IDENTIFYING AND CHARACTERIZING GENES AND TRANSCRIPTION FACTORS
INVOLVED IN COLORATION OF APPLE FRUIT

BY

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DISSERTATION

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ABSTRACT

Apple (*Malus × domestica* Borkh.) is one of the most highly cultivated fruit crops grown around the world and apple consumption has been increasing over the years. One of the most important determinants of fruit quality is skin color. Red coloration in apple fruit is attributed to anthocyanin accumulation. Anthocyanins are encoded by structural genes, in the anthocyanin biosynthetic pathway, which are highly regulated by transcription factors. In this thesis, studies were conducted to identify and characterize structural genes and associated transcription factors involved in the anthocyanin biosynthetic pathway.

Three genes encoding apple anthocyanin reductase gene (*MdANR*) were isolated, designated as *MdANR1*, *MdANR2a*, and *MdANR2b*. It is found that *MdANR2a*, and *MdANR2b* are in fact allelic. *MdANR1* and *MdANR2* were mapped to the apple linkage map on linkage groups (LG) 10 and 5, respectively. The functionality of *MdANR* gene was investigated following its overexpression in tobacco and found to influence flower color pigmentation and pattern. Overexpression of *MdANR* influenced other genes in the flavonoid biosynthesis pathway by down-regulating *chalcone isomerase* (*CHI*), *dihydroflavonol reductase* (*DFR*), and *leucoanthocyanidin reductase* (*LAR*) genes. Moreover, the observed loss of flower color in transgenic tobacco lines was attributed to reduction of anthocyanin pigments. This was likely due to down-regulation of tobacco *CHI* and *DFR* genes that are important in anthocyanin production. In addition, a new floral pigmented pattern was generated by incomplete inhibition of anthocyanin production. As expected, the epicatechin accumulated at higher levels in transgenic tobacco than in wild-type tobacco. However, higher amounts of catechin but lower levels of LAR, responsible for synthesis of catechin, were found in transgenic lines when compared to wild-type tobacco. Thus, it has been proposed that ANR plays a redundant role to that of LAR.

A novel MYB transcription factor (TF) gene, designated as *MdMYB11*, was isolated and genetically mapped onto LG15 of the apple genetic map. Alignment of deduced amino acid sequences of MdMYB11 to those of other R2R3 MYB TFs revealed that this new apple transcription factor contains the R2R3 conserved domain. Moreover, this TF is highly similar to Arabidopsis MYB subgroup 4, such as AtMYB3, 4, and 6, by which they negatively regulate

genes involved in monolignol biosynthesis. Functional analysis of *MdMYB11* was conducted via ectopic expression in tobacco. Expression of *MdMYB11* increased anthocyanin production in tobacco flowers by inducing several anthocyanin biosynthesis pathway genes, particularly those of *CHI*, *chalcone synthase* (*CHS*), and *UDP-glucose: flavonoid 3-o-glucosyltransferase* (*UFGT*). In addition, this TF functioned as a repressor of both *cinnamate-4-hydroxylase* (*C4H*) and *4-coumaroyl:CoA-ligase* (*4CL*) genes, both important in lignin biosynthesis, and possibly contributing to modulation of floral morphogenesis. Moreover, transgenic flowers had longer styles than those of wild-type flowers, suggesting that the *MdMYB11* gene might be involved in pistil development.

New candidate TF genes regulating apple fruit coloration were identified following global gene expression analysis of the apple transcriptome using an apple microarray. Comparison of gene expression in fruit peel of apple cv. Red Delicious subjected to continuous 'dark treatment' versus dark-grown fruit subjected to '14 h-light-exposure' identified 815 genes that were modulated. Following annotation (to the Arabidopsis Gene Ontology), these genes were classified into 19 categories, and were mostly involved in primary metabolism (17%) and transcription (12%). Of these, 18 genes encoded for putative TFs. Further identification of color-related TFs was conducted by comparison of expression profiles of fruit of red skinned apple cv. Red Delicious and non-red skinned apple cv. Golden Delicious, and using quantitative real-time (RT)-PCR (qRT-PCR). Two putative TF genes were found to be expressed at higher levels in fruit of 'Red Delicious' than that in 'Golden Delicious', thus suggesting that these TFs might be involved in fruit coloration.

Altogether, these findings have provided novel information and knowledge of the role(s) of genes and transcription factors involved in the anthocyanin biosynthesis pathway. Moreover, the regulator mechanism of fruit coloration has been further elucidated following transcriptome analysis of the apple genome and functional analysis of selected genes and transcription factors.

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CHAPTER 1
INTRODUCTION

1.1 Apple fruit quality

Apples are a member of Rosaceae family. The genetic evidence showed that *Malus sieversii* species is the progenitor of the cultivated apple (Harris et al., 2002). Nowadays, domesticated apple (*Malus x domestica*) is one of the most highly cultivated fruits grown around the world. Over 20,000 varieties of apple are currently known. The United States is the third largest producer and exporter according to USDA report (http://www.fas.usda.gov/htp/2010_Dec_fruit.pdf).

