**Title: Regulation of anthocyanin accumulation in apple by the transcription factor MdMYB10**

**ABSTRACT**

Anthocyanin concentration is an important determinant of the colour of many fruits. In apple (Malus x domestica), centuries of breeding have produced numerous varieties in which levels of anthocyanin pigment vary widely and change differently in response to environmental and developmental stimuli. The apple fruit cortex is usually colourless, although germplasm does exist where the cortex is highly pigmented due to the accumulation of anthocyanins, carotenoids or chlorophylls. From studies in a diverse array of plants species, it is apparent that anthocyanin biosynthesis is controlled at the level of transcription. In this thesis the transcript levels of the anthocyanin biosynthetic genes in a red-fleshed apple are compared with a white-fleshed cultivar. A MYB transcription factor (TF), MdMYB10, was isolated and is shown to be similar in sequence to known anthocyanin regulators in other species. Further, this TF is shown to induce anthocyanin accumulation in both heterologous and homologous systems, generating pigmented patches in transient assays in tobacco leaves and highly pigmented apple plants following stable transformation with constitutively expressed MdMYB10. Efficient induction of anthocyanin biosynthesis in transient assays by MdMYB10 was dependent on the co-expression of two bHLH proteins from apple, MdbHLH3 and MdbHLH33. A strong correlation between expression of MdMYB10 and apple anthocyanin levels during fruit development suggests that this TF is responsible for controlling anthocyanin biosynthesis in apple fruit; in the red-fleshed cultivar and in the skin of other varieties, there is an induction of MdMYB10 expression concurrent with colour formation during development.

Mutations in the genes encoding for either the biosynthetic or transcriptional regulation of the anthocyanin pathway have been linked to colour phenotypes. Generally this is a loss of function resulting in a reduction or a change in the distribution of anthocyanin. The upstream regulatory region of MdMYB10 was investigated and found to contain a rearrangement. This modification is responsible for increasing the level of anthocyanin throughout the plant to produce a striking phenotype that includes red foliage and red fruit flesh. It consists of a series of multiple repeats, forming a minisatellite-like structure that comprises five direct tandem repeats of a 23 base pair sequence. This MdMYB10 rearrangement is present in all the red foliage apple varieties and species tested, but in none of the white fleshed varieties. Transient assays demonstrated that the 23 bp sequence motif is a target of the MdMYB10 protein itself, and the number of repeat units correlates with an increase in transactivation by MdMYB10 protein. The repeat motif is capable of binding MdMYB10 protein in electrophoretic mobility shift assays. Taken together, these results indicate that a rearrangement in the promoter of MdMYB10 has generated an autoregulatory locus and this autoregulation is sufficient to account for the increase in MdMYB10 transcript levels and subsequent ectopic accumulation of anthocyanins throughout the plant.

Characterisation of MdMYB10 and the rearrangement in the promoter region has implications for the development of new varieties through classical breeding or a biotechnological approach. Understanding whether this mutation is simply an allele of other recently published apple MYB TFs, or if this is the only R2R3 MYB TF involved in apple anthocyanin response, is a challenge for future research.

**Declaration of work carried out by the candidate**

All work was carried out by the candidate except as noted below:

Chapter 3:

-All the apple EST sequences and EST-derived cloning reactions referred to in this and subsequent sections were carried out by the sequencing and cloning team, in particular, Dr Andrew Gleave and Sakuntala Karunairetnam, Plant & Food Research, Auckland. Section 3.2.1 and throughout the thesis.

-The phylogenetic tree of Arabidopsis MYB transcription factors was created by Dr Andrew Allan, Plant & Food Research, Auckland. Section 3.2.7.

Chapter 4:

-LC-MS assays and analysis was conducted by Janine Cooney, Plant & Food Research, Ruakura. Section 4.2.4.

-Stable tobacco transformation was performed by Kui Lin-Wang, Plant & Food Research, Auckland. Section 4.2.5.

-Apple transformation (35S:MdMYB10) and tissue culture was carried out by Sumathi Kutty-Amma, Plant & Food Research, Auckland. Section 4.2.7.

-Accelerated growth of transgenic apple trees was carried out at the National Climate Laboratories in Palmerston North and all plant husbandry was conducted by Cara Norling and Jonathon Crawford. Section 4.2.8.

Chapter 5:

-The mapping-based association study was conducted by Dr David Chagné, Plant & Food Research, Palmerston North. Section 5.2.8.

Chapter 6:

-Apple transformation (R6:MdMYB10) and tissue culture was carried out by Sumathi Kutty-Amma, Plant & Food Research, Auckland. Section 4.2.7.

In addition, some photographs and images were generated by Plant & Food Research staff as credited in the figure legends. Where training or assistance was supplied by colleagues, but the work carried out by the author, this is stated in the acknowledgements as customary.

**1 INTRODUCTION**

**1.1 Preface**

The biosynthesis and regulation of the anthocyanin plant pigments are one of the most intensely studied aspects of plant secondary metabolism. The variety of anthocyanins and the myriad roles they provide, both to the host plant and to the consumers of the plant, present a rich and fascinating area for study. The study of the biology of anthocyanins in plants has been helped by the ease of identifying phenotypic changes and mutants. Anthocyanin concentration is an important determinant of the colour of many fruits. In apple (Malus x domestica), centuries of breeding have produced numerous varieties in which levels of anthocyanin pigment vary widely and change in response to environmental and developmental stimuli. Anthocyanin accumulation is usually restricted to the skin of apple and these pigments provide essential cultivar differentiation for consumers and are implicated in the health attributes of apple fruit. The apple fruit flesh (cortex) is usually colourless, although germplasm does exist where the cortex is highly pigmented due to the accumulation of either anthocyanins and/or carotenoids.

From studies in a diverse array of plant species, it is apparent that anthocyanin biosynthesis is controlled at the level of transcription (Ramsay and Glover, 2005). Research into the activation and repression of anthocyanin biosynthesis in apple fruit has shown the presence of both developmental and environmental control mechanisms. The control of anthocyanin accumulation in apple is a key element in understanding and manipulating fruit colour. Identification of the factors that exert this control may provide the tools for moderating the extent and distribution of anthocyanin-derived pigmentation in fruit tissue, an important target for the introduction of new apple varieties. At the inception of this project, this regulation had not been characterised at the transcriptional level.

This thesis centres on the regulation of anthocyanin accumulation in apple and examines the role of a MYB transcription factor (TF) in determining anthocyanin-related phenotypic changes in apple trees and fruit. To provide a context for the areas covered in this thesis, the introduction will deal with three principal topics. Firstly, an overview of the origin of apples will be given and the commercial importance of the apple industry today will be discussed. Secondly, the types and uses of the major plant pigments will be outlined, with emphasis on anthocyanins. Finally, the regulation of anthocyanin production in plants will be covered and how this regulation might be manipulated in apple.

**1.2 The origins and domestication of apple**

**1.2.1 The centre of origin**

In 1931, the eminent Russian plant scientist, Nikolai Vavilov, wrote:

Alma-Ata translated means ‘Father of Apples’. Around the town and at a great distance along the mountain slopes, the overgrowths of wild apples are spread, creating massive forests here…..It is possible to see with our own eyes that we are in the remarkable centre of origin of the cultivated apple….Individual trees bear fruit which in quality is not inferior to that of cultivated forms. Some are of astonishingly large size and exceptional productivity.

Vavilov was instrumental in establishing the origin of many species (Vavilov, 1931) and his theory, concerning the geographical centre of origin of the apple, has provided the inspiration for others. Most notably the research of Aimak Dzhangaliev, who dedicated his career to the conservation of the wild apple species (Dzhangaliev, 2003).

Alma-Ata, now refererred to as Almaty, is the largest city in Kazakhstan and, until 1998, the capital. The mountains that Vavilov mentioned were the Tien Shan Mountains, located in central Kazakhstan (Figure 1-1). This region contains the centre of origin for Malus and related genera such as Pyrus, Sorbus and Cratageous as well as being the centre of origin for apricot (Karychev et al., 2005). It is here that Malus sieversii is found and there is genetic evidence that this species is the progenitor of the cultivated apple (Harris et al., 2002). Wild apples have been discovered in a region that stretches from Central Asia to Western China (Zhang et al., 1993; Watkins, 1995). Malus is classified as a member of the Rosaceae family, subfamily Maloideae, with 27 wild species that constitute the genus Malus (Way et al., 1991; Forsline et al., 2003). These species are extremely variable in tree form, fruit size and fruit colour (www.ars.usda.gov/main/.htm). Hybridisation of these species would have been possible by seed dispersal by birds as well as ruminants such as deer and antelope and bear (Juniper and Mabberley, 2006). The proximity of the Old Silk Road to the centre of origin is likely to have played an important role in the East-West dispersal of apple species into Europe and China.

**1.2.2 Domestication**

The process of apple hybridisation would have been accelerated by the increasing popularity of ancient trading routes, particularly the Old Silk Road. Horses, donkeys and mules would have eaten the fruit and distributed seed along the routes. This may have opened up hybridisation possiblities for species in geographically isolated areas. It is thought that in the Central Asian region there are as many as 6,000 varieties (Juniper and Mabberley, 2006). It is likely that apples for human consumption would have spread from Central Asia along the trade routes. Initially, domesticated apples would have been grown from seed and random hybridisations would have taken place, but with the advent of new technologies, such as grafting, it was possible to produce more uniform crops of the best selections (Juniper et al., 1998). Today there are more than 20,000 named varieties. Within the varieties the naming of the cultivated apple is somewhat controversial. It has been argued that the commonly used name Malus x domestica (Korban and Skirvin, 1984) is botanically incorrect and that the correct term is Malus pumila (Mabberley et al., 2001). Due to its continued widespread use, Malus x domestica has been used throughout this thesis.

**1.3 The apple industry**

**1.3.1 Worldwide apple production**

World fruit production stands at around 500 million tonnes, with apple one of the the most important crops, accounting for around 12% of total fruit production (Figure 1-2). Apple production value is estimated at US$10 billion (Food and Agriculture Organisation of the United Nations [www.faostat.fao.org]). Apple is the most versatile of the temperate fruit crops, capable of growing in diverse conditions and severe temperature extremes from high latitude regions to high elevations in the tropics (Forsline et al., 2003).

In economic terms, apple is the most important of the Rosaceae family. China accounts for 36% of total production, far in excess of the next largest producer, the USA, with 7.5% (Table 1-1). Turkey, France, Italy and Iran are all leading exporters for Northern Hemisphere production, whilst Southern Hemisphere producers and exporters are primarily Chile, Argentina, New Zealand and South Africa (O'Rourke, 2007). New Zealand accounts for less than one percent of world apple production and approximately 5% of total world exports.

