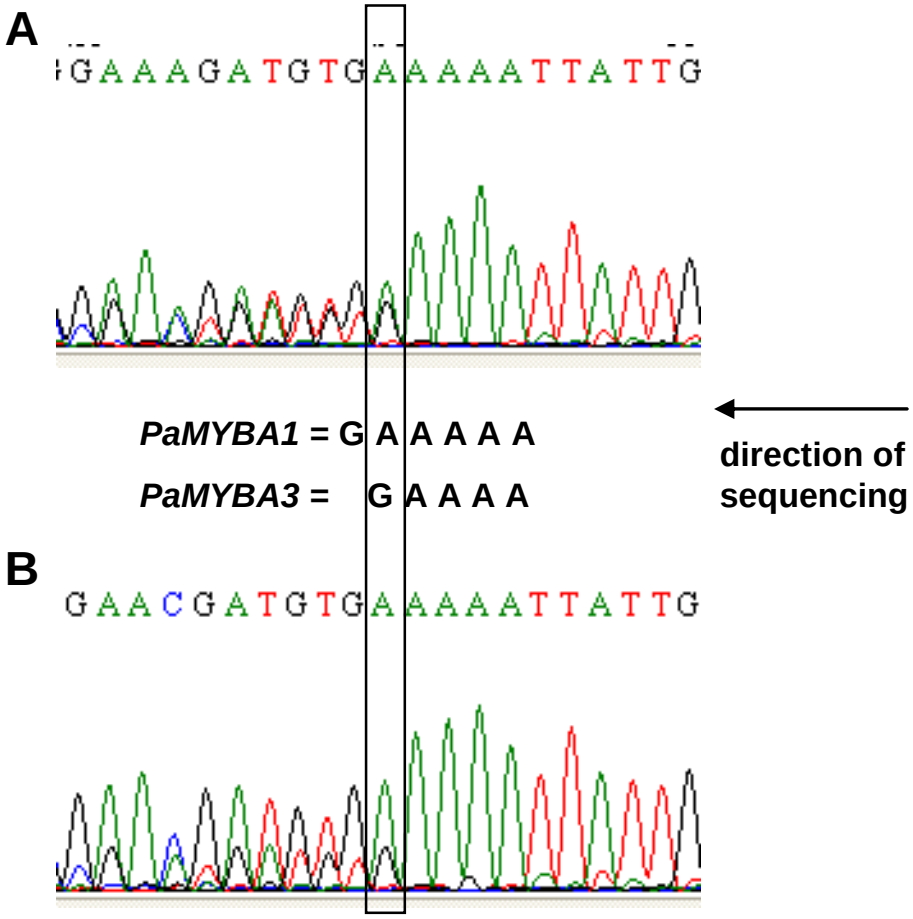


Figure 6.9 – The abundance of *PaMYBA3* in relation to *PaMYBA1* in cDNA varies amongst sweet cherry cultivars

A short section of *PaMYBA1/A3* including the polymorphic region was amplified from 'Yellow Glass', 'Ranier', 'Lapins' and 'Sam' colouring fruit cDNA by standard PCR. The PCR products were sequenced with a reverse primer (3' to 5') and the chromatograms were viewed with BioEdit Version 7.0.9.0 (Hall, 1999). The frameshift (see Figure 3.2) is boxed, where the green A signal represents *PaMYBA1* and the black G signal represents *PaMYBA3*. A) Section of the trace for 'Lapins' *PaMYBA1/A3* representing the relatively equal *PaMYBA1:A3* ratio (signal heights) seen in the traces from 'Yellow Glass', 'Ranier' and 'Lapins'. B) Section of the 'Sam' *PaMYBA1/A3* trace.

While colouring fruit cDNA from 'Yellow Glass', 'Ranier' and 'Lapins' seemed to contain approximately equal amounts of the two genes (as inferred from the very similar overlapping signal heights), the trace generated from 'Sam' suggested that *PaMYBA1* is prevalent over *PaMYBA3* in this variety. Unfortunately, however, the expression of *PaMYBA3* was unable to be quantified: expression could not be differentiated from that of *PaMYBA1* due to the almost identical sequences they display. Conversely, it is possible that this lack of distinction is resulting in the overestimation of *PaMYBA1* expression.

6.3 DISCUSSION

6.3.1 Light-induced changes to anthocyanin levels are specific to blush fruit

The colour enhancement of blush sweet cherry fruit by light has been well documented, but the response of red-fruited varieties to light has scarcely been published. The exclusion of sunlight from growing fruit of the red 'Lapins' and black 'Sam' sweet cherry varieties resulted in fruit that appeared no different to control fruit grown under normal light conditions, indicating anthocyanin synthesis in these varieties occurs independent of light. This finding is in agreement with reports that black 'Seneca' cherries (Kataoka *et al.*, 2005), as well as red 'Shiraz' grapes (Downey *et al.*, 2004) and highly coloured 'Red Delicious' apples (Robinson *et al.*, 1983) produce anthocyanins regardless of light intensity. In contrast, Patten and Proebsting (1986) reported that the artificial shading of red 'Bing' cherries reduced anthocyanin accumulation in these fruits. Varietal differences in susceptibility to light may explain why our findings and those of Kataoka *et al*. appear to contradict the light-dependent reduction in anthocyanin reported in 'Bing' cherries. Such variations in light response between cultivars have also been observed for apple (Arakawa, 1988) and grape cultivars (Jeong *et al.*, 2004). Bagged 'Lapins' fruit (0 DABR, initial Lenswood experiment) actually contained double the amount of anthocyanins and flavonols, and tannins, compared with control fruit. As flavonoid accumulation and expression of biosynthetic genes can be influenced by fluctuations in temperature in potato (*Solanum tuberosum*) and *Ginkgo biloba* (Korobczak *et al.*, 2005; Xu *et al.*, 2008) and water deficits in grapes (Castellarin *et al.*, 2007), the increased flavonoid levels may be caused by the heat and water stress experienced within the treatment bags.

Light treatment was unable to elicit the production of colour in the skin of the yellow-skinned variety 'Yellow Glass'. This observation is consistent with our finding that lack of *PaMYBA1* transcription is responsible for the absence of anthocyanin in 'Yellow Glass' fruit (Chapter 5). Thus, the genetic basis for non-coloured fruit is similar to white grapes, in which light cannot

stimulate anthocyanin production because functional anthocyanin regulators (VvMYBA proteins) are not present (Kobayashi *et al.*, 2004; Walker *et al.*, 2007).

In contrast with these varieties, and in agreement with the literature discussed below, we found skin colour in blush sweet cherry fruit to be light-dependent. Under normal growth conditions 'Ranier' fruit possesses yellow skin and flesh, with a generally non-uniform red blush on its skin. Depending on the cultivar and the degree of colouration, blush fruit generally contains 20 – 410µg g^{-1} for blush fruit (Gao and Mazza, 1995). The 'Ranier' control fruit utilised in this study, just prior to commercial harvest, contained approximately 100µg of anthocyanin g^{-1} fresh weight, falling within this reported range.

Recently, Mulabagal *et al*. (2009) have demonstrated that 'Ranier' fruit from trees grown beneath a physical barrier to natural radiation (other than light) contained no anthocyanin. We also found that 'Ranier' fruit within bagged bunches displayed no red pigmentation and contained no detectable anthocyanin. As anticipated, given the UV stimulation of colour in naturally grown blush cherry fruit both pre- and post-harvest (Arakawa, 1993; Taira *et al.*, 1995; Kataoka *et al.*, 1996; Kataoka *et al.*, 2005), re-introduction of bagged 'Ranier' fruit to sunlight resulted in the accumulation of anthocyanin. During seven days of light exposure, visible colour changes correlated with anthocyanin accumulation. However, the anthocyanin concentration in unbagged fruit only reached about one third of the concentration in control fruit, consistent with observations in apple over a seven day period (Wang *et al.*, 2000; Takos *et al.*, 2006b). It is possible that had the experiment been extended, unbagged 'Ranier' fruit would have continued to accumulate anthocyanin, as unbagged red Chinese sand pears (*Pyrus pyrifolia* Nakai) take at least ten days for anthocyanin to return and plateau at the level of control fruit (Huang *et al.*, 2009).

6.3.2 Other light-related physiological changes

In addition to changes in anthocyanin levels, light treatment of 'Ranier' fruit also resulted in the production of flavonols. Total flavonol concentrations in control fruit remained at 26.5µg g^{-1} during the experiment, while in unbagged fruit the concentration increased from 15.3 to 22.1µg g^{-1} (only 0 DABR data shown), but remained below levels in control fruit. The total tannin content of 'Ranier' fruit subjected to light treatment was, however, no different to that of control fruit. These findings are consistent with the reported decreases in flavonol concentrations of bagged apple fruit (Takos *et al.*, 2006b) and shaded grape berries (Downey

et al., 2004; Cortell and Kennedy, 2006), whilst in the same fruit tannin levels remain unaffected (Downey *et al.*, 2004; Takos *et al.*, 2006b).

A number of conflicting reports exist that describe the effect of light on fruit sweetness. Amongst fruit species, exclusion of light through bagging has been found to both increase (Noro *et al.*, 1989) and decrease (Wang *et al.*, 2002) the TSS content of apples, but had no effect on TSS in bagged fruits of sweet cherry (Taira *et al.*, 1995), plum (Zhang *et al.*, 2007) or pear (Huang *et al.*, 2009). The elevated TSS concentration of re-exposed bagged fruit when compared with control fruit is in contradiction to observations by Taira et al. (1995) that irradiation had no effect on the TSS of 'Satohnishiki' and 'Napoleon' sweet cherry fruit. These inconsistencies seem likely to be associated with differential responses to stresses such as high temperatures and humidity and low air-flow imposed by different light treatments.

6.3.3 Sunlight initiates the co-ordinate up-regulation of flavonol and anthocyanin biosynthetic genes

The expression of *PaCHS1*, a putative anthocyanin-related isoform of the first gene of the pathway, was analysed in 0 DABR and 4 DABR control and bagged fruit to determine whether any of the sweet cherry varieties were responding to light at a molecular level. In response to the introduction of light to dark-grown fruit, the unchanged fruit colour corresponded to relatively unchanged *PaCHS1* expression in yellow, red and black fruit. On the other hand, correlation of light-induced *PaCHS1* expression with both the development of skin pigmentation and accumulation of anthocyanins in skin tissue confirm that blush sweet cherries respond to light by up-regulation of the flavonoid pathway. This cultivar-specific light-induction of anthocyanin synthesis is similar to grape, in which diminished light intensity results in reduced anthocyanin levels and expression of anthocyanin biosynthetic genes in some cultivars (Jeong *et al.*, 2004; Matus *et al.*, 2009), but has no effect on others (Downey *et al.*, 2004). The major difference between anthocyanin synthesis in these two species is that light-sensitive grapes maintain some level of anthocyanin synthesis, whereas light-sensitive sweet cherries are unable to produce anthocyanin in the absence of light.