In the past three decades, the apple consumption per capita has increased due to a combination of factors including high population growth and diversity, rising incomes, new cultivars and products that meet consumer lifestyles and preferences, and recent concerns of a healthy diet. In order to meet public's demand, apple breeders have been continuously developing new varieties with high quality fruit.

The apple is self-incompatible so it is easy to intercross resulting in thousands of cultivars being produced. Breeding is undertaken using sexual hybridization by crossing parents with desired characteristics, followed by selection, and evaluation with the ultimate release of new apple cultivars. Since fruit characteristics influence fruit marketability, a major goal in apple breeding is to improve fruit quality including internal quality such as flavor, sugar and acid contents, and fresh texture, and external quality such as size, shape, and skin color. Fruit taste is very complex to analyze and highly subjective. It is characterized by a combination of sweetness, acidity, and flavor, which contribute to the overall organoleptic quality of fresh apple. Integration of plant physiology, biochemistry, and molecular biology of fruit characteristics has significantly provided useful information to improve fruit quality.

1.1.1   Sweetness

Soluble sugar mainly contributes to fruit sweetness. Glucose, sucrose, and fructose are predominant sugar in fruits. In term of sweetness, fructose is the highest,

followed by sucrose, and glucose is the lowest. During apple growth, sugar accumulation is in the form of sorbitol and sucrose derived from photosynthesis (Steenkamp et al., 1982; Yamaki, 2010). Sorbitol and sucrose are translocated to a sink organ such as fruit (Loescher, 1987) and assimilated in carbon metabolism to form fructose, glucose, sucrose, malic acid, and starch (Hansen, 1970). At ripening stage, starch is mostly breakdown to sucrose (Berüter and Feusi, 1997).

Sucrose is a disaccharide sugar composed of glucose and fructose. The synthesis of sucrose and hydrolysation of sucrose are reversible reaction. Hydrolyzation of sucrose by invertase (β-fructosidase) enzyme resulted in glucose and fructose while sucrose synthase (SUSY) enzyme catalyses the reverse reaction to synthesize sucrose. SUSY enzyme is encoded from *SUSY* gene. Although *SUSY* gene has not been cloned in apple, it was clone in tomato and the inhibition of the *SUSY* gene by antisense resulted in a reduction of sucrose unloading (D'Aoust et al., 1999). It has been reported in pear that SUSY has two isoforms (SSI and SSII) and they function in the utilization of carbohydrate temporally in young and mature fruit, respectively (Tanase and Yamaki, 2000).

Even though fructose can be derived from sucrose breakdown, it is mainly produced from the conversion of sorbitol to fructose by sorbitol dehydrogenase (SDH) in the sink (Berüter and Feusi, 1997; Yamaguchi et al., 1994; Oura et al., 2000). In apple, fructose was the most abundant in apple, followed by glucose and sucrose (Wu et al., 2007). It has been reported that the majority of the translocated carbohydrates are sorbitol (Klages et al., 2001); thus, fructose becomes a major sugar formed in the apple. SDH enzyme was highly active in apple leave, fruit, and shoot tips (Loescher et al., 1982; 1996; Nosarszewski et al., 2004; Zhou et al., 2006). *SDH* gene was isolated, actively expressed in developing apple fruit, and upregulated by feeding of exogenous sorbitol (Nosarszewski et al., 2004). Inhibition of sorbitol synthesis by antisense suppression of aldose-6-phosphate (A6PR) leads to significantly decrease in sorbitol concentration but increase in sucrose concentration in leaves while CO_2 assimilation and plant growth were not altered in transgenic apple (Cheng et al., 2005). In shoot tips of the transgenic apple, decreased in sorbitol synthesis, concentration of sorbitol was lower than that of sucrose

compared with the untransformed control (Zhou et al., 2006). This suggested the negative correlation of sorbitol and sucrose synthesis and accumulation. Nine apple SDH genes were identified from the 'Matsu' apple genome and their expressions were occurred in different tissues including five SDH genes in fruit, four in seed, and three, in cortex (Nosarszewski et al., 2004; Nosarzewski and Archbold, 2007). However, expression of four SDH genes was not correlated to SDH activity in apple leaves and fruits (Park et al., 2002) and in shoot tips and leaves of young tree, suggesting that other factors regulate the SDH activity after SDH expression (Wu et al., 2010).

1.1.2 Acidity

Organic acids are major contributors to fruit acidity. In apple fruit, the major organic acids were tartaric, quinic, malic, shikimic, citric and succinic acids; however, malic acid is predominant organic acid (Wu et al, 2007). Malic acid is in part of carbon metabolism and mainly synthesized in fruit.