**1.3.2 New Zealand apple production**

ties TM r improvement. This would involve the manipulation of pigmentation in the skin and/or flesh. The New Zealand apple industry is centered in the Hawkes Bay region in the North Island (61%) and the Nelson region in the South Island (26%), with a workforce of around 10,700 (Kerr et al., 2007). The major export destinations for New Zealand fruit and vegetables are Japan, the USA, the UK, the European Union (EU) and Australia. For apple exports, this profile changes slightly to reflect local production or import restrictions, with the UK, EU and USA providing the largest markets. Fresh apples valued at NZ$343 million were exported to 61 countries in 2007. Export varieties are predominantly ‘Braeburn’ (36%) and ‘Royal Gala’ (35%). Both varieties originate from New Zealand breeding programmes. In 2007, the comparative figures for kiwifruit and other fresh fruit exports from New Zealand were NZ$765 million and NZ$88.8 million respectively. While New Zealand horticultural exports have been steadily increasing in recent years (from NZ$118 million in 1988 to NZ$2.6 billion in 2007), the industry statistics for apple show a decline in production and export volumes and in the number of growers and area planted (Table 1.2). This has been party offset by large gains in productivity with yield in 2006 estimated at 47 tonnes per hectare which compares favourably with other competitors such as South Africa (33 tonnes per hectare) and Australia (20 tonnes per hectare) (Pipfuit New Zealand corporated, 2007, [www.pipfruitnz.co.nz](http://www.pipfruitnz.co.nz)).

The major competitors for the New Zealand export industry are other Southern Hemisphere producers which export to the Northern Hemisphere such as Chile, South Africa, Argentina and Brazil. The New Zealand industry has been aided by the introduction of New Zealand varie such as ‘Royal Gala’, ‘Braeburn’, the Pacific Series and SciFresh (JAZZ ). Competitors produce large quantities of varieties such as ‘Red Delicious’, ‘Granny Smith’ and ‘Golden Delicious’ but are increasingly growing quantities of the New Zealand introductions, ‘Royal Gala’ and ‘Braeburn’. New Zealand's competitiveness is reflected in its market returns. In the major markets of Europe, Asia and North America, New Zealand's share of returns is greater than its market share by volume. Increasing competition and a contracting market for apples (O'Rourke, 2007) has increased the need for the introduction of high quality, novel varieties, ideally with strong differentiation. Apple fruit colour could provide a product delineating feature for improvement. This would involve the manipulation of pigmentation in the skin and/or flesh.

**1.4 Plant pigments**

**1.4.1 Major plant pigments**

Excluding chlorophylls, there are three main classes of pigments for colouration in plants. These are the carotenoids, the betalains and the flavonoids (including anthocyanins). Carotenoids are isoprenoids and are essential for plant life, for example in photoprotection during photosynthesis (Green and Durnford, 1996). The carotenoid pathway has been extensively studied at the enzymatic level (Cunningham and Gantt, 1998; Howitt and Pogson, 2006). While there is evidence that the biosynthetic genes are regulated at the transcriptional level (Sandmann et al., 2006), the regulation of the pathway is still not fully understood. Carotenoids are widely distributed in plants, synthesised in chloroplasts and are lipid soluble. They confer a range of olours from red to orange in fruit, flowers and senescing leaves.

Betalains are limited to to just one order, the Caryophyllales, which includes including red beet (Beta vulgaris). They are derived from tryrosine, are water soluble and synthesised and stored in vacuoles (Grotewold, 2006). They confer yellow to red pigmentation and are more stable than anthocyanins, being less sensitive to pH. Unlike the flavonoids and carotenoids, betalains are not well characterized.

Anthocyanin pigments belong to the diverse group of ubiquitous secondary metabolites, collectively known as flavonoids. Flavonoid metabolism is one of the best characterised processes in plants (Harborne, 1967; Grotewold, 2006). The water soluble flavonoids are synthesised in the cytosol and localised to vacuoles. Flavonoids demonstrate a wide spectrum of colours from pale yellow to deep blue. They are believed to have a variety of functions, including defence and protection against light stress, and have been implicated in auxin transport (Brown et al., 2001; Peer and Murphy, 2007). The pigmented anthocyanin compounds play an important physiological role as attractants in plant/animal interactions (Harborne and Grayer, 1994; Koes et al., 1994). Plant pigments are not mutally exclusive and anthocyanins and carotenoids can co-exist, increasing the depth or variety of colour. However, anthocyanins and etalains have been shown to never co-exist (Stafford, 1994).

**1.4.2 Anthocyanins - uses and importance in plants**

The broad distribution of anthocyanins in angiosperms has led to extensive scientific characterisation (Harborne, 1988; Holton and Cornish, 1995; Harborne and Williams, 2000). Anthocyanins play a major role in reproductive tissue. In combination with other factors, such as fragrance, anthocyanins are the most obvious clue for pollinating insects and birds (Harborne and Grayer, 1994). Plants have adapted their anthocyanin composition on an ecological basis, with different colours present in different environments depending on the pollinators present (Harborne, 1993). Insect and bird-pollinated plants tend to have brightly coloured petals to advertise a reward, such as nectar (Koes et al., 1994). Similarly, anthocyanins perform a key role in seed dispersal, using pigmentation to attract fruit-eating animals (Cipollini and Levey, 1997)

Anthocyanins are also found in vegetative tissue, most noticeably in the senescing leaves of many deciduous trees in autumn (Lee, 2002). One possible explanation for this phenomenon is that anthocyanins play a role in protecting the photosynthetic apparatus during nutrient reabsorption (Hoch et al., 2003). This may be realised by light attenuation or the provision of antioxidant protection (Gould et al., 2002a). Similar mechanisms may be advantageous for the red leaves of understory plants in rainforests and for leaves under stress (Gould et al., 2002a).

Levels of anthocyanins are elevated in response to many environmental, developmental and pathological stimuli. In apple, pigment biosynthesis can be induced when fruit are exposed to light, particularly UV (Dong et al., 1998), a phenomenon also observed in other species such as maize (Piazza et al., 2002). Anthocyanin levels can be elevated by many different stress treatments including cold temperature storage of fruit (Dong et al., 1998). There is evidence that the anthocyanin biosynthetic enzymes are co-ordinately induced during development of apple fruit, with pronounced anthocyanin enzyme activity correlating with pigmentation increases in immature fruit, followed by a second peak of activity at ripening, depending on the cultivar (Lister and Lancaster, 1996). This suggests that the expression of the genes encoding the biosynthetic enzymes is co-ordinately regulated by one, or a few, regulatory proteins, such as TFs.

**1.4.3 Anthocyanins and human health**

Anthocyanin pigments have been implicated in a diverse range of health-promoting properties (Lila, 2004). The antioxidant properties of anthocyanins from various fruit towards free radicals and reactive oxygen species has been established (Smith et al., 2000; Chun et al., 2003), although the level of activity is dependent on the chemical structure and bioavailability. Recently, it has been suggested that the dietary antioxidant benefit may be conferred by the anthocyanin-related activation of endogenous antioxidant systems, rather than the anthocyanins themselves (Williams et al., 2004; Lotito and Frei, 2006). Anthocyanins may protect against age-related neurological disorders (Joseph et al., 1999) and anthocyanins have also been implicated in enhanced immune function (Wang and Mazza, 2002), restoration of vision disorders (Matsumoto et al., 2003) and anti-imflamatory properties (Shin et al., 2006). As part of a balanced diet, anthocyanins may also help reduce the incidence of obesity (Tsuda et al., 2003). Anthocyanins have been associated with a reduced incidence of coronary heat disease, termed the French Paradox (Renaud and de Lorgeril, 1992) and the prevention of atherosclerotic lesions in arteries (Tsuda et al., 2000). There have been numerous studies linking anthocyanins with a reduction in cancer initiation and proliferation (Smith et al., 2000).

Much of the evidence for the bioactivity and health properties of anthocyanins has been achieved using in vitro studies. Following absorption in the body, these molecules may be metabolised to the extent that plasma and tissues are not exposed to the original forms of the anthocyanins (Kroon et al., 2004). However, two recent studies have shown the beneficial effects of anthocyanins in vivo. A feeding trial using cancer susceptible mice demonstrated that tomatoes that had been genetically engineered with a high anthocyanin content significantly reduced the incidence of tumorigenesis and prolonged the animal’s lifespan (Butelli et al., 2008). A study in rats showed that feeding the animals with maize rich in anthocyanin, increased cardioprotection (Toufektsian et al., 2008). Both studies showed that plant-derived anthocyanins were beneficial, promoting the cause for anthocyanin-rich food in the human diet.

**1.5 The anthocyanin biosynthetic pat in biosynthetic pathway**

**1.5.1 Structural genes and biochemistry**

The predominant precursors for all flavonoids, including anthocyanins, are malonyl-CoA and pcoumaroyl-CoA (Dixon and Steele, 1999). From these precursors the enzyme chalcone synthase (CHS) forms chalcone, the first committed step in anthocyanin production and the establishment of the C15 backbone(Figure 1-3).

Chalcone is then isomerised by chalcone isomerase (CHI) to produce chalcone naringenin, and from there a hydroxylation step, via flavanone 3β-hydroxylase (F3H), converts naringenin to dihydroflavonol. The reduction of dihydroflavonol by dihydroflavonol 4-reductase (DFR) produces leucoanthocyanidin and this is converted into the coloured compound anthocyanidin by leucoanthocyanidin dioxygenase (LDOX). This is followed by a glycosylation step which is usually mediated by uridine diphosphate (UDP)-glucose:flavonoid 3-0-glucosyltransferase (UFGT). There may be further additions of sugars and acyl side groups following this, in some species. Differences in colour attributable to anthocyanins can be due to a number of factors including the number of hydroxyl groups on the B-ring, the sugars and acyl side groups (Harborne, 1967), the environment of the vacuole including its pH (Koes et al., 2005), or the accumulation of specific metal ions (Brouillard, 1988), or cellular ultrastructure (Noda et al., 1994). One of the most common anthocyanin pigments is cyanidin which, in the form of cyanidin 3-0-galactoside, is the pigment primarily responsible for red colouration in apple skin (Lancaster, 1992; Tsao et al., 2003). The enzymes operating in this biosynthetic pathway in pple have been well characterised (Honda et al., 2002; Kim et al., 2003).

**1.5.2 Transport and storage**

Many of the properties of anthocyanin cannot be realised until the anthocyanins are localised correctly in the cell, such as the relationship between the colour of anthocyanin compounds and the pH of the vacuole (Kitamura, 2006). The transport mechanism for subcellular sequestration of anthocyanins synthesised in the cytosol is less well understood than the biosynthesis, and different species may use different approaches. However, some of the mechanisms involved in trafficking are beginning to emerge (Grotewold, 2004). Transport of anthocyanins by glutathionine S-transferase (GST) proteins has been shown in Petunia (Mueller et al., 2000) and in maize (Goodman et al., 2004). An alternative non-GST linked mechanism has been shown as vesicle-mediated transport in Arabidopsis where endoplasmic–reticulum (ER)-derived vesicles transport anthocyanins to the vacuole (Poustka et al., 2007). A third method may involve ATPdependent ‘multidrug and toxic compound exclusion’ (MATE) transporters such as TT12 in Arabidopsis, although this was established for flavonoids, rather than nthocyanins.

There are a number of different vacuole types in plants (Marty, 1999), and it is not clear as to whether anthocyanins accumulate in one particular type. This may depend on the species or transport mechanism. In some species anthocyanin storage in flower petals has been identified as matrix-like inclusions in vacuoles, termed anthocyanin vacuolar inclusions (AVIs) (Markham et al., 2000).