Co-ordinate transcriptional regulation of the pathway has been previously demonstrated, as discussed earlier and reviewed by Davies and Schwinn (2003). Quantitative PCR was utilised to investigate the effect of light on the expression of multiple flavonoid pathway genes, leading to accumulation of flavonoid metabolites in 'Ranier' fruit. For the duration of the seven day experiment, no significant changes were seen for any of the genes analysed in

'Ranier' control or the bagged fruit baseline control. There was also no substantial change in the expression of *PaLAR2* upon exposure of bagged fruit to sunlight. Although the role of *LAR2* genes has not been determined, the closely related *LAR1* genes are involved in tannin synthesis. The constant level of tannin metabolites despite light treatment indicates that tannin synthesis in sweet cherry is a light-independent process, as has been concluded in both apples (Awad *et al.*, 2001; Takos *et al.*, 2006b) and grapes (Downey *et al.*, 2004). Following bag removal and subsequent exposure of fruit to sunlight, the expression of the shared pathway gene *PaCHS1*, the flavonol-specific gene *PaFLS* and the anthocyanin biosynthetic gene *PaUFGT* were all induced. Rapid induction of the transcripts from 0 to 1 DABR coincides with the requirement for the enzymes to synthesise flavonoids, resulting in the accumulation of anthocyanins and flavonols.

Together with the flavonoid metabolite levels, the real time PCR data suggests that in blush-skinned sweet cherries, the anthocyanin pathway is light-dependent and the flavonol pathway is light-inducible. Similar findings have been reported for many plants including Arabidopsis (Kubasek *et al.*, 1992; Hartmann *et al.*, 2005) and apple (Kondo *et al.*, 2002a; Takos *et al.*, 2006b), in which the increases in transcript levels correlate with the accumulation of anthocyanins and flavonols. Flavonol synthesis in grape berries is also affected by light via transcriptional activation of *CHS* and *FLS* genes {Downey, 2004 #2788; Matus, 2009 #2902; Czemmel, 2009 #2932.

6.3.4 Light-induced anthocyanin synthesis is regulated by PaMYBA1 in blush cherries

Dark-grown blush sweet cherry fruit displayed a rapid increase in *PaMYBA1* transcript abundance following light exposure. Given the demonstrated ability of PaMYBA1 to trans-activate *CHS* and *UFGT* promoters (Chapter 4), this suggests that in blush fruit, exposure to sunlight causes pigment production through trans-activation of anthocyanin biosynthetic genes. Expression of *PaMYBA1* was not induced by light in any of the other three cultivars studied; the response was solely observed in 'Ranier' fruit, where anthocyanin is produced only in skin. Therefore, to further understand flesh and skin colour and light-sensitivity differences between cultivars, expression of flavonoid structural and regulatory genes was analysed in skin and flesh tissues of 'Ranier' and 'Lapins' separately.

6.3.5 The flesh colour gene controls the expression of *PaUFGT*

In Chapter 5 we demonstrated comparable expression of the genes *PaUFGT* and *PaMYBA1* in whole fruit of 'Ranier' and 'Lapins'. Upon analysis of gene expression in skin and flesh

tissues separately, we found very similar levels of these genes in the skin of both varieties; however their expression in flesh differed. The absence of *PaUFGT* transcripts in 'Ranier' flesh indicate that this gene is the target for a positive regulatory "flesh colour gene" (*F*), which is defective in yellow flesh fruit. Although we are yet to determine the *F* gene, the expression of *PaMYBA1* in 'Ranier' flesh makes it highly unlikely that this is the flesh colour factor. Furthermore, this expression in flesh also rules out *PaMYBA1* as the minor skin colour factor, otherwise known as the blush gene (*B*), as it is anticipated this would display skin-specific expression.

6.3.6 PaMYBA1 is not the major skin colour determinant

Based on the gene expression analyses performed in this study and in Chapter 5, *PaMYBA1* cannot encode the major skin colour factor (*A*). Red skin colour is an incomplete dominant trait in sweet cherry (Fogle, 1958); however, *PaMYBA1* is expressed at a similar level in blush-, red- and black-skinned cultivars. Therefore, as we concluded in Chapter 5, a regulator other than *PaMYBA1* must be responsible for the lack of base colour pigmentation in 'Ranier' skin. The reduced abundance of the *PaCHS1*, *PaCHS3*, *PaDFR*, *PaF3'H* and *PaLDOX* transcripts in 'Ranier' skin compared with that of 'Lapins' points towards the transcriptional control of this anthocyanin gene set by the major skin colour factor. As red-skinned fruit develop colour in the dark, this major factor must be transcribed in the light and in the dark. The identity of this gene could be a regulatory co-factor, such as a bHLH or WD40 that is absent or non-functional in yellow- and blush-skinned fruits. Thus, determination of sweet cherry fruit colour might be similar to some varieties of morning glory (Park *et al.*, 2007), and petunia (Gerats *et al.*, 1985) in which bHLH proteins control differential flower colour as opposed to apple and grape where MYBs determine fruit colour (Section 1.4.2).

It is also possible that the major factor is a transcriptional repressor of anthocyanin biosynthetic genes. For example, in wild-type lettuce (*Lactuca sativa*) that accumulates anthocyanin, over-expression of the Arabidopsis transcriptional repressor *AtMYB60* was able to prevent the transcription of *DFR*, thereby inhibiting anthocyanin synthesis (Park *et al.*, 2008). The mechanisms by which transcriptional repressors might regulate anthocyanin synthesis in blush sweet cherry fruit will be discussed in Section 6.3.8.

6.3.7 A "blush gene" in fruit skin activates *PaMYBA1* in response to sunlight

Anthocyanin blush is known to develop on the skin of certain yellow-skinned sweet cherry cultivars in areas of light exposure (Marshall, 1954), an observation that is supported by our

findings. Using fruit bagging as a light treatment, we have demonstrated the light induction of anthocyanin biosynthetic genes by the anthocyanin regulator *PaMYBA1*, which is also light-induced. However, in Section 6.3.5 evidence that *PaMYBA1* is not the blush gene was presented. The induction of *PaMYBA1* only in 'Ranier' fruit, together with genetic evidence that blush is controlled by a separate gene to skin colour, lead us to believe that the light-responsive blush gene, active in 'Ranier' but not in 'Lapins' or 'Sam', is inducing the expression of *PaMYBA1* in our bagging experiments. Therefore, we propose that the blush gene encodes a transcription factor that acts upstream of *PaMYBA1*, and that it is probably part of the transcriptional cascade initiated by the perception of light by photoreceptors, as reviewed by Jiao *et al.* (2007). Transcription factors that regulate *MYB* levels have previously been described, and include VP1 (viviparous1) acting on *C1* in maize, and the WD40 protein AN11 upstream of *AN2* in petunia (de Vetten *et al.*, 1997; Suzuki *et al.*, 1997). The activation of *PaMYBA1* by the *B* gene would explain the differential response to light in blush versus red/dark cherries, despite the very similar sequences of *PaMYBA1* in these cultivars (Chapter 5). Alternatively, the light-induction of *PaMYBA1* specifically in the skin of 'Ranier' could result from light-responsive promoter elements, which are not present in the promoters of *PaMYBA1* genes of the red and black cultivars. In the future, investigation of proteins interacting with the *PaMYBA1* promoter, such as yeast one-hybrid analyses and electrophoretic mobility shift assays, could assist in elucidating the *B* gene.

Considering the hypothesis that *B* regulates PaMYBA1 in response to light, and given that *B* is thought to intensify skin colour in the presence of the major skin colour factor, *A*, there should theoretically be some degree of light-sensitivity in red fruit containing the blush gene. We did not observe any increase in anthocyanin concentration, nor *PaMYBA1* expression for either the red cultivar 'Lapins' or the black cultivar 'Sam'. This could mean that these cultivars do not possess dominant *B* alleles, and a more extensive search of red sweet cherry fruit may lead to the discovery of cultivars that are red when grown in dark conditions but deepen in colour upon re-exposure to light. Alternatively, *B* may be present in 'Lapins' and/or 'Sam' that enhances colour in light-treated fruit, but the already high anthocyanin concentrations and gene expression prevent any increases from being detected. It is also possible that 'Yellow Glass' possesses the *B* gene, but that a blush cannot result, as lack of *MYBA1* gene expression prevents the activation of anthocyanin biosynthetic genes (Chapter 5). Further research is required in this area, and will be discussed further in Chapter 7.

6.3.8 Roles for additional *PaMYBA* genes in anthocyanin regulation

The expression of *PaMYBA2* in yellow skin and flesh, combined with the lack of transcript detection in ripening 'Lapins' fruit indicate it is not a conventional activator of anthocyanin synthesis. In fact, the lack of significant accumulation of *PaMYBA2* transcripts in anthocyanin-accumulating tissues bears similarity to *AtMYBL2*, an Arabidopsis *R3-MYB* shown to negatively regulate anthocyanin synthesis via repression of *DFR* and *TT8* expression (Matsui *et al.*, 2008). *PaMYBA2* is generally expressed at very low levels and does not appear to contain characterised repression domains, such as the C1 motif required for effective repression of *AtC4H* by AtMYB4 (Jin *et al.*, 2000), the C2/EAR motif that can cause repression of anthocyanin synthesis in transgenic Arabidopsis plants when fused to AtPAP1 (Hiratsu *et al.*, 2003), or the recently reported R/KLFGV of AtMYBL2 (Ikeda and Ohme-Takagi, 2009) in its C-terminal region. Nonetheless, PaMYBA2 might indirectly repress anthocyanin synthesis through the sequestration of co-factors that normally interact with PaMYBA1, thereby preventing activation of anthocyanin biosynthetic genes.

In addition to PaMYBA2, the truncated PaMYBA3 might also act to prevent PaMYBA1-mediated activation of anthocyanin biosynthetic genes. The almost identical MYB domains of PaMYBA1 and PaMYBA3 suggest that these proteins would bind to the same target promoters, but PaMYBA3 is lacking the activation domain required for transcriptional initiation. Sequence similarities between these two genes prevent the detection of specific transcripts for either gene by quantitative PCR. Therefore, it is possible that a percentage of the *PaMYBA1* expression measured in 'Ranier' fruit is the result of *PaMYBA3* expression. Conversely, less competition for PaMYBA1 due to a lower level of *PaMYBA3* expression (Figure 6.9) might contribute to greater anthocyanin pathway activity and increased colour intensity in cultivars like 'Sam'. In the absence of gene expression data, the role of *PaMYBA3* can only be speculative. However, considering its expression in all of the tested cultivars *PaMYBA3* could contribute to the control of anthocyanin levels in ripening fruit, much like *FaMYB1*, which represses anthocyanin synthesis through reduced expression of flavonoid late biosynthetic genes when it is over-expressed in strawberry (Aharoni *et al.*, 2001).

Based on their preliminary expression profiles, the anthocyanin-like regulators *PaMYBA2* and *PaMYBA3* are unlikely to be critical determinants of sweet cherry fruit colour. However, they may play important roles in the combinatorial regulation of anthocyanin synthesis. More comprehensive gene expression analysis and resolution of the roles of these genes could

enhance knowledge of the complex control of anthocyanin biosynthesis in plants, and suggestions to this end will be made in Chapter 7.