Malate is synthesized via glycolysis and the pentose phosphate pathway (Blanke and Lenz, 1989) by using enzymes PEPcarboxylase (PEPC) (Chollet et al., 1996) and NAD-dependent malate dehydrogenase (MDH) (Miller et al., 1998). In plant, MDHs can be categorized to 5 classes based on coenzyme specificity, subcellular localization and physiological function (Ding and Ma, 2004). Apple cytosolic NAD-dependent malate dehydrogenase (*MdcyMDH*) gene was cloned and characterized, and the gene encoded an enzyme crucial for malate synthesis in the cytosol by catalyzing the reaction from oxalacetic acid (OAA) to malate in vitro (Yao et al., 2008; 2011). Malic acid (MA) is transported via vacuolar H^+-ATPase (VHA) and H^+-pyrophosphatase by generating the motive force organic acid transporters (Shiratake and Martinoia, 2007) and stored in the vacuole of apple parenchyma cells (Yamaki, 1984; Yamaki, 2010). MA was highly accumulated in the young fruit, and reduced during growth and ripening (Yao et al., 2011).

Malate can also be used as a substrate for respiration. Malate can support the TCA cycle and respiration directly or alternatively convert to pyruvate via NADP-dependent malic enzyme or via the pyruvate kinase pathway prior to enter the TCA cycle (Wang et

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al., 2010). In addition, malate can be a substrate for gluconeogenesis through OAA by decarboxylation of OAA to phosphoenol pyruvate (PEP) by ATP-dependent phosphoenolpyruvate carboxylase kinase (PEPCK) and fructose-1,6-bisphosphatase (FBP) (Bahrami et al., 2001; Gibson and McAlister-Henn, 2003).

The apple cultivar 'Usterapfel' has two known genotypes including a high-acid genotype (HA-fruit) and a low-acid genotype (LA-fruit) that differ in malic acid content (Beruter, 1998). Recent study suggested that the malate accumulation was restricted in apple parenchyma cells of LA-fruit affecting overall carbon partitioning by allowing malate to be more available for gluconeogenesis in LA-fruit (Beruter, 2004). The *MdcyMDH*, *MdPEPC* and *MdVHA-A* genes were implicated in malate synthesis and accumulation. Their gene expression patterns and MA accumulation pattern were tested in HA-fruit and LA-fruit throughout fruit development (Yao et al., 2009). The transcript level of *PEPC* and *VHA* were positively correlated with PEPC and VHA activities at most fruit developmental stages and malic acid accumulation in LA-fruit was high in young fruit and gradually reduced during fruit development whereas that in HA-fruit had two peaks at the early and latest stages of development. In contrast, the *MdcyMDH* transcript and the enzyme activity were high in LA-fruit at the ripening stage but its MA accumulation was low, suggesting post-transcriptional regulation may be involved (Mackintosh, 1998).

The low-acid character was known to be recessive in apple (Visser and Verhaegh, 1978) and citrus (Fang et al., 1997) but dominant in peach (Yoshida, 1970); thus, the mechanism determining low acidity should not be identical in different species. In apple, genetic linkage map of the cross 'Prima' × 'Fiesta' revealed the position of the *Ma* gene (Maliepaard et al., 1998). Two genetic markers including AFLP marker E31M38-0193 and SSR marker CHO5e04z were found to be linked to fruit acidity (Liebhard et al., 2003). Using cDNA-AFLP approach to screen genes differentially expressed between low and high acid apple, apple *Mal-DDNA* gene was identified and linked to fruit acidity (Yao et al., 2007). The *Mal-DDNA* transcripts were highly expressed in low-acid fruit whereas much lower in high-acid fruit, suggesting negative regulation of fruit acidity.

1.1.3   Flavor

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Flavor, referred to aroma, is an important characteristic of fruit quality although the aroma biosynthesis pathway is complex. Volatile compounds are contributed to overall flavor quality. In apple, over 300 volatile compounds have been complied including esters, alcohols, aldehydes, ketones, and sesquiterpenes (Dixon and Hewette, 2000). Although esters and alcohols are major volatiles, carboxyl esters are predominant including ethyl, butyl and hexyl acetates, butanoates, and hexanoates (Song and Forney, 2008). However, around 20 compounds are "character impact" compounds such as (E)-β-damascenone, (Z)-3-hexenal, 2-methylbutyl, hexyl acetates, and a number of esters including ethyl butanoate and 2-methylbutanoate (Fuhrmann and Grosch, 2002; Dixon and Hewette, 2000; Song and Forney, 2008). The contribution of these volatiles to aroma is dependent on the apple cultivar and its maturity, and volatile extraction procedures (Mehinagic et al., 2006). In addition, the volatiles present in variable proportions in each apple cultivar and no key characteristic compounds represents any given cultivar (Paillard, 1990)

The volatile esters are largely synthesized in fruit tissue, particularly in the skin (Guadagni et al., 1971; Defilippi et al., 2005). As volatile compounds composed of several chemical classes, they could be synthesized from amino acids, membrane lipids, and carbohydrates (Fellman et al., 2000; Pérez et al., 2002). In apple, esters are major compounds contributed to apple aroma; however, the ester biosynthetic pathway is poorly understood.