**1.6 Transcription factors**

Most properties of a cell are dependent on the genomic information it contains and the accessibility or transcription of this information. The transcription of genes is mainly controlled by a collection of cis-acting sequences. These elements are typically found in proximity to the coding sequence of the gene and multiple copies of numerous classes may be present. It is thought that the transcription of most genes is controlled by a combination of TFs and that these gene-specific TFs will be controlled by hierachical networks of other regulatory elements, including other TFs (Hobert, 2008). Transcription is generally initiated at the TATA-box in the proximal promoter region by the formation of the transcription initiation complex by general TFs and the recruitment of RNA polymerase II (reviewed in Zawell and Reinberg, 1995). This may be sufficient for low level transcription, but for specific, temporal and/or spatial transcription, other TFs are required. The proteins of these TFs bind to specific DNA sequences in the promoter regions of the target genes and can either enhance or repress transcription (Fickett and Wasserman, 2000).

TF proteins generally possess distinct functional domains, such as a DNA binding domain and a transcriptional activator domain. The DNA binding domain is highly conserved within the separate classes of TF and contains trans-acting amino acids that bind to the cis-acting sequences of target gene promoters. There may be multiple examples of these DNA domains, such as the large family of R2R3 domain MYB TFs. Activator domains may be less well conserved and are involved in the enhancement or repression of transcription, modulating the rate of mRNA synthesis by RNA polymerse II. However, transcription regulation may depend on the interaction of binding domains and activator domains of different TFs or on the homo- or heterodimerisation of the TF proteins. TFs may also regulate more than one gene, such as the multi-gene regulation by the Arabidopsis PAP1 MYB TF which enhances the transcription of a number of structural genes in the anthocyanin pathway (Tohge et al., 2005). Similarly, TFs may co-ordinate regulation in a combinatorial manner, with different classes of TF forming complexes at the protein:DNA or protein-protein level (Grotewold et al., 2000).

TFs are divided into families on the basis of the conserved DNA binding domain and, increasingly, on functional analysis. A genome-wide study in Arabidopsis showed that there were over 1,500 genes classified as TFs, divided into ~ 30 TF families, representing 6% of the Arabidopsis genome (Riechmann et al., 2000). The number of TFs in Arabidopsis is now over 2,290 (Guo et al., 2008). Considerable conservation of TFs is apparent even between animals and plants, suggesting a shared origin of regulatory circuitory. However, some TF families may be exclusive to plants, such as the DOF family. Studies into the evolution of these regulatory networks show that even closely related organisms may have evolved very different mechanisms for specific regulation (Tuch et al., 2008). The classes of TF directly involved in anthocyanin accumulation will be discussed in the next section.

**1.7 Regulation of anthocyanin biosynthesis by TFs**

**1.7.1 MYB TF regulation of anthocyanin levels**

A range of different TF classes are involved in flavonoid regulation including bHLH, WD40, WRKY, bZIP, HD-Zip, and MADS-box (Kubo et al., 1999; Martin et al., 2001; Davies and Schwinn, 2003; Ramsay and Glover, 2005). MYB TFs are found in all eukaryotes but are particularly abundant in plants and are thought to be one of the most important plant regulatory gene families (Jia et al., 2004). The term MYB is derived from the oncogenic avian retrovirus avian myeloblastosis virus (AMV), which carries an oncogene, v-myb, which, is itself a partial copy of a normal cellular gene found in animals, c-myb (Leprince et al., 1983; Klempnauer et al., 1986). Plant MYB TFs show structural similarities to mammalian versions and a 40% sequence homology to the products of myb proto-oncogenes from human, mouse, chicken and Drosophila (Paz-Ares et al., 1987), suggesting that these MYB genes were present before the divergence between plants and animals. However, there has clearly been a divergence in the physiological function of these genes as the anthocyanin-related MYB TFs in plants regulate a biosynthetic pathway that does not exist in mammals. In plants, MYB TFs have been implicated in controlling a diverse array of pathways and processes (Jin and Martin, 1999). One of the best characterized is the MYB-related control of phenylpropanoid secondary metabolism, including the biosynthesis of flavonoids and anthocyanins (Paz-Ares et al., 1987; Grotewold et al., 1994; Sablowski et al., 1994; Quattrocchio et al., 1998; Aharoni et al., 2001). Other characterized MYB TF regulation includes cell fate and development (Oppenheimer et al., 1991; Noda et al., 1994; Nesi et al., 2001; Kasahara et al., 2005), hormone response and signal transduction (Stracke et al., 2001; Abe et al., 2003). They are characterised by a structurally conserved Nterminal DNA binding domain consisting of single or multiple imperfect repeats, 1RMYB, R2R3MYB and 3R MYB (Stracke et al., 2001). These repeats each form three helixes, the third of which binds directly with the major groove of the DNA target (Williams and Grotewold, 1997). There are 126 R2R3 MYB TFs in Arabidopsis, which can be divided on the basis of their sequence into 24 sub groups (Stracke et al., 2001). Several anthocyanin pathway associated MYB TFs are of the two-repeat (R2R3) class.

There are numerous reports of the regulation of genes in the anthocyanin pathway by TFs (Allan et al., 2008). Collectively they have established that the components of the regulatory complex controlling anthocyanin biosynthesis are conserved in all higher plants (reviewed in Holton and Cornish, 1995). MYB TFs have been shown to play an important role in transcriptional regulation of anthocyanins (reviewed in Martin and Paz-Ares, 1997). Regulation appears to be specific to discrete subsets of structural genes, acting either early or late in the anthocyanin biosynthetic pathway (Davies and Schwinn, 2003). The point of this differentiation between early and late biosynthetic genes appears to be at F3H or DFR (Figure 1-3), depending on flavonoid co-production. Not all species show this distinction. In the leaves of perilla (Perilla frutescens) co-ordinate transcriptional regulation controls virtually all steps of anthocyanin biosynthesis from the genes encoding CHS to the final genes encoding anthocyanin transporter proteins (Saito and Yamazaki, 2002). In contrast, in grape (Vitis vinifera), specific regulation by MybA is restricted to the very late steps in anthocyanin production including UFGT, but not the earlier biosynthetic steps (Kobayashi et al., 2002). Anthocyanin regulation by MYB TFs has been most extensively studied in Arabidopsis, Petunia, maize and Antirrhinum.

In Arabidopsis, the PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1) MYB (Borevitz et al., 2000) falls into subgroup 10 (when the phylogeny of Stracke et al., 2001 is used) and demonstrates a high degree of amino acid conservation with other known MYB regulators of anthocyanin production. When PAP1 was over-expressed in transgenic Arabidopsis this led to up-regulation of a number of genes in the anthocyanin biosynthesis pathway, including CHS and DFR, and the plants were highly pigmented (Figure 1-4) (Borevitz et al., 2000). A more recent study using a combination of transcriptomics and metabolomics showed that the over-expression of PAP1 resulted in the activation of virtually all the anthocyanin pathway, together with related TFs and ancillary genes involved in anthocyanin transport (Tohge et al., 2005). A second MYB TF, PAP2, which is 93% similar at the protein level to PAP1, also elevated PAL, CHS, DFR and GST when over-expressed in tobacco (Borevitz et al., 2000) and may be a functional duplicate of PAP1. PAP1 has also been implicated in the sugar-induced accumulation of anthocyanin (Solfanelli et al., 2006) and is elevated in scenescing leaves (Pourtau et al., 2006).

In Petunia, regulation of the anthocyanin pathway is by the MYB Anthocyanin2 (AN2) or AN4 (Quattrocchio et al., 1999; Spelt et al., 2002). This is largely due to elevation of the late biosynthetic genes, while there is also evidence that AN2, in combination with other TFs, can co-ordinate multiple processes that include anthocyanin accumulation and acidification of the vacuole (Baudry et al., 2006). Two other MYB TFs in Petunia, PhMYB3 (Solano et al., 1995) and PhMYB27 (Spelt et al., 2000) have been reported to control the early anthocyanin biosynthetic genes and repression of the pathway respectively.

Several MYB genes are involved in anthocyanin regulation in Antirrhinum flower petals, where this also appears to be divided into regulation of the early and the late biosynthetic pathway. Transcript levels of early biosynthetic genes have been shown to be elevated by the MYB TFs AmMYB305 and AmMYB340 (Sablowski et al., 1994; Mol et al., 1998). The later biosynthetic genes are regulated by the MYB gene family Rosea1, Rosea2, and Venosa. A recent study showed that these three MYB TFs were responsible for differences in flower colour and patterning (Schwinn et al., 2006).

Maize provided the first studied example of a plant MYB (Paz-Ares et al., 1987). Reports have shown that C1 regulates several genes in the anthocyanin pathway in maize kernels (Paz-Ares et al., 1987; Goff et al., 1991). The control of maize anthocyanin is according to tissue type. A functional duplicate MYB to C1, the light-activated PL, controls anthocyanin accumulation in maize leaves (Cone et al., 1993; Gong et al., 1999), while the MYB TF, P, regulates flavonoid biosynthesis in floral organs (Grotewold et al., 1994). However, the MYB TFs are not solely responsible for anthocyanin regulation and at least two other classes of TF are required.

**1.7.2 Combinatorial TF regulation of the anthocyanin pathway**

All of the MYB TF examples from maize, Arabidopsis, Antirrhinum and Petunia have been shown to interact with other TFs (Table 1-3). The R2R3 MYB factors that regulate anthocyanin biosynthesis have been shown to interact closely with basic Helix Loop Helix (bHLH) TFs (Mol et al., 1996; Winkel-Shirley, 2001). Examples of interacting partners include the maize ZmC1 MYB TF and members of the maize R gene family of bHLH TFs, the Petunia AN2 MYB and AN1/JAF13 bHLH TFs and the Antirrhinum Rosea1, Rosea2 and Venosa MYB TFs and the Mutabilis and Delila bHLH TFs (Ludwig and Wessler, 1990; Goff et al., 1992; Goodrich et al., 1992; Mol et al., 1998; Schwinn et al., 2006). Over-expressing lines of PAP1 have elevated transcript levels of the TT8 gene which encodes a bHLH protein involved in regulating condensed tannin and anthocyanin biosynthesis (Nesi et al., 2000; Tohge et al., 2005). The dependency of these specific MYB-related proteins on a bHLH co-regulator to activate transcription is linked to the presence of a small number of amino acid residues (a signature motif) in the highly conserved R2R3 binding domain of the MYB proteins, as revealed by comparison between the structure of the maize P protein (which activates in a bHLHindependent manner) and the bHLH-dependent maize C1 protein (Grotewold et al., 2000). Substitution of just six amino acids from the R2R3 domain of MYB C1 into the corresponding positions in MYB P resulted in a mutant with bHLH-dependent behaviour, similar to C1. More recently it was suggested that this might be a key mechanism that permits MYB TFs to discriminate between target genes (Hernandez et al., 2004).

In contrast to PAP1 and other anthocyanin activating MYB TFs, FaMYB1, represses anthocyanin biosynthesis during the late development of strawberry fruit (Aharoni et al., 2001). FaMYB1 shares homology with MYB TFs that can activate anthocyanin genes and can interact with bHLH TFs such as the Petunia AN1 and JAF13 (Aharoni et al., 2001). However, despite the conservation of key residues between PAP-like activators and FaMYB-like repressors, phylogeny clustering and analysis shows that activators tend to fall in subgroup 10 while repressors fall in subgroup 4 (according to the classification of Stracke et al., 2001).