6.4 CONCLUSION

Light-dependent skin colouration in sweet cherry seems to be specific to blush-skinned fruit. Thus, factors and practices such as orchard exposure, row orientation, plant spacing and pruning may enhance the skin colouration of such varieties by reducing shading in the canopy. The parallel light induction of the anthocyanin and flavonol branches of the flavonoid pathway is a promising finding for the production of fruit with enhanced antioxidant content. Our evidence suggests that gradual colouration of sweet cherry skin upon re-exposure to light results from anthocyanin pathway initiation by PaMYBA1, evident by the up-regulation of *PaCHS1*, *PaUFGT* and *PaMYBA1* expression. Investigation of skin- and flesh-specific expression of *PaMYBA1* genes revealed that in sweet cherry, regulators other than MYB factors appear to determine fruit colour, though PaMYBA1, PaMYBA2 and PaMYBA3 could all play an important part in fruit colour.

Chapter Seven: General discussion

7. GENERAL DISCUSSION

The aim of this project was to investigate regulation of flavonoid biosynthesis in sweet cherry fruit. Numerous studies focusing on the regulation of phenylpropanoid biosynthetic pathways have revealed that the transcriptional control of the genes by MYB, bHLH and WD40 proteins is conserved throughout the plant kingdom (Du *et al.*, 2009). However, amongst different plant species the gene targets of functionally conserved MYB factors can vary considerably (Quattrocchio *et al.*, 1993). It was hypothesised that sweet cherry, much like apple and grape (Section 1.4.1), would possess multiple R2R3-MYB factors that are capable of controlling the expression of flavonoid branch-specific subsets of biosynthetic genes. Thus, we isolated putative regulators of anthocyanin, tannin and flavonol biosynthesis, and investigated their roles in the flavonoid pathway through molecular and functional characterisation. In addition, we explored whether transcriptional regulation of this pathway gives rise to the different fruit colour phenotypes observed within sweet cherry cultivars, as it does in apples (Takos *et al.*, 2006a; Espley *et al.*, 2007) and grapes (Walker *et al.*, 2006; Walker *et al.*, 2007).

7.1 FLAVONOID ACCUMULATION IN DEVELOPING 'LAPINS' FLOWERS AND FRUIT

Flavonoid composition of the ripe fruit of a number of sweet cherry varieties has been previously documented (Section 1.2.3), and the flavonoid species present in the ripe fruits studied in this investigation were consistent with these reports. However, the temporal accumulation of flavonols has not previously been documented. Total flavonol, tannin and anthocyanin concentrations were determined in 'Lapins' buds, flowers and developing fruit by HPLC. Flavonols and tannins were found to accumulate in the lead up to flowering, and in the very early stages of fruit development, consistent with their roles in pollen tube formation and predation prevention. For 'Lapins' fruit, no overlap was observed between the accumulation of these compounds with anthocyanin, which accumulated from around 58 DAFB to reach maximum detected levels in ripe fruit at 107 DAFB. This temporal separation might imply that the flux of the flavonoid pathway must switch to provide the anthocyanin branch with sufficient substrate for pigment production. In support of this theory, the high level of flavonoid pathway activity within the fruit of the black sweet cherry cultivar 'Sam' appears to provide enough substrate to support the concurrent production of all three classes of flavonoids in ripe fruit.

The accumulation of anthocyanins, tannins and flavonols during 'Lapins' development correlated well with the expression of genes encoding enzymes that are specifically required for their synthesis (including *PaUFGT*, *PaANR* and *PaFLS*, respectively). This correlation was expected given the extensive knowledge of the flavonoid pathway, as well as similar findings in fruits (Sections 4.1 and 5.1) and ornamental flowers (Mol *et al.*, 1998). Nonetheless, the abundance of structural gene transcripts supplied a useful comparative tool for the expression of the candidate flavonoid MYB TFs, providing supporting evidence for their assignment as candidates for flavonoid branch-specific regulation.

It was also interesting to note that, based on their expression profiles, the *PaCHS1* and *PaCHS3* isoforms seemed to be associated with the synthesis of anthocyanins, whereas *PaCHS2* was expressed during flavonol and tannin accumulation. Flavonol-specific *CHS* genes have been described for petunia, grape, gerbera and morning glory, as discussed in Section 4.3.4. Considering that the enzymatic reaction catalysed by CHS appears to be a key step in the synthesis of sweet cherry flavonoids (Section 5.3.4), further characterisation of these *PaCHS* genes may help to clarify their roles and assist in understanding the differences in flavonoid biosynthesis between blush and red/dark sweet cherry fruits. An initial starting point for characterisation would be to isolate and test the gene-specific promoters for trans-activation by the different sweet cherry MYB factors in a transient expression assay. Characterisation could also involve determination of potential differences in preference for CoA substrates (Christensen *et al.*, 1998), or even identification of flavonoid enzymes that might physically interact with the *PaCHS* gene products in a multi-enzyme complex to facilitate control of metabolic flux via substrate channeling (Burbulis and Winkel-Shirley, 1999).

7.1.1 Transcriptional regulation of flavonoid synthesis by MYB factors

Molecular and functional characterisation strongly supported *PaMYBA1* as a regulator of anthocyanin synthesis. The ability of *PaMYBPA1* to activate transcription from the promoters of tannin biosynthetic genes also strongly suggests that PaMYBPA1 can regulate tannin synthesis. Differences in the 'Lapins' developmental expression profile of *PaMYBPA1* compared with *PaLAR1* and *PaANR* may be explained by the broad regulatory capabilities of PaMYBPA1 that may extend beyond tannins, evident by its broad target set.

Despite the high degree of sequence similarity of the predicted PaMYBF1 protein with characterised flavonol regulators from Arabidopsis and grape, and despite the presence of C-

terminal motifs conserved between these MYB factors, the involvement of PaMYBF1 in regulation of flavonol synthesis was unable to be confirmed. The inability of PaMYBF1 to trans-activate the *VvFLS* promoter may stem from a lack of interaction, due to extensive differences between the sweet cherry and grape *FLS* promoters. The potential role of PaMYBF1 in regulation of flavonol synthesis could be further tested before excluding it as a candidate by trying to complement the flavonol deficient phenotype of the Arabidopsis *myb11 myb12 myb111* triple mutant (Stracke *et al.*, 2007). It is possible, however, that *PaMYBF1* has diverged from other flavonol regulators to play a different role in phenylpropanoid biosynthesis, and that another as yet unidentified MYB factor regulates *PaFLS* expression and consequently flavonol synthesis. The upcoming release of the sequenced peach genome may enable flavonol-like MYB TFs to be identified from peach, thus facilitating the isolation of a flavonol regulator from sweet cherry due to the synteny between these two species. Alternatively, microarrays could be used to identify MYB genes that are up-regulated around flowering and down-regulated during fruit development. Plant transformation, particularly the over-expression or silencing of *PaMYBF1* in a heterologous system, could provide clues as to which metabolites are affected by PaMYBF1 regulation, other then flavonols.

7.2 REVEALING THE GENETIC DIFFERENCE BETWEEN YELLOW CHERRIES AND ALL OTHER SWEET CHERRIES

Yellow sweet cherries, such as the cultivar ‘Yellow Glass’, are clearly impaired in the synthesis of anthocyanin, such that they cannot produce pigmentation under any circumstances. We have demonstrated that this fruit colour phenotype results from a lack of expression of *PaMYBA1*, a regulator of anthocyanin synthesis. In cDNA derived from the skin and flesh of sweet cherries, the *PaMYBA1* transcript was equally abundant in all anthocyanin-containing cultivars tested, regardless of colour intensity. However, the *PaMYBA1* transcript was not detected in ‘Yellow Glass’ fruit cDNA, signifying the genetic difference between these two distinct groups of cherries. As there are no anomalies present in the ‘Yellow Glass’ *PaUFGT* sequence, it is presumed that the absence of PaMYBA1 prevents the transcription of *PaUFGT* in yellow cherries, as indicated in the model presented in Figure 7.1. Thus, we have established that whilst *PaMYBA1* expression does not explain difference in colour intensity between blush, red and dark cherries, it is a critical factor for colour production in sweet cherry fruit.

The genetic separation of yellow cherries from all other anthocyanin-containing sweet cherries on the basis of *PaMYBA1* expression supports the observation that yellow fruit do not

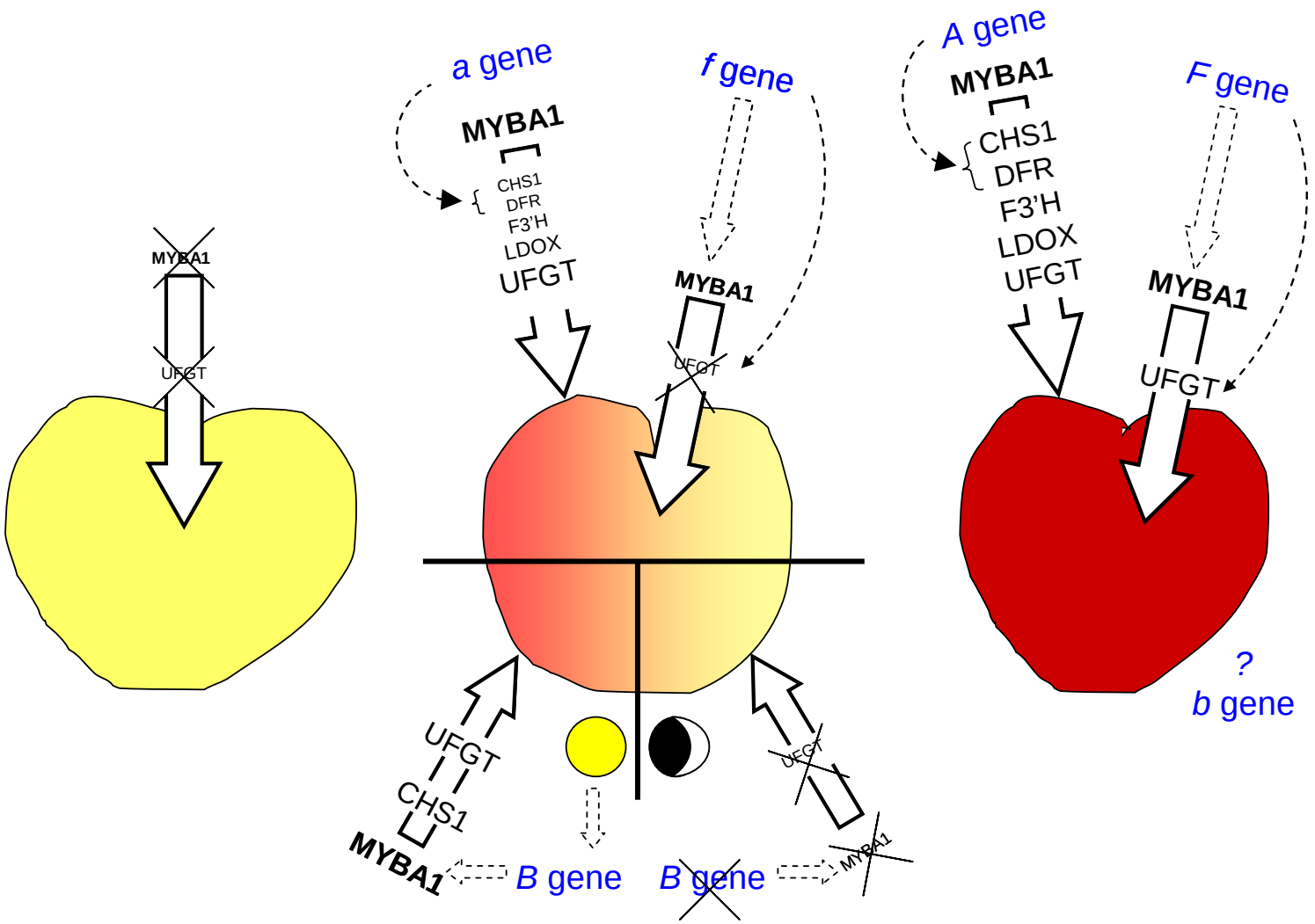
arise from crosses between blush and red sweet cherry cultivars (Schmidt, 1998). The exclusion of yellow-fruited cultivars from breeding programs means there is a lack of genetic material in which to study the interaction of *PaMYBA1* with other genes involved in fruit colour. Therefore, although they may not produce commercially desirable progeny, 'yellow x blush' and 'yellow x red' crosses may provide a means to study and better understand the genetic factors involved in sweet cherry colour.