Straight-chain esters in apple appeared to be synthesized from breakdown of fatty acid (FA) via lipoxygenase pathway (Rowan et al., 1999) whereas branch-chain esters derived from isoleucine (Rowan et al., 1996; Matich and Rowan, 2007). The C18:1 and C18:2 FA were increased rapidly during fruit ripening in association with aroma production; therefore, they were likely to serve as a precursor for aroma volatile biosynthesis (Song and Bangerth, 2003). In addition, ester could be generated as a result of esterification of alcohols, carboxylic acids, and acyl co-enzyme A (CoA). Beta-oxidation of fatty acid provides alcohol and acyl CoA to form ester. By a series of reduction, acyl CoAs are reduced to aldehydes by acyl CoA reductase, aldehydes are subsequently reduced to alcohols by the alcohol dehydrogenase (ADH) enzyme, and

converted to esters by the alcohol acyltransferase (AAT) (Bartley et al., 1985). AAT enzyme engaged in the final step of ester biosynthesis and appeared to be an important enzyme in this pathway. Genes encoding ATTs have been isolated from apple including *MpATT1* (Souleyre et al., 2005) and *MdATT2* (Li et al., 2006a). The apple *ATT* genes are expressed in different tissue depending on the apple cultivar and other *AAT*s are probably contribute to volatile ester biosynthesis with different characteristics (Souleyre et al., 2005; Li et al., 2006a).

Volatile biosynthesis is influenced by many factors such as ethylene-related fruit maturity and postharvest handling and storage. Apples are climacteric fruit that respiration and ethylene production are raised in association with ripening and storage. Ethylene plays an important role in ripening process of climacteric fruit and is strongly associated with aroma production (Defilippi et al., 2004; Barry and Giovannoni, 2007). Ethylene moderated the expression of the *AAT* gene whereas the expression of the *ADH* gene was not affected (Defilippi et al., 2005). Maturity, ethylene production, and volatile production are positively correlated (Song and Bangerth, 1996). The production of volatiles is increased during fruit ripening although immature fruit is preferred at harvesting time in order to increase storage time and avoid physical damage during transportation. However, immature fruits lose the capacity of volatile production more easily than mature fruits (Song and Bangerth, 1996; Harb et al., 2008). Therefore, harvesting fruit at maturity provides suitable aroma contents and the usage of ethylene suppression substances can alleviate ripening problem. The chemical substances such as aminoethoxyvinylglycine (AVG) and 1-methylcyclopropene (1-MCP) are used to delay fruit ripening by different mechanisms. The AVG inhibits ethylene biosynthesis by competitive inhibition of 1-aminocyclopropane- 1-carboxylic acid (ACC) synthase (Boller et al., 1979) whereas the 1-MCP binds to ethylene receptor (Sisler and Serek, 2003); however, they were found to inhibit volatile production (Mir et al., 1999; Fan et al., 1998; Li et al., 2006b). Application of AVG and 1-MCP had negatively impacted on the aroma biosynthesis by reducing the activities of lipoxygenase (LOX), alcohol dehydrogenase (ADH), and pyruvate decarboxylase (PDC) (Harb et al., 2010). Delay ripening can also be achieved by silencing approach. For example, antisense suppression

of ACC oxidase and/or ACC synthase production resulted in a reduction in fruit softening, sugars, and ester synthesis (Dandekar et al., 2004).

1.1.4   Fresh texture

Fruit texture is an important factor determining eating quality. According to sensory studies and consumer expectations, aroma, firmness, crispness, and juiciness are most relevant traits for apple (Harker et al., 2003; Hoehn et al., 2008), and crisp and juicy apple seem to be preferred (Hoehn et al., 2008). Understanding the biological basis of fruit ripening is crucial to improve texture and shelf life. Fruit ripening is related to fruit texture and programmed in developmental process. Fruit softening is attributed to modification of cell wall polysaccharide integrity (Fischer and Bennett, 1991). The majority of the cell wall polysaccharide is pectin existing in a wide range of acidic heteropolysaccharides (Willats et al., 2001). Several cell wall degrading enzymes such as polygalacturonase (PG), pectin methylesterase (PME), β-galactosidase, and expansin are contributed to the loss of cell wall integrity (Brummell and Harpster, 2001). It is implied that inhibition of these enzymes would prolong the softening process.

It has been known that fruit ripening is ethylene dependent for climacteric fruits such as apple (Barry and Giovannoni, 2007). Ethylene is synthesized from S-adenosyl-L-methionine via 1-aminocyclopropane-1-carboxylic acid (ACC) by two reactions in this pathway using enzymes ACC synthase and ACC oxidase (Bleecker and Kende, 2000). Low softening cultivar such as Fuji appeared to have homozygosity of two ethylene biosynthesis genes (*MdACS1* and *MdACO1*) resulting in low ethylene production (Costa et al., 2005). Another ripening-specific ACS gene (*MdACS3*) was identified and composed of three genes in the *MdACS3* family (a, b, c). Null allele of *MdACS3a* is unable to be transcribed leading to longer fruit firmness maintained, suggesting that the *MdACS3a* gene plays an important role in regulation of apple ripening (Wang et al., 2009).