An additional level of anthocyanin regulation involves a separate class of proteins, containing WD40 domains, which form complexes with MYB and bHLH proteins (Baudry et al., 2004). These WD40 proteins may facilitate the protein:protein interactions of other TFs, such as MYB and bHLH, and in these situations may not specifically bind the DNA of structural genes (Ramsay and Glover, 2005). Examples include an11 in Petunia (de Vetten et al., 1997) and TTG1 in Arabidopsis (Walker et al., 1999). However, a definitive role for a WD-repeat in the transcriptional complex regulating anthocyanin biosynthesis has yet to be demonstrated. Indeed, in maize, the WD repeat protein, PAC1, appears to function in regulating anthocyanin biosynthesis only in a subset of tissues (Carey et al., 2004). It may be that some bHLH and MYB proteins are more dependent on the participation of WD repeat proteins in the regulatory complex than others.

A model for the TF regulation of anthocyanin has been based on a consensus of reports (Figure 1-5) and comprises a ternary complex. However, this model is not necessarily complete. For example, a recent report showed that the Arabidopsis MYB TF, MYBL2, acts as a transcriptional inhibitor and may counter-balance the positive regulation of anthocyanin biosynthesis by the MYB/bHLH/WD40 complex (Matsui et al., 2008). While the combinatorial control of the anthocyanin pathway is complex, the plethora of previous research into this topic in a number of species eases the task of unravelling its regulation in other species, such as apple.

**1.8 Methods to manipulate anthocyanin accumulation**

**1.8.1 Breeding**

From the centre of apple’s origin discussed previously, intentional and unintentional selection of plants has been carried out for thousands of years. The introduction of grafting, from as early as 3,800 years ago, was instrumental in establishing cultivars and the Romans used this technology to create orchard economies (Harris et al., 2002). Since then, the breeding of new cultivars has flourished in many parts of the world, notably Western Europe, Asia and North America. Most cultivated apples are self incompatible and highly heterozygous. Apples are diploid (2n=34) with a relatively small genome of 750 Mb per haploid (Tatum et al., 2005). Modern breeding has relied on a relatively small number of parental varieties and such dependence may not be indefinitely sustainable (Noiton and Alspach, 1996).

New Zealand apple breeders have been highly successful in introducing popular new varieties. The Plant & Food Research (formerly HortResearch) apple breeding programme is multidisciplinary, using conventional interspecific breeding with market driven selections and the widespread use of genomic technologies for gene discovery and marker assisted selection. New Zealand introductions account for 11.5% of the world’s apple crop (O'Rourke, 2007), most notably ‘Royal Gala’ and ‘Braeburn’. A recent introduction, JazzTM (Figure 1-6), is expected to increase this figure further.

As an example of current breeding strategies, it is worth considering the route JazzTM has taken from initial crosses to market introduction and commercial viability. Plant & Food Research (then DSIR) made the initial crosses for what became JazzTM from the two most successful New Zealand-bred varieties, ‘Royal Gala’ (seed parent) and ‘Braeburn’ (pollen parent) in 1984 (Volz et al., 2004). From these crosses, 9,600 seedlings were planted out in 1988 and selections were made following evaluation of the fruit in 1990. Trial plantings were conducted in New Zealand in 1996, the USA in 1999 and France in 2000. Following the granting of Plant Patent (USA) and Plant Variety Rights (Europe), 45,000 trees were planted in orchards in New Zealand, France and the USA in 2003. The commercial success was established in 2005, some 20 years after the initial crosses were made.

**1.8.2 Genetic engineering**

There are a number of molecular-based approaches that offer alternatives to traditional breeding methods. For example, the genetic modification of flavonoid biosynthesis has been well documented (Dixon and Steele, 1999; Davies, 2000; Forkmann and Martens, 2001). For the anthocyanin pigments, there are many examples of the successful engineering of novel flower colours in diverse species including Dianthus (Zuker et al., 2002), Gerbera (Elomaa et al., 2003) and Chrysanthemum (Courtney-Gutterson et al., 1994). One of the most extensively studied species for flower colour modification is Petunia, and reports include the over-expression of an alfalfa CHS gene to turn white petals yellow (Davies et al., 1998), the introduction of suitable DFR genes to induce novel pelargonidin synthesis, creating orange flowers (Meyer et al., 1987; Tanaka et al., 1995) and a multi-gene approach to produce red flowers in tobacco by sensesuppression of endogenous genes (FLS and F3′H) and simultaneous over-expression of an introduced Gerbera DFR gene (Nakatsuka et al., 2007). One flower colour modification has been commercially released. The Australian-based Florigene company released a genetically modified carnation in 1996 (Lu et al., 2003). Since its introduction, some 75 million carnations, marketed as Moondust, Moonshadow, Moonlite and Moonshade and ranging in colour from blue-violet to deep purple, have been sold in Australia, Japan and the USA (Potera, 2007). Florigene is likely to introduce a genetically engineered blue rose in the near future.

Engineering of fruit pigmentation has been less extensively covered but studies in tomato have demonstrated success in pigment manipulation. These include a report on the transformation of tomato with the maize TFs Lc (bHLH) and C1 (MYB) (Bovy et al., 2002). Fruit showed an elevation of flavonoids, but not anthocyanins. Silencing of the DET1 gene in tomato also showed elevation in flavonoids (including modest gains in anthocyanin) and carotenoids (Davuluri et al., 2005). To date, the largest gain in anthocyanin levels in tomato has been due to the over-expression of the Antirrhinum TFs Delila (bHLH) and Rosea1 (MYB) (Butelli et al., 2008). This targeted approach resulted in a massive elevation of anthocyanin, producing purple coloured fruit with a high degree of antioxidant activity. There are numerous examples of the genetic modification of apples, largely targeting production traits such as disease resistance, dwarfing and fruit storage (reviewed in Bulley et al., 2007). At the inception of this project, there were no reported examples of pigment manipulation in apple by genetic engineering methods.

The modified carnations may have found some consumer acceptance and commercial success, but there are still considerable obstacles for the acceptance of genetically modified food crops, particularly in Europe (Laros and Steenkamp, 2004). This is also true in New Zealand which has served to strengthen the case for alternative approaches, such as intragenics and cisgenics (Kling, 2008). A brief definition of transgenics, intragenics and cisgenics are as follows. Transgenic plants are transformed with genes from non-crossable plants, viruses or bacteria and often include selection markers such as antibiotic or herbicide resistance. Intragenic plants are transformed with genes from the crop species itself or from crossable species that have been rearranged in vitro (Rommens, 2004). Intragenesis can be achieved by using marker free systems, such as Agrobacterium transformation of two T-DNAs, one with the gene of interest, the other with a marker that can be selected out (Rommens et al., 2004). Cisgenic plants are also transformed with genes from the crop species itself or from crossable species but only using the native gene’s promoter, terminator and intron regions (Schouten et al., 2006). Again, a markerfree system can be used. While intragenic plants would only be transformed using T-DNA border sequence derived from compatible plants, cisgenic plants, by definition at least, can contain non-coding DNA, including T-DNA borders, from non-crossable species (Schouten and Jacobsen, 2008). Both methods provide alternatives to standard transgenic techniques that may find more favour with legislators and the public.

Notwithstanding the details of the technology employed in the pursuit of GM fruit introductions, the target trait will also be influential in gaining acceptance (Gamble, 1999). A recent study into this acceptance reported that consumers in Europe and New Zealand were more likely to purchase GM apples if they had both a health and environmental benefit (e.g. pesticide-spray free) and a price advantage (Knight et al., 2007).

**1.9 The Plant & Food Research apple project**

**1.9.1 The Plant & Food Research apple EST collection**

A number of the facilities and tools available at Plant & Food Research were invaluable for the experimental work performed in this thesis, such as apple transformation technology and apple germplasm. An important tool used throughout this thesis was the Plant & Food Research EST collection. At the inception of this project, there were relatively few apple sequences in the public domain and so access to this collection was invaluable. The Plant & Food Research collection, comprising some 150,000 ESTs from ‘Royal Gala’ (estimated at half of the expressed genes in apple) was released in 2006 (Newcomb et al., 2006) and the number of sequences has now swelled due to a similar-sized release by the USA-based Apple EST Project (http://titan.biotec.uiuc.edu/apple/apple.shtml). All of these sequences, together with additional annotation, is available via the Genome Database for Rosaceae (GDR) (http://www.rosaceae.org) (Jung et al., 2008). Of great value for future research is the ongoing project to sequence the apple genome by the Istituto Agrario San Michele all'Adige (IASMA) in Italy (http://www.ismaa.it). Sequencing is now complete and annotation of the data is underway.

**1.9.2 Acquisition of the red-fleshed apple germplasm**

Between 1989 and 1996, the USDA sponsored a series of expeditions to Kazakhstan and Krygystan in Central Asia to collect germplasm from wild apple forests. This material was sourced from remote areas which were previously inaccessible to Western-based scientists (Forsline et al., 2003). This region contains Malus sieversii, the progenitor of the cultivated apple, Malus x domestica. The expeditions resulted in a large collection of apple species (including 949 accessions of M. sieversii), sampled across the entire range of the Tien Shan Mountains. This collection is now well characterised and provides a resource of apple species from diverse ecological sites. The collection is primarily housed at the USDA-Agricultural Research Services (ARS), Plant Genetics Research Unit (PGRU) in Geneva, New York ([www.ars.usda.gov/main.htm](http://www.ars.usda.gov/main.htm)).

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**1.9.3 The Plant & Food Research red-fleshed apple project**

To retain market share and premium prices, New Zealand breeders and growers recognise the need for new, high quality varieties. An international joint venture, PrevarTM (www.prevar.co.nz), was established in 2005 to promote the development of new varieties. The success of the Plant & Food Research JazzTM apple to leverage consistently high prices and the opportunity to licence the growth of trees in international markets, has provided a useful model for future introductions. An on-going Plant & Food Research breeding project for a red-fleshed apple was supported by PrevarTM as a target product. This project, using germplasm from the Central Asian collection, was designed to breed a high quality red-fleshed apple for the fresh and processed markets. The project was to eventually encompass researchers from numerous fields including breeding, mapping, physiology and molecular biology, and provided the framework for this thesis.

The Plant & Food Research seed collection from Kazakhstan and Krygystan was evaluated for unusual flesh colour. Seedlings that exhibited the desired traits were backcrossed with standard commercial varieties such as ‘Royal Gala’ and ‘Braeburn’ as well as more recent commercial releases by Plant & Food Research, including Pacific Rose™ and Jazz™ (Volz, 2006). Various breeding populations were developed at Plant & Food Research and subsequently used in mapping studies. One such population, consisting of 516 individuals, from crosses between a Pacific RoseTM, and an open-pollinated seedling of a red-fleshed cultivar, bearing red leaves, ‘Redfield’ (derived from Malus pumila ‘Niedzwetzkyana’) (Figure 1-8) is discussed in Chapter 4.

**1.10 Aims of the thesis**

The principal aim of this project was to isolate the TF that specifically regulated anthocyanin levels in apple and to characterise its functionality. This knowledge could then be transferred into the Plant & Food Research breeding programme for mapping candidate genes and in marker assisted breeding in an effort to accelerate the breeding programme. The secondary aim was to discover the mechanism that drives anthocyanin accumulation throughout the foliage and fruit of some apple varieties. This would advance the basic understanding of anthocyanin regulation and the response of plants to environmental and developmental signals.