7.3 INHERITED GENETIC FACTORS INFLUENCING THE ANTHOCYANIN PATHWAY DETERMINE SKIN AND FLESH COLOUR OF CHERRIES

Scientific observations of fruit colour segregation within sweet cherry populations have demonstrated that a number of genetic factors are involved in the determination of skin and flesh colour in sweet cherry fruit (Section 1.2.2). Expression analysis of flavonoid genes in separated skin and flesh of blush and red fruit, under normal conditions and in response to light treatment, have enabled the development of a working model for flavonoid pathway regulation in sweet cherry fruit (Figure 7.1).

7.3.1 Control of flesh colour in sweet cherry fruit

Sweet cherry flesh colour results from a single gene (which will be referred to as *F*), for which red flesh exerts dominance over yellow flesh (Crane and Lawrence, 1952). Comparison of gene expression within the flesh of ripening 'Ranier' (blush) and 'Lapins' (red) fruit revealed that the yellow flesh phenotype is attributable to a lack of *PaUFGT* expression, as pictured in the model in Figure 7.1. The mechanism by which *F* influences the expression of *PaUFGT* remains unclear, as *PaMYBA1* expression is only two-fold lower in 'Ranier' than in 'Lapins'. The identity of the *F* gene could be a *bHLH* or *WD40* encoding a co-factor of PaMYBA1, but preventing *PaUFGT* transcription when the *f* allele is inherited. To explore this possibility, it would be necessary to isolate sweet cherry *bHLH* and *WD40* genes, confirm their role in anthocyanin regulation, and analyse their expression in phenotypically different cultivars. Alternatively, the *F* gene could prevent anthocyanin synthesis in flesh by transcriptionally repressing *PaUFGT*, in a similar way that over-expression of *AtMYB60* in red lettuce represses *DFR* expression and anthocyanin synthesis (Park *et al.*, 2008). In the future, elucidation of the *F* gene could enable the development and application of molecular markers to predict flesh colour in sweet cherry seedlings, which will be discussed in further detail in Section 7.5.



Yellow fruit

Contains no anthocyanin

Blush fruit

Anthocyanin on skin only

Red/black fruit

Anthocyanin in skin and flesh

Figure 7.1 – A model for the regulation of anthocyanin synthesis in sweet cherry skin and flesh by inherited fruit colour factors

Arrows represent the anthocyanin pathway, and the location of the arrowhead indicates biosynthesis in either skin or flesh. Only the enzymes for which gene expression differs between phenotypes are listed. Text size for enzyme abbreviations indicates (approximate) relative transcript abundance of the encoding genes. A cross through a gene/enzyme represents a lack of expression. Fruit colour factors are shown in blue: *A* or *a* gene, major skin colour determinant; *B* or *b* gene, minor skin colour determinant (expressed as a blush in the absence of *A*); *F* or *f* gene, flesh colour determinant. For dashed arrows the targets of the genetic factors have been established, but the mechanism by which the factor causes gene expression to be altered remains unclear. The differential response of the skin of blush fruit to altered light conditions is depicted by the sun and moon in the bottom section of the fruit.

7.3.2 Control of skin colour in sweet cherry fruit

The major skin colour factor (*A*) confers dark skin colour in sweet cherry fruit, and is thought to be encoded either by *F* or a gene closely linked to *F* (Fogle, 1975). The data presented in this thesis, summarised in the working model (Figure 7.1), support skin and flesh colour being controlled by separate genes as *A* and *F* seem to facilitate regulation of different targets. As mentioned previously, *F* exercises control over *PaUFGT* in flesh, yet *PaUFGT* is not the target of the *A* gene, which is expressed at a comparable level in both the non-pigmented background skin colour of 'Ranier' and the red skin of 'Lapins' fruit. Comparative expression analysis in 'Ranier' and 'Lapins' skin demonstrated that the major identifiable difference in non-pigmented skin was the significant down-regulation of *PaCHS1*, *PaDFR* and *PaF3'H*. Therefore, we suggest that *a*, similar to *f*, might encode a non-functional transcriptional co-factor or negative regulator of these target genes. As WD40 proteins are often involved in multiple pathways in addition to anthocyanin synthesis, such as tannin synthesis and trichome formation (Koes *et al.*, 2005), it might be more likely that *A* encodes a *bHLH* gene. However, it is possible that sweet cherry may have evolved *WD40* genes that give rise to branch-specific WD40 proteins which, in theory, would explain why the tannin pathway continues to function in blush fruit.

From the model, it is evident that the possibility of *PaMYBA1* being the *A* factor is somewhat negated by the approximately equal abundance of the *PaMYBA1* transcript in the skin of these two visibly distinctive cultivars. However, as the quantitative PCR technique utilised in this study could not differentiate between the transcripts of *PaMYBA1* and the almost identical but truncated *PaMYBA3* (a potential competitive inhibitor, Section 6.3.8), *PaMYBA1* cannot be conclusively disregarded as the *A* gene. Thus, within the detected transcript abundance, the ratio of *PaMYBA3:A1* might be greater for 'Ranier', explaining the absence of background colour in the skin of blush fruit by co-factor sequestration or by competition for the binding sites of target promoters. The question of whether differential *PaMYBA3* expression affects fruit colour could be addressed by investigations at the protein level, assuming that differences in transcript abundance are reflected in protein concentrations. Using an antibody raised to the common DNA binding domain, the levels of the full-length PaMYBA1 and the truncated PaMYBA3 in blush, red and dark fruit could be ascertained and may provide further insight into the mechanism of action of the *A* gene.

In addition to PaMYBA3, it is proposed that PaMYBA2 may also act to prevent anthocyanin synthesis by competitive inhibition (Section 6.3.8).

7.3.3 Control of anthocyanin blush development in response to light

Anthocyanin blush, a red pigmentation on otherwise yellow sweet cherry skin, is believed to result from the incomplete dominance of the minor skin colour factor, *B*, and is visible when not epistatically masked by *A* (Section 1.2.2.2). The development of blush only on light-exposed skin sectors (Marshall, 1954) suggests that *B* is light-dependent. When grown in the absence of light, fruit from the blush cultivar 'Ranier' was devoid of anthocyanin and contained barely detectable levels of the anthocyanin regulator *PaMYBA1* and the anthocyanin biosynthetic gene *PaUFGT* (Figure 7.1). Re-exposure of dark-grown fruit to sunlight (light-treatment) induced the expression of both of these genes, as well as that of *PaCHS1*, in skin tissue. However, the expression of *PaMYBA1* in both 'Ranier' skin and flesh makes it unlikely that *PaMYBA1* is the skin-specific *B* factor. Thus, we hypothesise that initiation of anthocyanin synthesis occurs when the *B* gene is induced by sunlight and subsequently activates the downstream *PaMYBA1* (Section 6.3.7), enabling transcription of *PaCHS1* and *PaUFGT*. Nonetheless, we cannot rule out the possibility that *PaMYBA1* is the *B* gene, but that the flesh of blush-skinned fruit does not display a blush due to the absence of *PaUFGT* expression.

From genetic studies it has been suggested that in the presence of the *A* gene, *B* intensifies skin colour (Fogle, 1975). As *B* is expected to be light-dependent, skin colour intensification should occur only when fruit is exposed to light. During an experiment in the black 'Bing' cultivar, a reduction in anthocyanins was noted for shaded fruit (Patten and Proebsting, 1986). However, light-treatment of red 'Lapins' fruit or black 'Sam' fruit in this investigation did not alter the expression of *PaMYBA1* or *PaUFGT*, nor did it increase skin anthocyanins. These observations indicate that 'Bing' could possess a *B* allele, but that 'Lapins' and 'Sam' might be homozygous for *b*. Alternatively, it is possible that the relatively small increases in gene expression and anthocyanin accumulation are not evident as they are diluted by their already high levels in these fruits.

In addition to anthocyanin synthesis, flavonol synthesis was also enhanced by sunlight. The accumulation of flavonols in light-treated 'Ranier' fruit corresponded to the increase in expression of *PaFLS*. Flavonol synthesis has also been found to be affected by light in grape berries (Downey *et al.*, 2004), as a result of the light-inducible expression of *VvFLS* via the light sensitive TF *VvMYBF1* (Czemmel *et al.*, 2009). Although a flavonol *MYB* factor capable