In addition, ethylene is known to be involved in PG production. PG is one of the major enzymes involved in pectin depolymerization by biochemically catalyzing the hydro- lytic cleavage of α(1–4) galacturonan. PG is encoded by multi-gene family

(Hadfield and Bennett, 1998). The expression of apple PG1 (*Md-PG1*) gene assessed from 14 apple cultivars was related to ripening behavior (Wakasa et al., 2006). The lower the *Md-PG1* gene expression was associated with the lower firmness loss during fruit ripening.

Fruit texture is considered a quantitative trait and several quantitative trait loci (QTLs) were reported in several genetic mapping studies generating useful markers associated with fruit quality traits (Liebhard et al., 2003; Kenis et al., 2008). QTLs for firmness and flesh texture have been mapped on linkage groups LG01, LG08, LG10, LG15 and LG16 (King et al. 2000; Maliepaard et al. 2001; Seymour et al. 2002). Recently, Costa et al. (2010) mapped the Md-PG1$_{SNP}$ marker on to LG10 in which a QTL identified for fruit firmness is resided. Since fruit quality traits are controlled by polygenes, these markers would assist in genotype selection for breeding of better fruit quality traits.

Expansin is another major class of cell wall degradation enzymes and probably involved in breaking the non-covalent bonds between the hemicellulose matrix and the cellulose microfibril (Cosgrove, 1997; Cosgrove, 2000). Thus, the polymer structure is exposed and subsequently degraded by other cell wall enzymes. Seven apple expansin genes (*MdExp*) were isolated; however, only *MdExp3* and *MdExp7* genes were likely associated with fruit softening (Wakasa et al., 2003; Costa et al., 2008). An SSR marker for *MdExp7* gene has been mapped to LG01 where a major QTL for fruit firmness has been identified (Costa et al., 2008).

Identification of genes encoding other cell wall degradation enzymes has been reported. By using homology-based cloning, a pectin methylesterase (*MdPME1*), a pectate lyase (*MdPL1*), an α-l-arabinofuranosidase (*MdAF1*), an endo-1,4-β-glucanase (*MdEG1*), two xyloglucan endotransglucosylase/hydrolases (*Md-XTH1* and *Md-XTH2*), and an alpha-expansin (*MdEXPA3*) genes were isolated from over-ripe fruit cDNA library (Goulao et al., 2008). However, none of the clones were fruit specific when determining their expression in various plant tissues.

1.1.5   Fruit skin color

The apple skin color is an important determinant of fruit quality. Generally, red-skin fruit is preferred by consumers as it is perceived to correlate with better taste and flavor (King and Cliff, 2002). The red pigment in apple fruit is a result of anthocyanin accumulation. Anthocyanin pigments have been known to have antioxidant properties and implicated in various health-promoting properties (Lila, 2004). Recent studies suggested that the dietary antioxidant benefit might be not a direct result of the anthocyanins themselves but the anthocyanin-related activation of endogenous antioxidant systems (Williams et al., 2004; Lotito and Frei, 2006). Numerous studies have indicated that the anthocyanins may protect against or reduce the incidence of many health-related problems such as age-related neurological disorders (Joseph et al., 1999), cardiovascular diseases (Mennen et al., 2004; Toufektsian et al., 2008), obesity (Tsuda et al., 2003), and various cancers (Gallus et al., 2005; Rui et al., 2005). Anthocyanins also have an ability to promote immune function (Wang and Mazza, 2002), restore vision disorders (Matsumoto et al., 2003) and protect against inflammation (González-Gallego et al., 2010; Jung et al., 2009). Anthocyanin biosynthesis has been well characterized and transcriptionally controlled by transcription factors. More detail in fruit coloration and its regulation can be found in the following sections.

1.2 Coloration in plants

1.2.1 Plant pigments

Accumulation of flavonoids (including anthocyanins), carotenoids, and betalains contribute to development of various colors in flowers and fruits. While the first two classes are ubiquitous, betalains are limited to one group of angiosperms, Caryophyllales; e.g., beetroot (*Beta vulgaris*), and they have not been found in combination with anthocyanins (Stafford, 1994; Tanaka et al., 2008).

These pigments are synthesized via different pathways in different tissues, and stored in different organelles. Carotenoids are lipid soluble, derived from isoprenoids, synthesized in chloroplasts, and widely distributed in plants (DellaPenna, 1999; Grotewold, 2006). Betalains and anthocyanins are water-soluble and stored in vacuoles

(Tanaka et al., 2008). Betalains are derived from tyrosine and synthesized in vacuoles, while, anthocyanins are derived from flavonoids, and synthesized in the cytosol (Grotewold, 2006; Tanaka et al., 2008).

Pigmentation in flowers and fruits play important roles in attracting insects. The main role of flower pigmentation is to attract pollinators as color patterns of flowers that are distinctive against their background allow for easy recognition. However, pollinators perceive colors in different ways. For instance, red color is visible to hummingbirds, but it is colorless to bees (Kevan et al., 1996). For fruit skin, color attracts animals to aid in seed dispersal (Schaefer et al., 2004; Lev-Yadun et al., 2009; Lomáscolo and Schaefer, 2010) and also can determine the fruit quality. Thus, inactivation of some structural genes involved in coloration may influence plant/animal interactions; i.e., type of pollinators resulting in genetic isolation and possibly speciation (Vickery, 1995; Owen and Bradshaw, 2011). Beside coloration, flavonoids are believed to play several roles including plant defense against light stress and pathogen attack (Treutter, 2005).