**2 MATERIALS AND METHODS**

**2.1 Bacterial strains and genotype**

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**2.2 Plasmids used in this study**

Plasmids used in this study were either available in the public domain, commercially available or created at Plant & Food Research and are shown in Table 2-1.

**2.3 Plant material**

A fruit development series was constructed from apple fruit collected at 6 time points during the season from spring through to late summer, 2003-2004: Stage 1, 7 days after full bloom (DAFB), stage 2, 40 DAFB, stage 3, 67 DAFB, stage 4, 102 DAFB, stage 5, 130 DAFB and stage 6, 146 DAFB, from trees at the Plant & Food Research orchard (Nelson, New Zealand). Samples were separated into skin and cortex, and the core was discarded. Each sample time point was made up of six apples and the leaves of 2 genotypes; the white-fleshed commercial cultivar Malus x domestica ‘Sciros’ (Pacific Rose™, derived from a cross between ‘Gala’ and ‘Splendour’) and an open-pollinated red-fleshed seedling of Malus x domestica ‘Red Field’ (a cross between 'Wolf River' and Malus pumila ‘Niedzwetzkyana’) (Brooks and Olmo, 1972). For the first developmental fruit time point (7 DAFB), successful excision of skin from cortex was not possible, so this time point was excluded. All samples were snap frozen in liquid nitrogen and stored at -80 ºC. Other plant material as follows:

Apple: Malus x domestica ‘Royal Gala’.

Arabidopsis: Arabidopsis thaliana (ecotype Columbia Col-0).

Tobacco: Nicotiana tabacum ‘Samsun’ and leaves of Nicotiana benthamiana.

**2.4 Oligonucleotides**

Oliginucleotide primers were designed to sense (S) or anti-sense (AS) strands of DNA sequence and were commercially synthesised (Invitrogen, NZ). Incorporation of restriction sites into primer sequences for subsequent cloning reactions is noted in the description column in the following tables.

**2.5 Growth media**

Growth media were sterilised by autoclaving at 120 ºC, 20 min. If necessary the media pH was adjusted using NaOH or HCl. Selection antibiotics were added as required.

Luria-Bertani (LB) broth: 1% (w/v) bactotryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, pH 7.0.

LB plates: LB broth, plus 1.5% (w/v) bacto agar.

SOC media: 1.6% (w/v) Bactotryptone, 1% (w/v) yeast extract , 0.5% (w/v) NaCl, 10 mM MgCl2, 10 mM MgSO4, 20 mM glucose, pH 7.0.

ZY base media: 1.6% (w/v) Bactotryptone, 1% (w/v) yeast extract.

ZYM-5052 Auto-induction media: 1.6% (w/v) Bactotryptone, 1% (w/v) yeast extract, 1 mM MgSO4, 0.1% (v/v) 1000 x base metal mix, 2% (v/v) 50 x 5052, 5% (v/v) NPS.

**2.6 Antibiotic stock solutions**

Ampicillin 100 mg ml-1

Chloramphenicol 35 mg ml-1

Kanamycin 50 mg ml-1

Spectinomycin 100 mg ml-1

Streptomycin 50 mg ml-1

**2.7 Solutions and common buffers**

5052: 25% (w/v) glycerol, 2.5% (w/v) glucose, 10% (w/v) α-lactose.

Acetosyringone: 200 mM, dissolved in dimethyl sulfoxide (DMSO).

EMSA binding buffer: 10 mM tris, 50 mM KCl, 2.5 mM DTT, 1 µg poly(dI-dC), 10 ug BSA, 4% glycerol.

His Trap binding buffer: 30 mM imidazole, 0.5 M NaCl, 5 mM dithiothreitol (DTT), 20 mM sodium phosphate, pH 7.4.

His Trap elution buffer: 500 mM imidazole, 0.5 M NaCl, 5 mM dithiothreitol (DTT), 20 mM sodium phosphate, pH 7.4.

Infiltration buffer: 10 mM MgCl2, 10 µM acetosyringone.

NPS (20 x): 6.6% (v/v) (NH4)2SO4, 13.6% (v/v) KH2PO4, 14.2% (v/v) Na2HPO4.

Phosphate Buffer Saline (PBS): 6.3 mM Na2HPO4, 0.2 mM KH2PO4, 2.6 mM KCl, 138 mM NaCl, pH 7.4.

RNA extraction buffer: 2% (w/v) CTAB (hexadecyltrimethylammonium bromide), 2% (w/v) PVP (polyvinylpyrrolidinone), 100 mM Tris-HCl (pH 8.0), 25 mM EDTA, 2.0 M NaC1, 0.5g/L spermidine (+ 2% (v/v) β-mercaptoethanol added just before use).

SDS loading buffer: 250 mM Tris-HCl, 8% (w/v) SDS, 40% (v/v) glycerol, 20% (v/v) 2- mercaptoethanol, 0.004% (w/v) bromophenol blue, pH 6.8.

Semi-dry transfer buffer: 25 mM Tris, 192 mM glycine, 10% (v/v) methanol.

SSTE: 1M NaCl, 0.5% (v/v) SDS, 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0.

TBS: 30 mm Tris, 150 mM NaCl.

TBS-Tween 20: 30 mM Tris-HCl, 150 mM NaCl, 0.05% (v/v) Tween 20, pH 7.5.

Trace metals mix (1000 x): 0.1 M FeCl3, 1 M CaCl2, 1 M MnCl2, 0.2 M CoCl2, 0.1 M CuCl2, 0.2 M NiCl2, 0.1 M Na2MoO4, 0.1 M Na2SeO3, 0.1 M ZnSO4, 0.1 M H3BO3.

**2.8 Radioisotopes, chemicals and enzymes**

All chemicals used were of molecular biology grade, typically purchased from Sigma-Aldrich (USA) or BDH (United Kingdom). Radioisotopes ([32P]-γ-ATP) were purchased from Perkin Elmer (USA). Commonly used enzymes were as follows:

Ligation: Rapid DNA ligation kit (Roche, Germany).

Restriction digestion: Enzymes, buffers and BSA from New England Bioloabs (USA).

Reverse transcriptase: Transcriptor Reverse Transcriptase (Roche, Germany) or Superscript III cDNA synthesis kit (Invitrogen, USA).

SYBR green master mix: LightCycler FastStart SYBR Green Master Mix (Roche, Germany).

T4 DNA polymerase: Promega, USA

T4 polynucleotide kinase (PNK): New England Bioloabs (USA)

Taq polymerase: Platinum taq (Invitrogen, USA), PWO DNA polymerase (Roche, Germany) or TaKaRa LA Taq (Takara, Japan).

**2.9 Manipulation of bacteria**

**2.9.1 Transformation of E. coli (DH5α and BL21)**

Commercially available competent E. coli were transformed by the freeze-thaw method. A 5 μl aliquot of plasmid DNA or ligation reaction mixture was added to 50 μl thawed cells in a 1.5 ml tube and gently mixed. The cells were incubated on ice for 30 min before heat-shock at 42 ºC for 40 s (DH5α) or 20 s (BL21) in a waterbath, followed by a further 3 min on ice. A 0.95 ml aliquot of LB or SOC media was added to each tube and incubated for 1 h at 37 ºC with shaking at 250 rpm. Aliquots of 30 μl and 100 μl of the transformation mixture were spread onto LB agar plates containing the appropriate antibiotic. The plates were incubated at 37 ºC for 16-20 h.

**2.9.2 Transformation of Agrobacterium GV3101 and LBA4404**

Binary vectors were transferred into Agrobacterium tumefaciens (Agrobacterium) GV3101 (for Arabidopsis and tobacco) and LBA4404 (for apple) by electroporation. Competent cells (50 μl aliquots) were thawed on ice and plasmid DNA (50-200 ng in 1 μl water) was added, gently mixed and pipetted into a pre-chilled electroporation cuvette (0.2 cm gap, Bio-Rad Laboratories, USA). The cuvette was placed in a GenePulser (Bio-Rad Laboratories, USA) and electroporation was carried out at a voltage of 2.5 kV (capacitance 25 μFd, resistance: 400 Ohms) with a typical pulse time of 7-9 ms. The cells were recovered by addition of 1 ml LB, transferred to 1.5 ml tubes and incubated at room temperature, with shaking (60 rpm) for 2 h. Aliquots of 10 μl and 100 μl of the transformed bacteria were spread onto separate LB plates containing the appropriate antibiotics. Plates were grown at 28 °C for 48 h.

**2.10 Manipulation of plants**

**2.10.1 Cultivation of tobacco, apple and Arabidopsis**

Arabidopsis, tobacco and apple plants were maintained in the glasshouse using standard growth conditions and media with the aid of Julie Nicholls and G. Wadasinghe, Plant & Food Research, Auckland. Glasshouse conditions varied depending upon the season, but typical conditions for short days were 10 h light, 14 h dark, and for long days 16 h light, 8 h dark. Light intensity also varied, but a typical measurement on an overcast day was 150-200 μE m-2 s -1.

For the sterilisation of Arabidopsis seed, 20 mg of seed (approximately 1000 seeds) were washed in a bleach solution (1.5% (v/v) available chlorine containing 0.01% (v/v) Triton-X) for 30 min with vortexing every 2 – 3 min. The seed was then rinsed several times with distilled water, and resuspended in 0.1% (w/v) agarose. Transgenic seed was plated onto ½ x MS media, containing 50-100 μg ml-1 kanamycin. Plates were transferred into growth rooms with a 12 h light/12 h dark cycle, and light intensity of 50 μE m-2 s -1. After approximately 2 weeks, seeds that had germinated into plants were transferred to fresh kanamycin containing media for a further week’s growth. Plants were then transferred into soil and grown in a humidity chamber for 2-3 days, before relocation to the glasshouse. Arabidopsis plants and seeds were analysed for phenotypic variation caused by anthocyanin accumulation.

Tobacco seed sterilization was conducted by Kui Lin-Wang, Plant & Food Research, Auckland, using similar methods.

**2.10.2 Apple tree grafting and accelerated growth conditions**

After tissue culture, transformed plantlets of ‘Royal Gala’ transgenic lines were micro-grafted onto ‘M9’ rootstock, adapted from a previously described protocol (Lane et al., 2003) and grown in a PC2 containment glasshouse. The scions were tissue cultured transgenic shoots, approximately 3 cm long. Scions were prepared by cutting a section at the base of the stem and cutting back most of the expanded leaves. M9 rootstocks were prepared by pruning growing shoots to approximately 10 cm from the base, making two short cuts through the bark and peeling the bark back for scion insertion. The scion was inserted under the bark of the rootstock, held in place with a short strip of masking tape and covered with a small polythene bag to prevent excessive moisture loss. The grafted plants were placed in a shaded area for 10 to 14 days until leaf expansion was detected on the scions. The bags were removed and the plants gradually exposed to higher light levels. Once growth was established the trees were transferred to the National Climate Laboraties at Plant & Food Research in Palmerston North where they were maintained by Cara Norling. Growth conditions are shown in Table 2-5. Additional treatments included a copper spray before & after flowering, regular application of half strength Hoagland’s nutrient solution, three Wuxal liquid fertiliser applications during early flowering, application of growth regulator (100 ppm) (Regulox, Bayer, UK) applied to active shoots at tree top level (not near fruit) and two Wuxal root feeds, which were applied during late flowering.