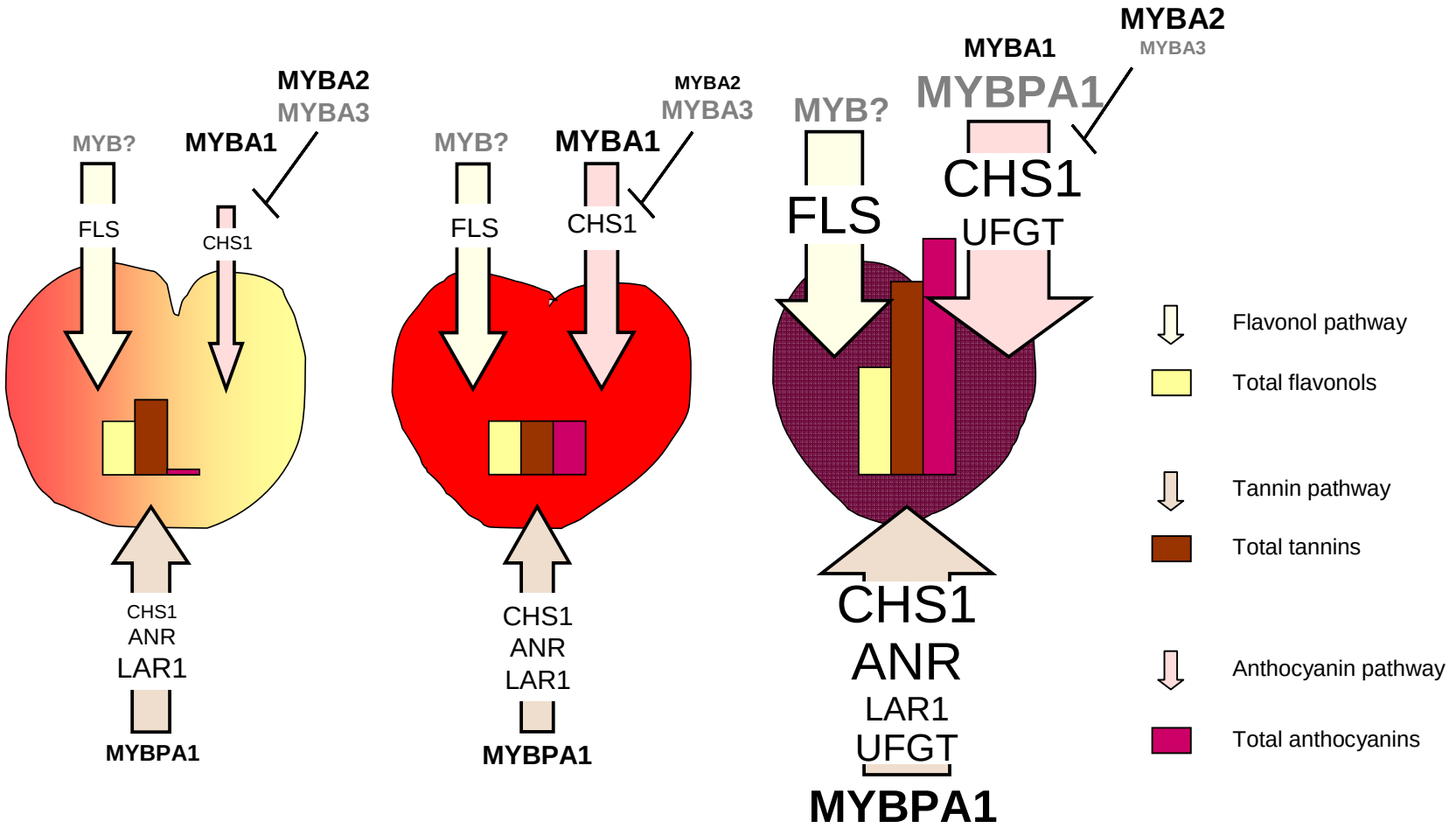
of activating *PaFLS* has not yet been isolated, it is predicted that the promoter of this *MYB* will contain light responsive elements that would facilitate this gene up-regulation.

7.4 CULTIVAR-SPECIFIC DIFFERENCES IN FLAVONOID GENE EXPRESSION RESULT IN FRUIT WITH ALTERED FLAVONOID ACCUMULATION

Variations in the accumulation of flavonoids within cultivars belonging to the same plant species can arise from different levels of expression of flavonoid structural and regulatory genes (Section 5.1). These variations can occur even when cultivars share a common parent, such as 'Ranier' and 'Lapins' which are both progeny of 'Van' crosses. The data presented in Chapter 5 has been summarised to show flavonoid genes that appear to be up- or down-regulated in colouring whole fruit (skin and flesh) of different coloured sweet cherry cultivars (in reference to the red 'Lapins', Figure 7.2). Additionally, we have attempted to represent how pathway flux might be altered in the absence of significant changes in gene expression, providing further insight into how this complex flavonoid pathway regulation might be achieved.

7.4.1 Comparison of anthocyanin pathway activity

The differences in anthocyanin accumulation in the ripe fruit of the sweet cherry cultivars 'Ranier', 'Lapins' and 'Sam' can be seen easily. The reason for this is that, compared with 'Lapins', ripe 'Ranier' fruit contains about 64-fold less anthocyanins per fruit, while 'Sam' possesses 4 times more anthocyanins per fruit (Figure 7.2). We therefore predicted that the transcript abundance of genes required for anthocyanin synthesis, including the anthocyanin regulator *PaMYBA1*, would reflect these differences. However, *PaMYBA1* was actually expressed at a slightly higher level in colouring 'Lapins' fruit, 2-fold higher than in 'Ranier' and 'Sam'. The differences in anthocyanin accumulation seem to be determined largely by the abundance of *PaCHS1*, for which 'Ranier' and 'Sam' respectively possessed 10-fold lower and higher expression than 'Lapins'. Furthermore, *PaUFGT* was three times more abundant in 'Sam' than in both 'Ranier and 'Lapins' colouring fruit.



Blush fruit

Anthocyanin on skin only

Red fruit

Anthocyanin in skin and flesh

Black fruit

Anthocyanin in skin and flesh

Figure 7.2 – A diagrammatic summary of flavonoid gene expression and resulting flavonoid accumulation in colouring sweet cherry cultivars displaying a range of colour phenotypes

Arrows represent the flavonol, tannin and anthocyanin branches of the flavonoid pathway, and the size of the arrow approximately corresponds to the amount of metabolite accumulation (columns within the cherries). Only the enzymes for which gene expression differs between phenotypes are listed. Text size for enzyme abbreviations indicates (approximate) relative transcript abundance of the encoding genes. MYB regulatory factors influencing a pathway branch are shown at the beginning of the arrow. Grey text colouring signifies further work required to confirm the predicted role. Note that transcripts for *MYBA1* and *MYBA3* could not be distinguished by quantitative PCR, thus text sizes for MYBA3 are merely speculative.

In addition to PaMYBA1, PaMYBPA1 has been shown to activate transcription from the apple *CHS* and *UFGT* promoters (Figure 4.7). This fact, together with the detection (relative to 'Lapins') of 2-fold less and 20-fold greater expression of *PaMYBPA1* in 'Ranier' and 'Sam' fruit, suggest that the observed differences in anthocyanin accumulation may be driven by *PaMYBPA1* rather than *PaMYBA1*. Phylogenetic analysis supports the possibility that PaMYBPA1 acts broadly to regulate the synthesis of flavonoids other than tannins, as the PaMYBPA1 predicted protein is most similar to VvMYBPA1 but also clusters with the general phenylpropanoid regulators VvMYB5a (Deluc *et al.*, 2006) and VvMYB5b (Deluc *et al.*, 2008), and also with maize C1, an anthocyanin regulator capable of controlling *AtBAN* (*ANR*) transcription (Baudry *et al.*, 2004) in Arabidopsis protoplasts. Investigation of *PaMYBPA* copy number suggested the presence of a second gene within sweet cherry. Although this gene has not yet been identified, it could regulate flavonoid biosynthesis in a manner more specific to tannins.

The presence of transcriptional repressors or competitive inhibitors of PaMYBA1 could also contribute to the differences in anthocyanin accumulation, despite similar levels of *PaMYBA1* expression. Expression of the anthocyanin-like regulator, *PaMYBA2*, could barely be detected in developing 'Lapins' fruit, yet its expression was 3-fold higher in colouring 'Ranier' fruit than in 'Lapins'. Consequently, it seemed feasible that PaMYBA2 might be competing with PaMYBA1 for co-factors and promoter binding sites, thereby reducing anthocyanin accumulation (Section 6.3.8). However, the 11-fold up regulation of *PaMYBA2* in 'Sam' is not consistent with this theory. The role of *PaMYBA2* remains unclear and further investigations, such as those proposed for *PaMYBF1* in Section 7.1.1, might help to clarify its potential role in regulation of anthocyanin synthesis.

In addition to PaMYBA2, another anthocyanin-like MYB factor was identified that may compete with PaMYBA1. PaMYBA3 is a truncated version of PaMYBA1 lacking the C-terminal domain, but *PaMYBA3* has been deemed a separate gene to *PaMYBA1*, rather than an allele, due to its presence being detected in all of the cultivars tested (Section 3.2.2.1.1). We suggest that differential levels of *PaMYBA3* expression might contribute to variations in anthocyanin accumulation between the fruits of 'Ranier', 'Lapins' and 'Sam'. While analysis of the expression of this gene has not been possible here, its quantification (Section 7.3.2) would be a useful addition to the working model of flavonoid regulation. The *F* and/or the *A* factor being encoded by a transcriptional repressor is a distinct possibility (Section 7.3.1 and

7.3.2). If *PaMYBA3*, or another negative regulator present within sweet cherry, represents *F* or *A*, this could explain the large variations in anthocyanin accumulation in 'Ranier', 'Lapins' and 'Sam' fruit that occur despite relatively consistent *PaMYBA1* expression across these cultivars. The inheritance of a single copy of a semi-dominant anthocyanin repressor could also explain the intermediate red colour phenotype of 'Lapins'.

7.4.2 Comparison of tannin pathway activity

Similar to anthocyanins, tannins were also significantly more abundant in 'Sam' fruit (Figure 7.2). While total tannins and tannin gene expression did not vary greatly in the colouring fruit of 'Ranier' and 'Lapins', 'Sam' accumulated almost 4-fold more tannins per fruit in both colouring and ripe fruit. Between the green fruit and colouring fruit stages tannin levels declined on a per fruit basis in 'Ranier' and 'Lapins', whereas 'Sam' fruit continue to actively synthesise tannins during this time (Figure 5.6a). This large increase in tannins correlates with increases in the expression of *PaCHS1* (10-fold), *PaANR* (19-fold) and *PaLAR1* (13-fold). It is highly likely that this up-regulation in expression results from the greater than 20-fold higher level of *PaMYBPA1* expression in colouring 'Sam' fruit compared with that of 'Ranier' and 'Lapins', as PaMYBPA1 is capable of trans-activating the promoters of the apple and grape homologues of these genes when transiently expressed in grape suspension cells.

7.4.3 Comparison of flavonol pathway activity

In comparison to elucidation of anthocyanin and tannin synthesis, control of flavonol synthesis in sweet cherry fruit is still not well characterised. Within the colouring fruit of 'Ranier', 'Lapins' and 'Sam', total flavonol levels were found to be approximately similar, not differing by more than 1.5-fold. In 'Ranier' fruit the expression of *PaFLS* was 2-fold lower than that of 'Lapins' but did not result in a significant difference in total flavonol levels per fruit (Figure 7.2). However, the consequences of the 40-fold greater abundance of *PaFLS* expression in colouring 'Sam' fruit were seen in ripe fruit, which contained more than twice the flavonols per fruit of 'Ranier' and 'Lapins' ripe fruit. Thus, it is expected that the MYB factor regulating the expression of *PaFLS* (shown in Figure 7.2 as MYB'?') would also be expressed at a higher level in 'Sam' than the other cultivars, although this factor has not yet been isolated.

7.5 IMPLICATIONS OF THE RESEARCH

This research has demonstrated that regulation of flavonoid biosynthesis in sweet cherry fruit is considerably more intricate than in grape, or even apple, due to the involvement of multiple inherited skin and flesh factors that influence colour. Although the model of regulation presented here is not complete, the addition of the molecular information obtained from this research to knowledge of colour inheritance will aid considerably future efforts to identify the colour determinants of sweet cherry skin and flesh. The elucidation of these genes responsible for cherry colour could pave the way for the development of molecular colour markers, enabling MAS of seedlings with the desired phenotype. The use of MAS to select seedlings with desirable characteristics would considerably reduce costs associated with screening, such as financial, time and space requirements. Furthermore, this work contributes to our current knowledge of how plants control secondary metabolism via transcriptional regulation.