1.2.2   Flavonoid-derived coloration

Various organic compounds are present in plants, and they are generally classified as either primary or secondary metabolites. Primary metabolites are compounds that are found across the plant kingdom and play essential roles in physiological mechanisms such as respiration, photosynthesis, as well as growth and development. Many secondary metabolites are distributed among a limited number of species in the plant kingdom despite their structural diversity.   In recent years, secondary metabolites have gained interest as they play several key roles in protecting plants against herbivores and microbial infection, and attracting seed-dispersing animal for pollination (Dewick 2002; Treutter, 2005). Moreover, secondary metabolites are deemed new potential sources of natural medicinal drugs, antibiotics, insecticides, and herbicides (Dewick 2002). In the area of human nutrition research, long-term intake of some secondary metabolites has been reported to reduce the incidence of cancer such as prostate cancer, stomach cancer, breast cancer, and colorectal cancer (Kyle et al., 2010; Luo et al., 2010; Rossi et al., 2010; Steinbrecher et al., 2009) and many chronic diseases such as cardiovascular

disease, and obesity (Conforti and Menichini, 2011; Santos et al., 2010; Survay et al., 2011).

Among diverse groups of secondary metabolites, flavonoids are widely found throughout the plant kingdom (Harborne, 1994). Their functions in plants are varied and include UV protection, pigmentation, and disease resistance (Winkel-Shirley, 2001a; Treutter, 2005). More importantly, there is increasing evidence on the benefits of flavonoids in human health (Chong-Han, 2010; Liu, 2003; Lotito and Frei, 2006; Rudkowska and Jones, 2007; Ververidis et al., 2007).

Within a diverse group of flavonoid compounds, anthocyanins, condensed tannins (CTs), and flavonols are ubiquitously found in higher plants (Tanaka et al., 2008). The most conspicuous class of flavonoids is anthocyanins due to their role in contributing to the wide range of colors. The majority of flower pigments are anthocyanins. Anthocyanins can be found not only in flowers and fruits, but also in leaves, particularly during senescence. They are also present in stems, roots, and sometimes in fruit flesh and seeds (Tanaka et al., 2008). The divergence of this group of flavonoids in types and functions that exist in plants raises questions on the mechanisms of action of these metabolites, their roles in generating arrays of colors, as well as their synthesis, expression, and regulation.

A variety of colors of anthocyanins may be due to the number of hydroxyl groups on the B-ring, sugars, and acyl side groups. When the number of hydroxyl groups on the B-ring and attached aromatic acyl groups increase, flower color is more likely to be blue. In addition to the structural features of anthocyanins, other factors that determine floral hues are co-pigmentation, vacuolar pH, and cell shape (Tanaka et al, 2005; 2008). For example, co-pigmentation with flavonols influences color (Goto and Kondo, 1991; Fossen et al., 2000).

Coloration of apple fruit skin is mainly attributed to the accumulation of anthocyanins, a class of flavonoid-pathway secondary metabolites. Five polyphenolic groups including hydroxycinnamic acids, flavan-3-ols/procyanidins, anthocyanins, flavonols, and dihydrochalcones are mainly found in various apple cultivars (Mazza and Velioglo, 1992; Schieber et al., 2001; Tsao et al., 2003). Many phenolics mostly forms

complex with sugar moiety, particularly galactose, glucose, rhamnose, xylose, arabinose, and rutinose (Mazza and Velioglo, 1992; Merken and Beecher, 2000; McRae et al., 1990; Guyot et al., 1998). However, cyanidin 3-galactoside is predominant and found only in red apple skin (Lancaster, 1992; Tsao et al., 2003).

1.2.3   Factors affected coloration in apple

Efforts have been undertaken to improve coloration of apple skin and to understand the mechanism underlying pigment formation. Two major factors that influence coloration have been proposed including both internal and external controls.

Coloration is genetically determined by various structural genes (Winkel-Shirley, 2001a). The biosynthetic pathway of anthocyanins is known, and several genes coding for enzymes in the biosynthetic pathway of anthocyanin have been isolated, cloned, and characterized (Table 1.1). Expression profiles of these enzymes during fruit development have been investigated. Honda et al. (2002) reported that induction of most genes along the anthocyanin biosynthetic pathway increased accumulation of anthocyanin in apple skin. In addition, the anthocyanin biosynthetic enzymes are induced coordinately during fruit development with high enzyme activities observed in immature fruit, and again at ripening, depending on the cultivar (Lister et al., 1996).