**2.11 Transformation technology**

**2.11.1 Transformation of Arabidopsis**

The floral dip method was used for Agrobacterium–mediated transformation of Arabidopsis thaliana (Clough and Bent, 1998). Arabidopsis ecotype Columbia (Col-0) plants were grown from seed for approximately 5 weeks, under long day conditions, to promote flowering. Agrobacterium containing a binary vector for plant transformation was inoculated into 10 ml of LB media containing the appropriate antibiotics for 24 h at 28 ºC, with shaking at 200 rpm. These cultures were used to inoculate a further 100-200 ml of LB media, with antibiotics, which were grown for 24 h at 28 ºC, with shaking. The cells were collected by centrifugation (3,500 x g, 10 min, 4 ºC) and resuspended to a final A600 of 0.8, in 5% (w/v) sucrose solution with 0.05% (v/v) Silwet L-77 (OSi Specialties, USA). Arabidopsis plants, showing a number of immature flower clusters, were used for the dipping procedure. The entire above ground portion of each plant was dipped into the Agrobacterium suspension, and gently agitated for 3-5 s. The dipped plants were then placed in humidity chambers in reduced light for 2-3 d, before being allowed to flower and set seed as normal.

**2.11.2 Transformation of tobacco**

The stable transformation of N. tabacum was kindly carried out by Kui Lin-Wang, Plant & Food Research, Auckland, based on a method described by Svab et al., (1975).

**2.11.3 Dual luciferase assay of transiently transformed tobacco leaves**

N. benthamiana plants were grown under glasshouse conditions in full potting mix, using natural light with daylight extension to 16 h, until at least 6 leaves were available for infiltration with Agrobacterium. Plants were maintained in the glasshouse for the duration of the experiment. Transformed Agrobacterium cultures containing reporter cassettes and candidate TFs were cultured on LB agar plates containing the appropriate antibiotics and incubated at 28 °C. A 10 μl loop of bacterium was re-suspended in 10 ml of infiltration media (10 mM MgCl2, 0.5 μM acetosyringone), to an OD600 of 0.2, and incubated at room temperature without shaking for 2 h before infiltration. Infiltrations were performed according to the methods of Voinnet et al. (2003), using a 1 ml syringe with no needle attached. Approximately 150 μl of this Agrobacterium mixture was infiltrated at six points into a young leaf of N. benthamiana and transient expression was assayed 3 days after inoculation. Six technical replicates of 3 mm diameter leaf discs were excised from each plant using a leaf hole-punch and buffered in Phosphate Buffer Saline (PBS). Plate-based assays were conducted using a Berthold Orion Microplate Luminometer (Berthold Detection Systems, Germany) according to the manufacturer’s specifications for the dual luciferase assay, using the Dual Glow assay reagents (Promega) for firefly luciferase and Renilla luciferase. Luminescence was calculated using Simplicity version 4.02 software (Berthold Detection Systems, Germany).

**2.11.4 Induction of anthocyanins by transient transformation of tobacco**

N. tabacum were grown as above and maintained in the glasshouse for the duration of the experiment. Agrobacterium cultures were incubated as for the dual luciferase assay and separate strains containing one of the MdMYB10 promoter fusions (35S:MdMYB10, R1:MdMYB10 or R6:MdMYB10) and 35S:MdbHLH3 were mixed (500 μl each) and infiltrated into the abaxial leaf surface as for the luciferase assay. Six separate infiltrations were performed into N. tabacum leaves (two plants per treatment) and changes in colour were observed. For the quantitative measurements of colour change, readings were taken daily using a Minolta CR-300 (Konica Minolta, USA) chroma meter (calibrated to D65 light) using the L\*a\*b\* system (CIE, 1986). Pigmentation was typically visible four days after infiltration. The level of pigmentation increased throughout the experimental period; digital photographs and microscope images were taken eight days after infiltration. To control for leaf-to-leaf variability, at least 2 leaves were infiltrated, and each leaf included positive (Agrobacterium cultures carrying 35S:MdMYB10 and 35S:MdbHLH3) and negative (Agrobacterium with empty vector) controls.

**2.11.5 Transformation of apple**

The transformation of apple was kindly carried out by Sumathi Kutty-Amma, Plant & Food Research, Auckland. Transgenic Malus x domestica ‘Royal Gala’ plants were generated by Agrobacterium-mediated transformation of leaf pieces, using a method previously reported (Yao et al., 1995).

**2.12 Microscopy**

Apple leaf sections from 35S:MdMYB10 and control lines were sandwiched in cork (Cutler et al., 2008) and sectioned using a Vibratome 1000 (Vibratome, USA). Sections were mounted on glass slides with a drop of glycerol (due to the high solubility of anthocyanins in water) and examined in bright field using an Olympus Vanos AHT3 microscope (Olympus Optical, Japan).

**2.13 Analysis of photosynthetic activity**

The fluorescence yield (F) and maximal yield (Fm′) were measured using a Pulse-Amplitude Modulation (PAM) Chlorophyll Fluorometer (Walz GmbH, Germany), according to the manufacturer’s instructions. Leaves were ‘relaxed’ in the dark for >10 min prior to analysis. A darkened situation was maintained throughout the measurement period. Six readings at 1 min intervals were taken at each of the 6 light levels. Chlorophyll fluorescence was measured at 660 nm to 760 nm. True light levels were measured with a lightmeter (Li-COR, USA) and calculations adjusted accordingly, using a correction factor of 0.345. The overall quantum yield of photochemical energy, the photosynthesis yield, was expressed as:

Yield (Y) = (Fm′ – F)/Fm′ = ΔF/Fm,

The ETR was calculated as follows:

Yield (Y) x Photosynthetic active radiation (PAR) = Electron Transport Rate (ETR)

ETR was then multiplied by 0.5 (a single electron requires the absorption of two quanta) and by 0.84 (the fraction of incident light absorbed by a leaf).

**2.14 Volatile analysis**

Aroma compounds were extracted from whole apples. For each replicate, apple fruit at a similar maturation stage and of a similar total weight (approximately 250 g) were removed from cool store and allowed to warm to room temperature. Fruit were placed in 1 litre Quickfit® Erlenmeyer flasks which were fitted to a headspace adptor with an air inlet and outlet. Aromas were sampled with Chromosorb 105TM (Sigma, USA) packed cartridges. After equilibriation of the closed system for 20 min at room temperature, the samples were flushed with clean air for 40 min at 25 ml min-1, trapping through the cartridge. For the identification and confirmation of compounds, a mass spectrometer coupled to a gas chromatagragh was used (Pegusus® III GCTOF-MS, Leco, USA) according to the method described by Matich et al. (2003). Component identification was carried out using the Leco ChromaTOF software v. 3.34 (optimised for Pegasus®) and the in-house Plant & Food Research compound database. Quantification was carried out using direct comparison of authentic standards of a known volume.

**2.15 Identification of anthocyanin compounds**

Tobacco or apple samples (of < 3 g) were freeze-dried and ground before re-suspension in 5 ml methanol and 0.1% HCl. Samples were sonicated for 5 min and anthocyanins extracted at room temperature for 2 h in the dark. Samples were centrifuged at 3500 rpm for 2 min and aliquots of 1 ml were dried down to completion overnight in a pre-chilled Labconco Centrivap Concentrator (Labconco, USA). Samples were re-suspended in 20% methanol.

Anthocyanins were characterized by HPLC on a 250 x 4.6 mm, Synergi, 4m particle size, PolarRP, 80 Å pore size, ether-linked phenyl column (Phenomenex, New Zealand). This was fitted to a Shimadzu analytical HPLC (Shimadzu Scientific Instruments, USA) with a column oven, autosampler, vacuum solvent degasser and diode-array detector. Solvents were (A) acetonitrile with 0.1% formic acid and (B) acetonitrile/water/formic acid, 5:92:3. The flow rate was 1.5 ml min-1 and the column temperature was 45 ºC. The content of solvent A was 0% at time zero and ramped linearly to 17% at 17 min, 20% at 20 min, 30% at 26 min, 50% at 28.5 min, 95% between 32 and 35 min, then back to 0% between 36 and 42 min. Quantification of reaction products was at 520 nm for anthocyanins and 280 nm for other phenolics. Spectra were recorded from 240-600nm in 4 nm steps. The sample injection volume was 40 µL. Anthocyanins were identified as previously described (Stevenson et al., 2006), with reference to standards of anthocyanin compounds. Peaks predicted to represent anthocyanins were confirmed by LCMS, which was kindly carried out by Janine Cooney, Plant & Food Research, Ruakura.

**2.16 DNA technology**

**2.16.1 Plasmid DNA isolation**

Plasmid DNA was purified from overnight LB cultures (typically 3 ml) of E. coli using the Qiagen Qiaprep Miniprep kit (Qiagen, Germany), according to the manufacturer’s instruction. Typically, concentration of recovered DNA was estimated at 200 ng µl-1.

**2.16.2 Plant genomic DNA isolation**

Genomic DNA was isolated from apple using the Dneasy Plant Mini Kit (Qiagen, Germany), according to the manufacturer’s instructions.

**2.16.3 Isolation of MdMYB10 by degenerate PCR and 5′ RACE**

A degenerate primer with 32 fold degeneracy (section 2.4) was designed to amplify MYB TFs from apple cDNA at the R2R3 site, based on sequence homology with known MYB regulators of anthocyanin biosynthesis in other species. The degenerate primer was designed in reverse orientation and used in conjunction with oligo dTs to amplify target mRNA. The predicted size was between 600 and 800 bp, based on homologous sequences. PCR conditions were as follows: 95 °C for 5 min followed by 35 cycles of 95 °C (30 s), 55 °C (30 s) and 72 °C (60 s) with a final extension at 72 °C for 5 min. The PCR products were cloned into pGEM-T Easy and sequenced (Appendix 9.3). Sequence analysis enabled the design of nested primers for 5′ RACE (section 2.4). A GeneRacerTM kit (Invitrogen, USA) was used to amplify 5′ cDNA ends, according to the manufacturer’s instruction. PCR products were cloned into pGEM-T Easy and sequenced.

**2.16.4 Isolation of MdMYB10 promoter region by genome walking**

Upstream DNA, immediately adjacent to the transcription start site of MdMYB10, was isolated from apple genomic DNA by PCR genome walking based on the GenomeWalker™ kit (BD Biosciences Clontech, USA) protocol. Genomic DNA was extracted from Malus x domestica ‘Sciros’ (Pacific Rose™, derived from a cross between ‘Royal Gala’ and ‘Splendour’). Seven libraries were constructed from high quality gDNA preparations by digestion with seven restriction enzymes (DraI, Ecl13611, EcoRV, ApaI, ScaI, SspI and StuI), leaving blunt ends, to which the GenomeWalker™ adaptor was ligated. Nested primers to the coding region of MdMYB10 were designed (section 2.4) and used in conjunction with adaptor primers for primary and secondary PCR, following the manufacturer’s method for GenomeWalker™. The PCR products were cloned using the TOPO TA Cloning® kit (Invitrogen, USA) according to the manufacturer’s instructions and the sequences aligned using Vector NTI v9.0 (Invitrogen, USA). This resulted in approximately 800 bp of confirmed upstream sequence. Since this did not reach the target of at least 1 kb of promoter region, a second genome walk was performed, with nested primers based on the sequence from the first walk (section 2.4) and resulted in a total of 1.7 kb of upstream sequence adjacent to the transcription start site. The PCR products were cloned using the TOPO TA Cloning® kit (Invitrogen) and the sequences aligned using Vector NTI v9.0 (Invitrogen, USA). Promoter fragments from genomic DNA was subsequently isolated from Malus x domestica 'Granny Smith', Malus x domestica ‘Royal Gala’ and Malus x domestica ‘Red Field’ OP, using forward and reverse primers (section 2.4).