From a functional food perspective, there are two main outcomes of this research that may be of particular interest when trying to optimise intake of health-promoting flavonoids. Firstly, careful consideration of row orientation and spacing of blush sweet cherry trees by growers could increase the exposure of fruit to sunlight, thereby potentially increasing total anthocyanins and flavonols. Secondly, certain sweet cherry cultivars such as ‘Sam’ contain much higher levels of flavonoids that other cultivars. It might be beneficial to ascertain whether high levels of flavonoid compounds are common to all black sweet cherry cultivars. If this is the case, then perhaps the current plantings of black sweet cherry cultivars could be extended, and the fruit marketed as having additional benefits over other types of cherries. Additionally, the finding that enhanced flavonoid accumulation in ‘Sam’ fruit could be caused by increased *PaMYBPA1* expression provides an interesting potential target for genetic modification.

7.6 SUMMARY

This research represents the first molecular investigation of regulation of flavonoid biosynthesis in sweet cherry. We have discovered that in the absence of a functional PaMYBA1 regulator of anthocyanin synthesis, cherries are yellow rather than red. As a result of these investigations into flavonoid pathway regulation during fruit development, and in response to sunlight, this research has identified some molecular targets of the genetic determinants of sweet cherry skin and flesh colour, and enabled a working model for the regulation of flavonoids in sweet cherry to be proposed. This information moves us closer to knowing the identity of these inherited factors. However, the model presented here is based

on only one representative cultivar for each colour phenotype. Further experimentation, together with hypotheses testing in additional sweet cherry cultivars, would be useful to clarify this model, especially where genetic factors and their modes of action are yet to be identified. A better understanding of flavonoid biosynthesis and its regulation in sweet cherry fruit may provide growers, breeders and scientists with tools to deliver fruit that is of a very high quality to consumers.

Appendices

APPENDIX 1: BUFFERS, SOLUTIONS AND MEDIA

All chemicals were analytical or molecular biology grade and obtained from BD Biosciences (http://www.bdbiosciences.com) or Sigma-Aldrich (http://www.sigmaaldrich.com). Solutions were made with SNW and, if necessary, autoclaved on a fluid cycle at 121°C for 20 min.

- **AIX + agar plates:** 100µg/ml Ampicillin, 18.75µg/ml IPTG, 56µg/ml X-Gal (final concentrations) in LB medium + agar
- **DNA tracking dye (5X):** 50% glycerol, 1mM EDTA pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol
- **GC medium/agar:** 0.3% (w/v) Gamborg's B-5 basal medium, 3% (w/v) sucrose, 0.025% (w/v) casein hydrolysate, 0.93µM kinetin, 0.54µM NAA, pH 5.7-5.8. Agar 0.8% (w/v)
- **GeneScreen hybridisation buffer:** 33.84ml SNW, 4g dextran, 4ml 10% SDS. Heat to 65°C for 30 min then add 2.32g NaCl
- **GPS buffer:** 0.2 M Glycine, 0.1 M sodium phosphate (dibasic), 0.6 M NaCl, pH 9.5
- **Hot borate buffer:** 0.2M disodium tetraborate, 30mM EGTA, 1% (w/v) SDS, 1% (w/v) deoxycholic acid. Warm at ~55°C for 30 min, pH 9.0
- **LB medium/agar:** 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, pH 8.0. Agar 1.5% (w/v)
- **Phloroglucinol buffer:** 0.3g Ascorbic Acid, 2.5g Phloroglucinol, 430ul conc. HCl, make to 50mls with methanol. Store in dark in refrigerator (4°C) for up to three weeks
- **SOC medium:** 2% tryptone, 0.5% yeast extract, 10mM NaCl, 2.5mM KCl, 10mM $MgCl_2$, 10mM $MgSO_4$, 20mM glucose
- **SSC (20X):** 3M NaCl, 0.3M sodium citrate dihydrate, pH 7.5
- **STE (30%):** 30% (v/v) ethanol, 0.1 M NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA
- **TAE (10X):** 0.4M Tris, 0.2M Acetic acid, 0.01M EDTA, pH 8.0
- **TBE (10X):** 1.1M Tris, 0.9M Boric acid, 0.025M EDTA, pH 8.0
- **TE:** 10mM Tris-HCl pH 7.5, 1mM EDTA pH 8.0

APPENDIX 2: PRIMER SEQUENCES

Primers were synthesised by Geneworks (Adelaide, Australia)

F = forward primer, R = reverse primer

Quantitative Real Time PCR primers are listed in Tables 3.6 and 3.7

Appendix 2a: Degenerate primers

Degenerate primers for isolation of regulatory genes are listed in Table 3.1

D = A/G/T, H = A/C/T, I = inosine, M = A/C, N = A/C/G/T, R = A/G, S = C/G, W = A/T, Y = C/T

Primer name	Primer sequence (5' - 3')
CHS dF1	TTYTGYACNACIYSIGGIGTIGAYATG
CHS dF2	GAYTGGAAYYSIYTITTYTGG
CHS dR	NGGICCRAAICCRAAIARIACNCCCAA
FLS dF	GGNGTNGTIGCICAYACIGAYATG
FLS dR	NGGIGGYTCIARRAAIACIGGCCA
LAR dF1	ITICCIWSIGARTTYGGICAYGAY
LAR dF2	TGYTGYAAIWSIATIGCIISNTGG
LAR dR	YGTRCAICCITTIATRAAIATRTCRTG
ANR dF1	ARGATCCIGARAAIGAYATGATHAA
ANR dR1	CAIACRTCYTCIACRTGIGYNAT
ANR dR2	ARYTCIGGIACISWIGTRTTNAC

Appendix 2b: 3'RACE primers

All primers are forward primers, with the exception of B26

Primer name	Primer sequence (5' - 3')
CHS1 3'F1	GCCCAGCAATTCTAGACCAAG
CHS1 3'F2	ATGTCCAGTGCCTGTGTGTTA
CHS2 3'F1	GCCCAGCAATTCTAGACCAAG
CHS2 3'F2	ATGTCCAGTGCCTGTGTGTTA
CHS3 3'F1	TTGGATGAGGTTAGGAAGAGG
CHS3 3'F2	ATGTCGAGTGCTTGTGTGCTG
F3'H 3'F2	AGAGGTTTGATGCCTTCTTGAC
F3'H 3'F3	AGACCGGCTTGTAACTGAATTG
FLS 3'F	CTGCACAGAACCACAGTGAAC
FLS 3'F2	GACAAGACAAGAATCTCATGG
DFR probe F	GATCAAGCCAACAATAAATGGG
LDOX probe F	ACTCAAGAGTTGAGACCTTGGC
UFGT probe F	ATCACCAAGAATGCCAACATC
LAR exon1 F2	ACCCTTCTAAGGCTGACA
LAR2 QRT F1	TCAGAGTTTGGGCACGATGTG
LAR 3'F1	ACGATGAAAACAATCCACGAC
LAR 3'F2	GTCCGAACAATCAACAAATCTG
ANR 3'F1	GACTTCCTCATAAATCATGCC
ANR 3'F2	TGAAAGGTATGCAACTACTATCAGG
MYBA1 3'F1	AGGGTTTGCAGAGGATGAAG
MYBA1 3'F2	GCTTTTAGAAAACAGGTGGTC
MYBA2 3'F1	ATCAAGAGAGGAGACTTTATGG
MYBA2 3'F2	GGTCATTGATTGCTCAAAGAC
MYBPA F1	CCTTGTTGTTCTAAGGTTGGTTTG
MYBPA F2	CTCCTTAGGTGTGGGAAGAG
MYB12 EST F1	GAYAAYGARATAAARAAYTAYTGG
MYB12 EST F2	GHTGGGCCATGAAGAAGAACA
TT2 repressor 3'F1	AAGAACTACTGGAACACCCACATAAG
TT8 3'F1	AGCAGCTTAGGGAGCTGTATG
TT8 3'F2	ATACAGACGGTGGTTTGCATTCC
TT12 3'F1	TAGTGTCCAACCCGTCATTTC
B26	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT

Appendix 2c: Genome Walker primers

All primers are reverse primers, with the exception of AP1 and AP2

Primer name	Primer sequence (5' - 3')
F3'H GW1	AGCTGAGCCAAGTTCACTGTCTTTGAC
F3'H GW2	GGAGAACAAATGGACTGAGCTGATCTTC
FLS GW GSP1	CTCCATTTGATCACCAATGTGGATGAC
FLS GW GSP3	TTTTCAGATAGGCAACATGCCACGTC
FLS GW GSP4	CTGCATGGATTTTTAACCGTATGTGC
FLS 5'R	ACGAGAATGGTGACGATGGAC
DFR GW1	TCTTTGGACTCAAAATCCATAGGAGTGG
DFR GW2	GAATGGCTTCATCAAAGCTTCCCTC
LDOX GW GSP1	GCTTGCTTCCATAGCCTTGAATTTTACC
LDOX GW GSP2	CCTGGTCATTGGCATACTTCTCCTTTTG
LDOX GW GSP3	GTGCAGGCCTATGCTCTTTTCTTATGG
LDOX GW GSP4	TGGAGGTGCTTATATTTCAAGGGGAAC
LDOX GW GSP5	CACACATTTGGCTACCCTGAACAAAGAG
UFGT GW GSP1	TGAGTCCAAGTTTCCAAAGATGACACC
UFGT GW GSP2	CTTTGATTCGTACTTTGGACATTCCTG
UFGT GW GSP3	CTCTAGAGAGAGACTGGTGGCTGGCTTAG
UFGT GW GSP4	CGGGTGGTTGTTTCTCTCTCTAAATATCG
LAR2 QRT R1	GAAGGATGCTTGTTGTCGAAG
LAR2 GW GSP1	ACTTGACGCTTCTCCAAATACATGGTG
LAR 5'R	ATCCAAGGGTGGGAGAACATC
LAR 5' primary	GCCATCACCATAGATGTGGAAG
ANR GW GSP1	CCCTTGAACTGCTGGTTTAATCATATCC
ANR GW GSP3	CGCGTTTAACTGTTTTGGCTTTCACAC
ANR GW GSP4	TTATGAGCTGTTGGGCCACTTGTTTAG
ANR GW GSP5	AGGACTAGAAGGAATTATGAGACTGGT
AN2 5' AD	CACCTTTTTCAGGCGAGAATCCGTCC
AN2 5' R1	AGCTCCTCCTGCACCTGTTC
AN2 5' R2	CCCTGCTTTGTAAGGAACTTG
AN2 5' R3	CTTTGTAAGGAACTTG
MYBPA GW1	CAGAAGTGAATGTAGTCTGACAATCAGG
MYBPA GW2	TATGTCTGGTCTTAAATAGTTCATCCACC
MYB12 GW GSP1	TAATGTCCTTCTGTCCCTTGTTGTGACG
MYB12 GW GSP2	TGCTGGTAGGCTACTCATTTGATCACTAC
TT2 GW primary	CCAGTRGTTSTTKATSTCRTTG
TT2 QRT R1	GAAGCTGCAAAAGAAATTGTGG
TT8 5' R	CTCCAACAACCCGTCACTCTC
TT8 5' R2	CTCCATTGTAGTACCCATCTGACCATAC
AP1	GTAATACGACTCACTATAGGGC
AP2	ACTATAGGGCACGCGTGGT