It has long been known that environmental conditions such as light and temperature affect color formation in apple. For instance, UV-B irradiation and low temperature stimulated anthocyanin production (Saure, 1990). Biochemical studies indicated that enzymes in the anthocyanin biosynthetic pathway were highly induced by UV-B irradiation and low temperature (Dong et al, 1998; Kim et al., 2003; Ubi et al., 2006; Ban et al., 2007; Steyn et al., 2009). Light exposure induced coloration was also observed in other plant species such as maize (Piazza et al., 2002; Ferreyra et al., 2010). This demonstrated that most of genes involved in anthocyanin biosynthesis were coordinately induced under UV-B irradiation and low temperature.

Thus, it is likely that regulatory genes are induced by environmental factors, and act upstream of the anthocyanin biosynthesis cascade (Davies and Schwinn, 2003). So far, anthocyanin biosynthesis genes are largely regulated at the transcriptional level, and

consequently expression patterns of regulatory genes determine pigmentation patterns (Holton and Cornish, 1995; Ramsay and Glover, 2005).

1.3 Flavonoid biosynthetic pathway

Flavonoids and other flavonoid-related compounds, such as stilbenes, are synthesized via a complex network of routes based primarily on the shikimate, phenylpropanoid, and malonate pathways (Winkel-Shirley, 2001a; Tanaka et al., 2008). Flavonoids, including anthocyanins and condensed tannins (CTs), as well as stibenes are synthesized from malonyl-CoA, derived from the malonyl pathway, and *p*-coumaroyl-CoA, derived from the phenylpropanoid pathway. Enzymatic steps in sequential reactions along with several reported enzymes involved in flavonoid synthesis are illustrated in Figure 1.1. A summary of flavonoid biosynthesis pathway genes that have been isolated and characterized are presented in Table 1.1 (modified from Winkel-Shirley B, 2001b).

1.4 Anthocyanin-regulating transcription factors

Anthocyanin synthesis is derived from the coordination of genes encoding enzymes in the anthocyanin biosynthesis pathway. It has been reported that regulation of these genes likely occurs at the transcriptional level by transcription factors. These transcription factors controlling anthocyanin biosynthesis have conserved domains in all higher plants (Holton and Cornish, 1995). Generally, an R2R3 MYB and a basic helix-loop-helix (bHLH) are major transcription factor families that control expression of anthocyanin biosynthesis pathway genes.

Transcription factors (TFs) may regulate coordinately with different classes of TF in a combinatorial manner by forming complexes at protein:DNA or protein-protein level (Grotewold et al., 2000). Across all plant species reported to date, anthocyanin biosynthesis is regulated by a basic helix-loop-helix (bHLH), and an R2R3 MYB TF along with a WD40 protein (Stracke et al, 2001; Allan et al, 2008). Interaction of bHLH and R2R3 MYB was studied in maize and indicated that the bHLH stabilized or permitted the R2R3 MYB to activate transcription, and enhanced the activity by interacting on promoters containing a cis-regulatory element conserved in several

anthocyanin biosynthetic genes (Hernandez et al., 2004). WD40 is also an important regulatory protein in the regulatory complex. The R2R3 MYB/bHLH/WD40 protein complex is not limited to regulation of anthocyanin, but also important in the control of various developmental processes (Ramsay and Glover, 2005). Even though MYB, bHLH, and WD40 can form a protein complex, as revealed by yeast two-hybrid assays, ectopic expression in transgenic *Arabidopsis* plants has indicated that MYB is sufficient to activate anthocyanin biosynthesis genes, possibly through the complex formation with endogenous bHLH and WD40 (Borevitz et al., 2000). Therefore, MYB transcription factors therefore play important roles in the control of anthocyanin accumulation. Some of the MYB, bHLH, and WD40 proteins that regulate flavonoid biosynthesis are listed in Table1.2.

1.4.1 MYB transcription factors

MYB TFs are reported to be important regulators of biosynthesis pathways in plants (Dubos et al., 2010). MYB TFs are abundant in plants although they are present in all eukaryotes (Jia et al., 2004). MYB TFs have diverse functions, as they are involved in regulating gene expression of several pathways related to cellular morphogenesis, cell cycle, disease resistance, and secondary metabolism, as well as anthocyanin biosynthesis pathway (Jin and Martin, 1999). MYB TFs are structurally characterized by conserved DNA-binding domain consisting of single or multiple imperfect repeats. The R2R3 MYB family is the largest family characterized in plants and has been reported to be associated with the anthocyanin biosynthesis pathway (Allan et al., 2008). In *Arabidopsis*, 339 members of R2R3 MYB TF-encoding genes have been identified (Feller et al., 2011) and they have been classified into 25 subgroups (Dubos et al., 2010).