**2.16.5 Cloning**

Unless stated otherwise, all general cloning procedures including restriction digestion, ligation and DNA purification were carried out according to standard protocols (Sambrook et al., 1989). For all generic sub-cloning of PCR products for sequencing and downstream applications, the pGem-T Easy vector (Promega, USA) was used and ligations carried out according to the manufacturer’s instructions. All vectors for plant transformation were sequenced to confirm authenticity. The plasmids referred to in this thesis are summarized in Table 2-6 and a description of the cloning strategies for each construct follows.

AtDFR:LUC: The promoter sequence for the Arabidopsis DFR gene was cloned into the pGreen 0800-LUC vector by Roger Hellens, Plant & Food Research, Auckland, as described in Espley et al. (2007).

MdDFR:LUC: A 1.3 kb promoter fragment from the apple DFR1 gene was isolated by genome walking and was inserted into the cloning site of pGreen 0800-LUC. The sequence was modified to introduce an NcoI site at the 3’ end of the sequence, allowing the promoter to be cloned as a transcriptional fusion with the firefly luciferase gene (LUC). The isolation and cloning were carried out Roger Hellens, Plant & Food Research, Auckland.

Plant & Food Research TF library clones: EST-derived constructs, including all the TFs used in the transient assay screen, were cloned by the Plant & Food Research cloning team (Andrew Gleave and Sakuntala Karunairetnam). ESTs were cloned into the pART7/27 binary vector system (Gleave, 1992) with the appropriate plant regulatory signals including the CaMV35S promoter and octopine synthase terminator. For clones lacking suitable restriction sites for conventional cloning, Gateway (Invitrogen, USA) recombinant technology was used to clone into pHEX vectors with the same regulatory signals as pART7/27, as described in Hellens et al., (2005).

35S:MdMYB10: The MdMYB10 cDNA sequence was isolated from pGEM-T Easy by sequential restriction digestion, using the unique sites for HindIII (situated at the extreme 5′ end of the MdMYB10 5′ UTR) and SalI (located in the pGEM-T Easy MCS). The pSAK277 binary vector was linearised using the unique sites for HindIII and XhoI. Restriction digestion by XhoI was not possible for MdMYB10 as a XhoI site was located in the coding region. Digestion by SalI or XhoI leaves compatible overhangs for directional cloning. However, the restriction site is lost when the vector and insert are ligated (Figure 2-1).

R1:MdMYB10 and R6:MdMYB10: Genomic DNA of the promoter regions of MdMYB10, R1 and R6, and the 5′ region of the coding sequence were amplified by PCR using the primers RE187 and RE188 (section 2.4). These primers included the restriction enzyme sites SacI and XhoI respectively and produced a fragment that included the promoter and the 5′ region of the coding sequence to ensure the sequences was retained in the correct frame. The pSAK vector, 35S:MdMYB10, was digested with SacI and XhoI and the vector backbone purified from agarose gel. Directional cloning of the insert into the linearised vector and was performed using SacI and XhoI restriction site overhangs.

R1:LUC and R6:LUC: The R1 and R6 promoter fragments were isolated from pGEM-T Easy plasmids using restriction digestion with Pst1 and SacII. The pGreen 0800:LUC vector was similarly digested and purified from agarose gel (Figure 2-2). The vector and insert were ligated and sequence verified. All plant transformation using pGreen required the co-transformation of the pSOUP helper plasmid (Hellens et al., 2000), which provides trans-acting replication functions.

R2:LUC, R3:LUC, and R4:LUC: The difficulty of a PCR-based approach for the generation of repetitive DNA fragments necessitated the synthesis of the truncated minisatellite repeat unit. These were designed as R2 (the primary repeat unit and one other), R3 (the primary repeat unit and two others) and R4 (the primary repeat unit and three others). Double stranded DNA synthesis was carried out by GeneArt, Germany (Figure 2-3). The synthesised fragments were designed with DraI and SpeI restriction sites for cloning into R1:LUC. A residual SpeI site was carried over from the cloned pGEM-T Easy fragment in R1:LUC (Figure 2-4). The SpeI fragment was removed by restriction digestion prior to DraI digestion. However, a DraI site in the vector backbone of pGreen was overlooked, necessitating the use of partial digestion techniques. Following sequence verification, the SpeI fragment was then re-ligated and positive clones sequenced.

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R1+:LUC: An inverse PCR approach was used for the R1+:LUC construct with the inclusion of unique restriction sites (BamHI and SacI) for the cloning of non-specific DNA (from pGEM T Easy, Promega) using the primers RE212 and RE213 (section 2.4). PCR was perfomed using TaKaRa LA Taq (Takara, Japan) under the following conditions:

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R1ΔA, R1ΔB, R1ΔC, R1ΔD, R6ΔA, R6ΔB, R6ΔC and R6ΔD: Promoter deletions were performed on R1 and R6 sequences using the following restriction enzymes; ΔA, HindIII (retaining the 5′ fragment only), ΔB, SpeI, ΔC, SspI and ΔD, HindIII (retaining the 3′ fragment only) (Figure 2- 5).

R0, R1-R, R1-MS, R5, and R6-MS: Deletion of the first repeat unit and microsatellite (R0:LUC) was based on the R1:LUC promoter construct and performed using a partial restriction digest with DraI. The linear fragment was further digested with BsgI and treated with T4 DNA polymerase (Promega) prior to re-ligation. For the construction of R1-R and R1-MS, R0 was digested with BsgI and annealed oligos were directionally cloned in with an AA (underlined below) overhang: R1-R, 5′- CTATATATCGAGTGTGTGTGTGTGTGTGTATTTCACAACTGTTGGAATGTTTGAA - 3′; R1-ms, 5′- CTATATATCGAATTTCACAACTGTTGGAATGTTTGAA -3′. The R6 versions were based on R1-R and R1-MS. The minisatellite repeat unit from R6 was amplified by PCR using the primers RE162 and R6 Reverse (section 2.4) and cloned in at the BsgI restriction site to create R5 and R6-MS. All clones were positively identified by bi-directional sequencing.

pET:MdMYB10, pET:MdMYB10CO, pET:MdMYB10ΔC1: For the construction of the protein expression vectors, the MdMYB10 coding sequence was amplified by PCR using the primers RE185 and RE195 (section 2.4) and cloned into pET30a(+) using the restriction sites Nco1 and Sac1. Between the T7 promoter and the coding sequence lies an in-frame 6 x His tag for purification by immobilised metal affinity chromatography (IMAC). Due to the poor expression levels in E. coli from this construct, a codon optimised version of MdMYB10 (MdMYB10CO) was synthesised (GeneArt, Germany). A comparison of the native and codon optimised sequences can be found in Appendix 9.1. The codon optimised MdMYB10 was mobilised into the pET30a(+) expression vector using the restriction sites Kpn1 and Sac1. A third variant was made using a truncated version of the MdMYB10 coding sequence, pET:MdMYB10ΔC1, with a C-terminal deletion. The deletion was made by restriction digest of the codon optimised full length sequence with BglII and ligating into a BglII-linearised pET30a(+) vector. The sequence retained 162 aa, including the R2R3 binding domain.

**2.16.6 PCR**

General PCR was performed using 50 µl reactions in a MasterCycler Gradient (Eppendorf, Germany) with the following conditions for Platinum taq (Invitrogen, USA) or PWO DNA polymerase (Roche, Germany) based reactions:

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**2.16.7 DNA Sequencing**

DNA sequencing was carried out by the Allan Wilson Centre Genome sequencing service (AWCGS), Palmerston North, using capillary separation on an ABI3730 Genetic Analyzer (Applied Biosystems, USA).

**2.17 Database mining**

**2.17.1 DNA Sequence analysis**

DNA sequence from the Plant & Food Research EST database and sequenced products were analysed with Vector NTI version 9.0.0 (Invitrogen, USA) and DNA sequence contigs were edited and compiled using Vector NTI version 9.0.0 ContigExpress and AlignX.

**2.17.2 Phylogenetic analysis**

Apple EST sequences were trimmed of vector, adapter and low quality sequence regions and uploaded to Vector NTI version 9.0.0. EST clustering was performed using Vector NTI AlignX program. Alignments were then exported as MSF format files to GeneDoc version 2.6.002 (http://www.psc.edu/biomed/genedoc/). Trees were generated by re-aligning exported files in CLUSTALX (v1.81) using the default settings (Thompson et al., 1997). The circular tree (Figure 3-9) was generated by Andrew Allan, Plant & Food Research, using MEGA version 2.1 (Kumar et al., 2001).

**2.18 DNA sequences deposited in Genbank**

DQ267896.1: Malus x domestica ‘Red Field’ MYB10 mRNA, cds

DQ267897.1: Malus x domestica ‘Pacific Rose’ MYB10 mRNA, cds

DQ267898.1: Malus x domestica ‘Granny Smith’ MYB10 mRNA, cds

EU518248.1: Codon optimised MdMYB10 cDNA

EU518249.1: MdMYB10 R1 - Promoter region and 5' UTR of MdMYB10 gene

EU518250.1: MdMYB10 R6 - Promoter region and 5' UTR of MdMYB10 gene

**2.19 RNA technology**

**2.19.1 RNA isolation**

RNA procedures were performed using ribonuclease (RNase) free water prepared by a Nanopure water purification system (Barnstead, USA) or solutions made with RNase free water. RNA was isolated from the leaf material of Arabidopsis and tobacco using the RNeasy Plant Mini Kit (Qiagen, Germany), according to the manufacturer’s instructions.

Due to the high phenolic content in apple fruit, RNA from apple was isolated by a method based on Chang et al. (1993). For each sample, 15 ml RNA extraction buffer with 2% (v/v) ßmercaptoethanol was pre-heated to 65 ºC. Typically 6-10 g fruit tissue was ground in liquid nitrogen and added to the extraction buffer and homogenized using a polytron. An equal volume of chloroform/isoamyl alcohol was added, thoroughly mixed and the homogenate was centrifuged for 10 min at 10,000 rpm at room temperature to separate the phases. The top aqueous phase was passed through Mira cloth and centrifuged for 10 min at 10,000 rpm at room temperature to separate the phases. This was repeated (without Mira cloth filtration) twice more and the aqueous phase removed. LiCl was added to a final concentration of 2 M and the sample was stored at 4 ºC for at least 2 h or typically overnight. The sample was centrifuged at 4 ºC for 20 min at 10,000 rpm to pellet the RNA and the supernatant was removed. The pellet was disolved in 500 µl SSTE and transferred to a clean microcentrifuge tube. An equal volume of chloroform/isoamyl alcohol was added and the phases were separated by centrifugation at 13,000 rpm at 4 ºC. The aqueous phase was removed, two volumes of EtOH added, and the sample was stored at -80 ºC for 30 min to precipitate the RNA. The sample was centrifuged at 13,000 rpm for 20 min at 4 ºC to pellet the RNA and the supernatant discarded. The pellet was then dried and resuspended in 200 µl RNAse free water. RNA integrity and concentration were assessed by gel electrophoresis and fluorometric quantitation.