Appendix 2d: Primers to generate probes for Southern blotting

Primers were designed to amplify approximately 500bp fragments (Table 2.2)

Gene	F primer sequence (5' - 3')	R primer sequence (5' - 3')
CHS	CCATCGACGGACATCTCCGTG	TTCAAGCCCACGCTGTGAAGCAC
FLS	TGGTACGACGTTAAGTACATC	CTCACCAACACATACAACTTCC
DFR	GATCAAGCCAACAATAAATGGG	ATGTTTCTCAGCAGGAATTGG
LAR	ACGATGAAAACAATCCACGAC	GCATTTGGCACGAGGATAAA
ANR	CGGAGAAGGACATGATTAAACC	CAGGAACACTGGTATTGACAGC
LDOX	ACTCAAGAGTTGAGACCTTGGC	TAATCAGCAGGTGTTTGAGGC
UFGT	ATCACCAAGAATGCCAACATC	CACCATCCCATTACTTGCTTTTAC
MYBA1 (R2R3)	CAATGTGTGAAATCAGAACC	CACCCAAGTTATAGCCCTCCATC
MYBA1 (AD)	CTATCTGTCGAAGGTGGTCATTG	GCCATCAAACATTACACACC
MYBA2	GGTCATTGATTGCTCAAAGAC	AATCCAGACTAGGTCTCTTCAC

Appendix 2e: General primers

Role	Primer name	Primer sequence (5' - 3')
Sequencing	SP6 (pDRIVE)	CATACGATTTAGGTGACACTATAG
	T7 (pDRIVE)	TAATACGACTCACTATAGGGAGA
	OCS R (pART7)	GGCGGTAAGGATCTGAGCTA
	35S F (pART7)	CAATCCCACTATCCTTCGCAA
Cloning into pLuc	*MYBA1* F *Xho* 1	AAGCTCGAGAGATGGAGGGCTATAAC
	MYBA1 R Xba 1	CACTCTAGACTCTACCTAGTCCTTCTG
	MYBPA F *Xho* 1	TGAGTGACTCGAGATATGGGAAG
	MYBPA R *Xba* 1	TTTCTAGATTAGATCAGCAGTGACTC
	MYBA2 F *Xho* 1	AAGAAACTCGAGATATGGAGGGT
	MYBA2 R *Xba* 1	CTTACTTTCTAGACTATTCTTCATTCG
	MYB12 F2 *Xho* 1	AAGAGAGAGTTGCTCGAGAAATGG
	MYB12 R2 *Xba* 1	GTAAATCTAGAATTTCAAGACAGAAGC
	MYBR F *Xho* 1	GATTCTCGAGAAATGGGAAGATCTCC
	MYBR R *Xba* 1	AACCATCTAGAGTCTCATTTCATCTCC
cDNA synthesis	oligo $dT_{(18)}$	TTTTTTTTTTTTTTTTTT

APPENDIX 3: PCR TEMPLATES, PARAMETER VARIATIONS AND PRIMER SETS

	Gene abbreviation	Tissue isolated from	PCR variations [$MgCl_2$]	T_{ext}	1° PCR primers	Nested primers
Degenerate PCR	*CHS1*	Stella gDNA		55°C*	*CHS* dF1/dR	*CHS* dF2/dR
	CHS2	Stella gDNA		55°C*	*CHS* dF1/dR	*CHS* dF2/dR
	CHS3	Stella gDNA		55°C*	*CHS* dF1/dR	*CHS* dF2/dR
	FLS	Stella gDNA	2.0mM	45°C*	*FLS* dF/dR	n/a
	LAR	Stella gDNA	2.0mM	45°C*	*LAR* dF1/dR	*LAR* dF2/dR
	ANR	Lapins stage 1 fruit cDNA		55°C*	*ANR* dF1/dR2	*ANR* dF1/dR1
	MYBA1	Stella cDNA 2004	2.0mM	37°C*	*AN2* dF/dR	n/a
	MYBA2	Lapins leaf gDNA	1.5mM		*AN2-2* 5'UTR F/*AN2* 5'R1	*AN2* intron1 seq2 F/*AN2* 5'R1
	MYBPA			55°C*	see 3' RACE	n/a
	MYBF	Lapins open cluster cDNA	2.5mM	45.5°C*	*MYB12* EST F1/R1	n/a
	MYBR	Lapins stage 1 fruit cDNA	3.0mM	37°C*	*TT2* dFjp/dRjp	n/a
	TT8	Lapins shuck split cDNA	2.0mM	37°C*	*TT8* dF/dR	n/a
	TT12	Lapins tight cluster cDNA	1.5mM	50°C*	*Ppersica* v2 116 F1/R1	n/a
3' RACE	*CHS1*	Lapins young leaf cDNA		48°C	*CHS1* 3'F1/**B26**†	*CHS1* 3'F2/**B26**†
	CHS2	Lapins young leaf cDNA		48°C	*CHS2* 3'F1	*CHS2* 3'F2
	CHS3	Lapins young leaf cDNA		48°C	*CHS3* 3'F1	*CHS3* 3'F2
	F3'H	Stella wildtype cDNA		50°C/55°C	*F3'H* 3' F2	*F3'H* 3'F3
	FLS	Lapins stage 1 fruit cDNA		32°C/35°C	*FLS* 3'F	*FLS* 3'F2
	DFR	Lapins young leaf cDNA		52°C	*DFR* probe F	n/a
	LDOX	Lapins young leaf cDNA			*LDOX* probe F	n/a
	UFGT	Lapins young leaf cDNA			*UFGT* probe F	n/a
	LAR1	Lapins young leaf cDNA			*LAR* exon1 F2	*LAR2* QRT F1
	LAR2	Lapins stage 1 fruit cDNA		47°C	*LAR* 3'F1	*LAR* 3'F2
	ANR	Lapins stage 1 fruit cDNA		50°C	*ANR* 3'F1	*ANR* 3'F2
	MYBA1	Stella ripe fruit cDNA		50°C	*MYBA1* 3'F1	*MYBA1* 3'F2
	MYBA2	Lapins young leaf cDNA		52°C/55°C	*MYBA2* 3'F1	*MYBA2* 3'F2
	MYBPA	Lapins young leaf cDNA		50°C	*MYBPA* F1	*MYBPA* F2
	MYBF	Lapins open cluster cDNA			*MYB12* EST F1	*MYB12* EST F2
	MYBR	Lapins young leaf cDNA		54°C	*TT2* repressor 3'F1	n/a
	TT8	Lapins shuck split cDNA		35°C	*TT8* 3'F1	*TT8* 3'F2
	TT12	Lapins young leaf cDNA		50°C	*TT12* 3'F1	n/a
Genome Walker PCR	*F3'H*	Lapins GW libraries 1-6			*F3'H* GW1/**AP1**#	*F3'H* GW2/**AP2**#
	FLS	Lapins GW libraries 1-6			*FLS* GW GSP1	*FLS* 5'R
		Lapins GW libraries 1-6			*FLS* GW GSP3	*FLS* GW GSP4
	DFR	Lapins GW libraries 1-6			*DFR* GW1	*DFR* GW2
	LDOX	Lapins GW libraries 1-6			*LDOX* GW GSP1	*LDOX* GW GSP2
					LDOX GW GSP3	*LDOX* GW GSP4
					LDOX GW GSP4	*LDOX* GW GSP5
	UFGT	Lapins GW libraries 1-6			*UFGT* GW GSP1	*UFGT* GW GSP2
					UFGT GW GSP3	*UFGT* GW GSP4
	LAR1	Lapins GW libraries 1-6			*LAR2* QRT R1	*LAR2* GW GSP1
	LAR2	Lapins GW libraries 1-6			*LAR* 5' primary	*LAR* 5' R
	ANR	Lapins GW libraries 1-6			*ANR* GW GSP3	*ANR* GW GSP1
		Lapins GW libraries 1-6			*ANR* GW GSP5	*ANR* GW GSP4
	MYBA1	Lapins GW libraries 1-6			*AN2* 5' AD	*AN2* 5' R1
		Lapins GW libraries 1-6			*AN2* 5' R2	*AN2* 5' R3
	MYBA2	Lapins GW libraries 1-6			*AN2* 5' R2	*AN2* 5' R3
	MYBPA	Lapins GW libraries 1-6			*MYBPA* GW1	*MYBPA* GW2
	MYBF	Lapins GW libraries 1-6			*MYB12* GW GSP1	*MYB12* GW GSP2
	MYBR	Lapins GW libraries 1-6			TT2 QRT R1	TT2 GW primary
	TT8	Lapins GW libraries 1-6			TT8 5' R	TT8 5' R2

PCR conditions listed vary from standard parameters listed in Section 2.5.2.

Some primer names do not match gene names as the later was changed at a later date.

* Annealing temperature only altered from 52°C for the 33 cycles after the initial 2 cycles at 37°C

† B26 (reverse) primer used for all 3' RACE reactions. Forward primers were nested

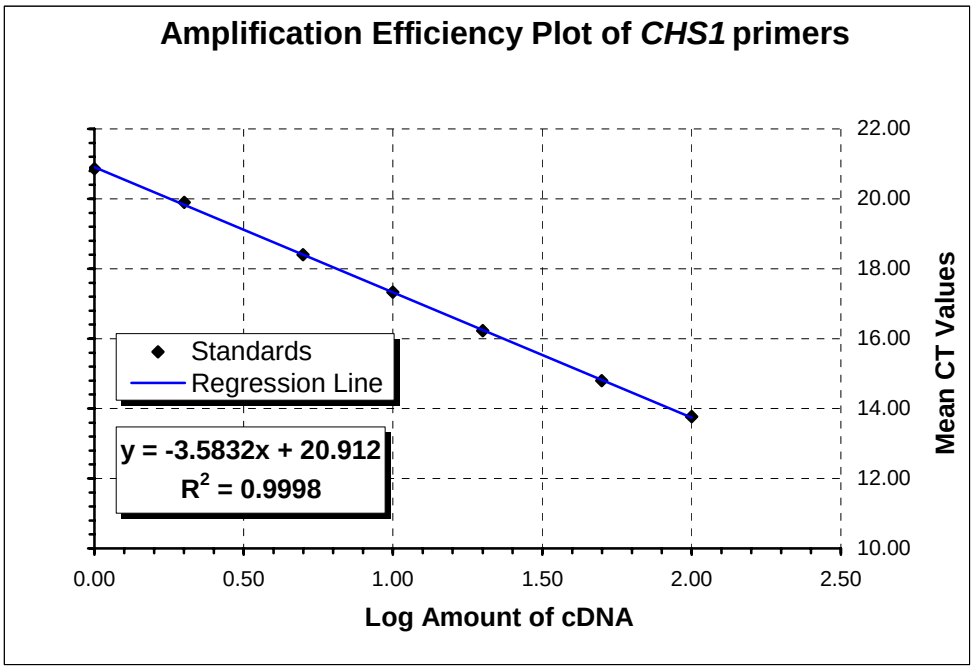
For Genome Walker PCRs, 1° reactions used adapter primer (AP)1 and 2° reactions used AP2

APPENDIX 4: ACCESSION NUMBERS OF FLAVONOID PATHWAY GENE 5'UTRS

5'UTRs (including promoter regions) were isolated by Genome Walking, from the gene sequences listed in Table 2.1.