**2.19.2 cDNA synthesis**

The quantity of cDNA template for individual qPCR reactions should be constant. Therefore, the conditions for each first strand cDNA synthesis reaction were strictly controlled to ensure maximum uniformity between samples. RNA samples were treated with DNase (DNA-freeTM, Ambion, USA), according to the manufacturer’s instructions and concentrations were determined using a NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, USA). The amount of RNA used for each cDNA synthesis was kept constant at 1 μg for each sample. Reactions were performed using a Superscript III cDNA synthesis kit (Invitrogen, USA), according to the manufacturer’s instructions. To maximize the RNA conversion both random hexamers and oligo(dT)s were used in each reaction. To offset the variability of the efficiency of individual reactions, three replicate reactions for each RNA sample were synthesised and pooled before qPCR analysis.

**2.19.3 Real time (qPCR) expression analysis**

Real time or quantitative PCR (qPCR) was performed on cDNA isolated from apple and tobacco to detect relative transcript expression levels of both native genes and transgenes. The primer sets used for each gene are listed in Table 2-2. Genes encoding apple anthocyanin pathway enzymes and regulators were identified by homology in the Plant & Food Research EST database and, in the case of possible isoforms, selection was made according to the expression profile and library tissue. Gene specific primers, corresponding to these genes were designed using Vector NTI version 9.0.0 (www.invitrogen.com) to a stringent set of criteria, including a Tm of between 59.5ºC and 60.5ºC, an amplicon length of between 100 and 200 bp, avoidance of GC clamp at 3′ end and limited predicted secondary structure or dimer formation. Adherence to these criteria enabled the application of universal reaction conditions.

To verify reaction specificity, RT-PCR reactions were carried out according to manufacturer’s instructions (Platinum Taq, Invitrogen, USA), with a thermal profile as follows; pre-incubation at 95 °C for 5 min followed by 35 cycles of 95 °C (30 s), 60 °C (30 s) and 72 °C (30 s) with a final extension at 72 °C for 5 min. PCR products were visualised by gel electrophoresis.

Amplification and analysis by qPCR was carried out using the LightCycler System (Roche LightCycler 1.5, Roche, Germany). All reactions were performed with the LightCycler FastStart SYBR Green Master Mix (Roche, Germany) with a thermal profile as follows; pre-incubation at 95 °C for 5 min followed by 40 cycles of 95 °C (5 s), 60 °C (5 s) and 72 °C (8 s). Reactions were performed in triplicate using 2 μl 5x Master Mix, 0.5 μM each primer, 1 μl diluted cDNA and nuclease-free water (Roche, Germany) to a final volume of 10 μl. A negative control with no cDNA template (NTC) was included in each run. Fluorescence was measured at the end of each annealing step. Amplification was followed by a melting curve analysis with continual fluorescence data acquisition during the 65 °C to 95 °C melt (Figure 2-6).

The raw data was analysed with the LightCycler software version 4 and expression was normalised to a reference gene (e.g. Malus x domestica Actin, MdActin, Genbank accession CN938023) with one sample acting as calibrator with a nominal value of 1 (e.g. Pacific Rose™ leaf sample). A comparison of frequently used reference genes confirmed that MdActin was suibtable for the development series, with Cp values changing by < 2. For each gene, a standard curve was generated with a cDNA serial dilution and the resultant PCR efficiency calculations (ranging between 1.839 and 1.945) were imported into relative expression data analysis (Figure 2-7). The relative expression ratio of a target gene is calculated on the reaction efficiency (E) and the crossing point deviation of the target gene and reference gene, normalised to the calibrator sample. The equation can be represented as:

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PCR products were sequenced to confirm authenticity.

**2.20 Protein technology**

**2.20.1 Expression of MdMYB10 protein**

A full length version of MdMYB10 cDNA (amino acids 1-243) and a carboxy-terminal deletion of the MdMYB10 cDNA was made (retaining amino acids 1-162, including the R2R3 DNAbinding domain) and cloned into pET-30. Protein expression was performed in E. coli BL21 cells (Stratagene) containing the relevant plasmid. A single colony of bacteria was used to inoculate 5 ml of LB media, containing 50 μg ml-1 of kanamycin and 35 μg ml-1 of chloramphenicol, which was incubated at 37 ºC overnight, with shaking at 220 rpm. A 500 x dilution of the overnight culture was used to inoculate 500 ml of ZY autoinducible media (with the same antibiotics) (Studier, 2005) and the culture was grown at 37 °C for 2 h at 300 rpm. The temperature was then lowered to 16 °C and incubation continued for a further 60 h at 300 rpm.

**2.20.2 Purification of MdMYB10 protein**

Cells were harvested by centrifugation (3,500 g) and re-suspended in 1 x His Trap binding buffer (30 mM imidazole, 0.5 M NaCl, 5 mM dithiothreitol (DTT), 20 mM sodium phosphate, pH 7.4), and EDTA-free inhibitor cocktail tablets (Roche, Germany). Cells were disrupted with two passes through an EmulsiFlex-C15 high-pressure homogenizer (pressure setting 15,000-20,000 psi) (Avestin, Canada) and were then pelleted at 15,000 rpm and the supernatant filtered through a 0.25 μm filter (Millipore, USA) before loading onto a pre-charged and equilibrated 5 ml HisTrap HP column (GE Healthcare, UK) charged with Ni2+. Bound proteins were washed following the manufacturer’s specifications and eluted using a continuous 0-500 mM imidazole gradient at 2 ml min-1. Chromatographic peaks corresponding to recombinant proteins were collected (8-12 ml total volume) and concentrated to 1 ml in 50 ml 10,000 MWCO concentrators (Vivascience, Germany). Fractions containing recombinant proteins were confirmed by SDSPAGE and Western analysis. Some samples were further purified by size-exclusion chromatography using a Superdex gel filtration 200 column (GE Healthcare, UK) connected to an ATKA FPLC (GE Healthcare) and eluted in 20 mM Tris HCl, pH 7.0.

Concentration of protein was estimated using flouromentic quantitation (Quant-iTTM Protein Assay Kit, Invitrogen, USA) or micro-fluidics based protein chip assays (ExperionTM Pro260 Analysis kit, Bio-Rad, USA) according to the manufacturer’s instructions.

**2.20.3 Denaturing gel electrophoresis**

Fractions from the protein purification steps were added directly to SDS-PAGE sample buffer, in a ratio of three parts of protein sample to one part buffer. After mixing, the samples were placed in a heating block at 98 ºC for 5 min, and then allowed to cool at room temperature before loading. Samples were loaded on a SDS-PAGE gel, consisting of a 10% (w/v) polyacrylamide separating gel and a 4% (w/v) polyacrylamide stacking gel (Schägger and von Jagow, 1987). NuPAGE® Novex ® Bis-Tris mini gels (Invitrogen, USA) were also used, according to the manufacturer’s protocol.

Following loading, the gel was placed in a Mini-Protean 3 electrophoresis cell (Bio-Rad Laboratories, USA) using anode and cathode buffers (Schägger and von Jagow, 1987). Up to 20 μl of sample in buffer was loaded in each well of an SDS-PAGE gel. Gels were electrophoresed at a constant current of 90 mA for 1 h, or until the loading dye had reached the bottom of the gel. For the estimation of protein size, pre-stained protein standards were also loaded, SeeBlue Plus2 (Invitrogen, USA) or Precision Plus Protein Dual Colour (Bio-Rad Laboratories, USA). Following electrophoresis, gels were either stained with Coomassie Brilliant Blue G250 protein stain (Neuhoff et al., 1988) or transferred to a membrane for Western blotting.

**2.20.4 Analysis of protein by Western blot**

Gels were transferred to Immobilon-P PVDF membrane (Millipore, USA) by semi-dry electophoresis (Trans-Blot Semi-Dry Cell, Bio-Rad Laboratories, USA) at a constant 5 V for 4 h or 20 V for 90 min. After soaking in methanol for 10 s, to enable aqueous buffers to enter the pore structure, the membrane was dried at 37 ºC for 10 min. For Western analysis a rapid immunodetection method was used without initial blocking. The primary (Anti-His6 mouse monoclonal antibody, Roche, Germany) and secondary (Anti-mouse IgG goat alkaline phosphatase, Sigma, USA) antibodies were diluted 1:1000 and 1:2000 respectively in 1% BSA, 0.05% TBS-Tween 20. Between each antibody detection step, the membrane was washed three times in TBS, for 30 s each wash with gentle agitation. For visualization, 5-bromo-4-chloro-3- indolyl phosphate (BCIP) was applied to the blots and incubated for 10 min. To stop the reaction, blots were washed with H2O.

**2.20.5 Electrophoretic mobility shift assays**

Complementary oligonucleotides for electrophoretic mobility shift assays (EMSA) were annealed. Oligos were heated to 95 ºC for 5 min to remove any secondary structure and then placed in a thermal block for 30 min at the predicted Tm of the primer pair and then slowly cooled to room temperature overnight. To make probes, the annealed oligos were labelled with [ 32P]-γ-ATP (Perkin Elmer, USA) using T4 polynucleotide kinase (New England Biolabs, USA). Unincorporated labelled nucleotides were removed using ProbeQuant G50 micro columns (GE Healthcare, UK) according to the manufacturer’s instructions.

For the binding assay 0.2 - 0.9 µg of purified recombinant MdMYB10 protein was mixed with 0.05 – 0.1 pmol of double stranded, labelled DNA probe in binding buffer (10 mM tris, 50 mM KCl, 2.5 mM DTT, 1 µg poly(dI-dC), 10 µg BSA, 4% glycerol) and incubated for 30 min at 25 ºC. Cold competitor DNA was also made from annealed complimentary oligos as above, and were added at 20 or 200-fold excess versus the radiolabelled probe.

The bound complexes were resolved by electrophoresis on native 5% polyacrylamide gels. The recipe, sufficient for two gels, was as follows: 12.5 ml filtered TBE, 8.4 ml H2O, 4.1 ml 40% (w/v) polyacrylamide, 250 µl 10% (w/v) ammonium persulfate (APS) and 18.5 µl TEMED. The total binding reaction volume (20 µl) was loaded with 5 µl loading buffer (50 mM Tris HCl, 10% (v/v) glycerol, 0.01 % (w/v) bromophenol blue, 5 mM DTT). The gel was placed in a Mini-Protean 3 electrophoresis cell (Bio-Rad Laboratories, USA) and electrophoresis was conducted in filtered 0.5% Tris-Borate EDTA (TBE) buffer (pH 8.3) at a constant 100 V for 60 min at 25 ºC. The gel was dried in a gel drier (Atto Corporation, Japan) for 40 min at 80 ºC under vacuum before autoradiography with an intensifier screen at -80 ºC.