Gene 5'UTR	GenBank accession
PaF3'H	GU938694
PaFLS	GU938695
PaDFR	GU938696
PaANR	GU938697
PaLDOX	GU938698
PaUFGT	GU938699
PaMYBA1	GU938700
PaMYBA2	GU938701
PaMYBPA1	GU938702
PaTT8	GU938703

APPENDIX 5: AMPLIFICATION EFFICIENCY OF Q-PCR PRIMERS



Amplification efficiency (E_{target}) plot generated using the Q-Gene software application (Muller, 2002). Note that $E_{target} = 10^{-1/slope}$

APPENDIX 6: PROTEIN ACCESSIONS AND CITING ARTICLES

Flavonoid branch	Species	Protein	Accession	Reference
Anthocyanin	*Antirrhinum majus*	VENOSA1	ABB83828	Schwinn *et al*., 2006
		ROSEA1	ABB83826	Schwinn *et al*., 2006
	Arabidopsis thaliana	PAP1/MYB75	AAG42001	Borevitz *et al*., 2000
		PAP2/MYB90	AAG42002	Borevitz *et al*., 2000
		PAP3/MYB113	AAG38380	Gonzalez *et al*., 2008
		PAP4/MYB114	AAG38381	Gonzalez *et al*., 2008
	Gerbera hybrida	MYB10	CAD87010	Elomaa *et al*., 2003
	Gossypium hirsutum	MYB10	ABR01222	Hsu *et al*., 2008
	Malus x *domestica*	MYB1	ABK58138	Takos *et al*., 2006a
	Malus x *domestica*	MYBA	BAF80582	Ban *et al*., 2007
	Malus x *domestica*	MYB10a	ABB84755	Espley *et al*., 2007
	Petunia x *hybrida*	AN1	AAG25927	Spelt *et al*., 2000
		AN2	AAF66727	Quattrochio *et al*., 1999
	Prunus avium	MYB10	ABX71493	
	Prunus armeniaca	MYB10	ABX71490	
	Prunus cerasus	MYB10	ABX71494	
	Prunus domestica	MYB10	ABX71492	
	Pyrus communis	MYB10	ABX71487	
	Vitis vinifera	MYBA1	ABL14063	Walker *et al*., 2007
		MYBA2	ABL14065	Walker *et al*., 2007
		MYBA3	ABL14067	Walker *et al*., 2007
Tannin	*Arabidopsis thaliana*	TT2/MYB123	CAC40021	Nesi *et al*., 2001
		TT8	CAC14865	Nesi *et al*., 2000
	Brassica napus	TT2-2	ABI13039	Wei *et al*., 2007
	Gossypium hirsutum	MYB38	AAK19618	
	Lotus japonicus	*TT2a*	AB300033	Yoshida *et al*., 2008
		TT2c	AB300035	Yoshida *et al*., 2008
	Oryza sativa	Rc	ABM68351	Sweeney *et al*., 2007
	Prunus dulcis	MYB185	ABR13320	
	Populus tremuloides	MYB134	ACR83705	Mellway *et al*., 2009
	Populus trichocarpa	MYB115	EEE81917	
		MYB201	EEE99191	
	Vitis vinifera	MYBPA1	CAJ90831	Bogs *et al*., 2007
		MYBPA2	ACK56131	Terrier *et al*., 2009
	Zea mays	C1	AAA33482	Paz-Ares *et al*., 1987
Flavonol	*Arabidopsis thaliana*	MYB11	CAB83111	Stracke *et al*., 2007
		MYB12	ABB03913	Mehrtens *et al*., 2005
		MYB111	AAK97396	Stracke *et al*., 2007
	Lotus japonicus	MYB12	BAF74782	
	Malus x domestica	MYB22	AAZ20438	
	Solanum lycopersicum	MYB12	ACB46530	Luo *et al*., 2008
	Sorghum bicolor	Y1	AAX44239	Boddu *et al*., 2005
	Vitis vinifera	MYBF1	ACV81697	Czemmel *et al*., 2009
Other	*Antirrhinum majus*	MYB308	P81393	Jackson *et al*., 1991
	Arabidopsis thaliana	MYB4	CAC40021	Jin *et al*., 2000
		MYBL2	AAS09989	Matsui *et al*., 2008
		GL1	NP_189430	Larkin *et al*., 1994
		TT12	CAC36941	Debeaujon *et al*., 2001
	Eucalyptus gunnii	MYB1	CAE09058	Legay *et al*., 2007
		MYB2	CAE09057	Goicoechea *et al*., 2005
	Fragaria ananasa	MYB1	AAK84064	Aharoni *et al*., 2001
	Gossypium hirsutum	MYB9	AAK19619	
	Humulus lupulus	MYB1	BAF46265	
	Malus x domestica	MYB2	unpublished *	
	Populus trichocarpa	MYB221	ACN97176	
	Prunus persica	Prunus_v2_contig 1162 (TT12-like)	BU043948[#] AJ533665[#]	
	Vitis vinifera	MYB4		
		MYB5a / MYBCs1	AAS68190	Deluc *et al*., 2006
		MYB5b	AAX51291	Deluc *et al*., 2008
		AM1	ACN91542	Cutanda-Perez *et al*., 2009
	Zea mays	MYB31	CAJ42202	
		MYB42	CAJ42204	

* Sequence courtesy of Dr Adam Takos

[#] ESTs from the Genome Database for Rosaceae (GDR)

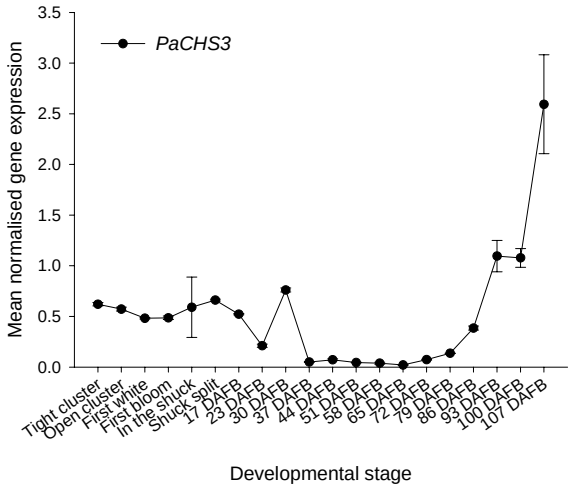
Accession for sweet cherry sequences can be found in Table 2.1. References refer to functional characterisation

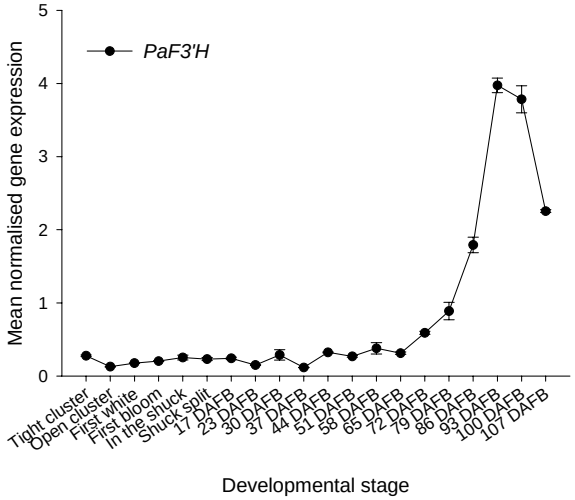
APPENDIX 7: REFERENCE PROTEINS FOR MOTIF IDENTIFICATION

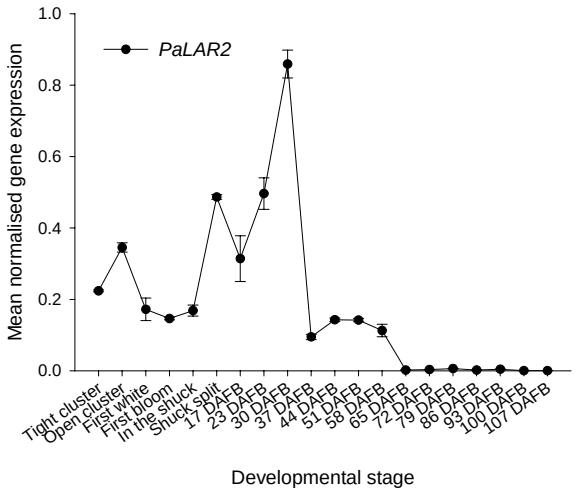
Motif names	Reference proteins
SG6 (mod1)	AtPAP1, PhAN2, MdMYB1, VvMYBA1, PaMYBA1, PaMYBA2
SG6-A1*	PaMYBA1, P. communis MYB10, P.cerasus MYB10, PdMYB10
SG6-A2^	PaMYBA2, P. armeniaca MYB10, P. avium MYB10
SG5-2, SG5-3, SG5-4, SG5-5	PaMYBPA1, VvMYBPA1, PdMYB185, PtMYB115, PtMYB201
SG5a (mod2)	VvMYBPA2, GhMYB38, PtMYB134, LjTT2a, LjTT2c, AtTT2, BnTT2-2
SG7-2 (mod1)	AtMYB11, AtMYB12, AtMYB111, VvMYBF1, MdMYB22, PaMYBF1, LjMYB12, SlMYB12, GhMYB1
SG4 (mod1), C1/SG4-2 (mod1), SG4-3	PaMYBR, AtMYB4, VvMYB4, PtMYB221, HlMYB1, MdMYB2, GhMYB9, EgMYB1, AmMYB308, ZmMYB31, ZmMYB42, FaMYB1

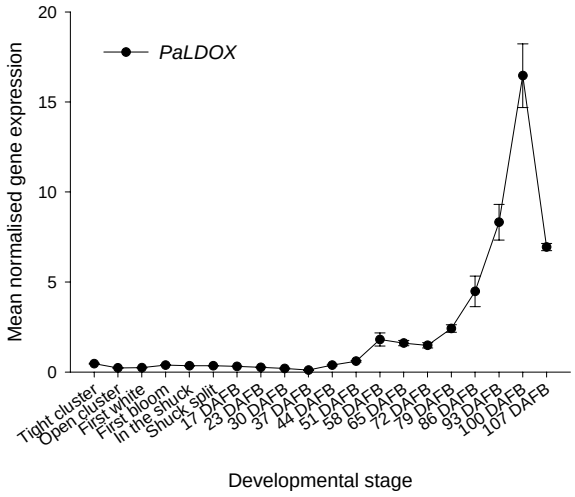
For protein accession numbers, see Appendix 6

APPENDIX 8: EXPRESSION OF ADDITIONAL GENES IN DEVELOPING 'LAPINS' FRUIT









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