Like other TFs, MYB TFs may act as either activators or repressors depending on whether they are involved in upregulation or downregulation of transcription initiation (Allan et al., 2008). For example, the production of anthocyanin pigment 1 (PAP1, OR AtMYB75) MYB of Arabidopsis and anthocyanin2 (AN2) of petunia activate anthocyanin accumulation by upregulating several genes in the anthocyanin biosynthesis pathway (Borevitz et al., 2000; Quattrocchio et al., 1999; Tohge et al., 2005). On the

other hand, MYB TFs acting as repressors in the flavonoid biosynthesis pathway have been identified in several plant species, for example *Arabidopsis* AtMYBL2, AtMYB4, AtMYB6, AtMYB7, and AtMYB32 (Dubos et al., 2008; Gao et al., 2011; Jin et al., 2000; Preston et al., 2004), strawberry FaMYB1 (Aharoni et al., 2001), snapdragon AmMYB308 and AmMYB330 (Jin et al., 2000; Tamagnone et al., 1998), eucalyptus EgMYB1 (Legay et al., 2010), and maize ZmMYB31 (Fornal et al., 2010).

In apple, there are at least three MYB transcription factors regulated anthocyanin structural genes including *MdMYB10*, *MdMYB1*, and *MdMYBA* (Allan et al., 2008). However, it is questionable whether they are alleles due to various reasons. First, their sequences are similar; in fact, *MYB1* and *MYBA* sequences are identical (Ban et al., 2007). Second, genetic markers for *MYB10* and *MYB1* genes are located on the same linkage group 9 of the apple genetic map at the similar location (Chagné et al., 2008). These genes are reported to be allelic using an allele-specific PCR primer approach (Lin-Wang et al., 2010). Thus, the current knowledge is not sufficient to completely understand the regulation of coloration of MYB TFs in apple. Due to the complexity of the anthocyanin biosynthetic pathway, it is possible that there are additional MYB TFs regulated genes involved in coloration. Further studies should be pursued to discover new regulatory genes, and study their mechanism of regulation.

1.4.2   Other TFs

Besides the MYB TF family, bHLH, WD40, WRKY, bZIP, HD-Zip, and MADS-box TF are also partly involved in flavonoid regulation (Kubo et al., 1999; Davies and Schwinn, 2003; Ramsay and Glover, 2005). Among these factors, bHLH and WD40 TF are the most studied. The bHLH TF and WD40 protein family have a variety of functions in plants (Allen et al., 2008; Tanaka et al., 2008). There are 162 members of bHLH TF-encoding genes divided into 12 subgroups found in Arabidopsis (Heim et al., 2003; Feller et al., 2011). The structure of the bHLH domain is composed of about 18 basic amino acids followed by two hydrophobic α-helices separated by an intervening loop. The initial evidence revealing the role of bHLH TF in transcriptional regulation of flavonoid

biosynthesis is the identification of RED (R) that activates accumulation of anthocyanin in the aleurone layer of maize kernels (Dellaporta et al., 1988).

The role of bHLH TF in regulation of anthocyanin biosynthesis pathway genes has been reported to frequently interact with R2R3 MYB TFs (Allen et al., 2008). The co-regulation of MYBs and bHLHs in color-related pathway genes has been reported in several species, such as ZmC1 MYB and ZmB bHLH in maize, and AN2 MYB and AN1 and JAF13 bHLHs in petunia (Mol et al., 1998; Goff et al., 1992). The co-regulation of bHLH is reported to be dependent on MYB proteins via its linkage to a small motif in the R2R3 binding domain (Grotewold et al., 2000).

1.5 Manipulation of the flavonoid biosynthetic pathway

The genetics and biochemistry of flavonoid synthesis were extensively studied in many plant species since the early experiments of Gregor Mendel on flower color in peas. Elucidation of flavonoid structures combined with the biochemical synthesis pathway have aided in associating single genes with particular structures. Moreover, mutations in flavonoid genes have been studied, as these are viable and easy to identify (Winkel-Shirley, 2001a). Mutants of different compounds of the flavonoid biosynthesis pathway have been identified and characterized prior to corresponding gene isolation and functional analysis.

Nowadays, isolation of genes involved in the flavonoid biosynthesis pathway has been moving at a rapid pace using genomic approaches. Isolation of flavonoid biosynthetic genes has been pursued using a variety of methodologies including protein purification, transposon tagging, differential screening, and polymerase chain reaction (PCR) amplification (Winkel-Shirley, 2001b). Isolated flavonoid genes are subjected to restriction to confirm their function by restriction fragment length polymorphism (RFLP) mapping, complementation, or expression in heterologous systems (Winkel-Shirley, 2001b). In addition, homologous genes from different species can be readily isolated once the gene of interest has been isolated from one species by simply using the original clone as a molecular probe.

There are three species including maize (*Zea mays*), snapdragon *(Antirrhinum majus),* and petunia *(Petunia hybrida)* that have been used for studying and elucidating the anthocyanin biosysthetic pathway and isolating genes involved in the flavonoid biosynthesis (reviewed in Mol et al., 1998). Yet, transcriptional regulation of flavonoid-related genes has not been well understood. Anthocyanin biosynthesis could be genetically modified by inhibiting or enhancing anthocyanin production.

1.5.1   Inhibition of anthocyanin biosynthesis

Inhibition of anthocyanin production could be achieved by two basic approaches: i) inhibiting production of a key biosynthetic enzyme; and ii) introducing an enzyme that competes with an anthocyanin biosynthetic enzyme for substrate (Davies, 2009).

The first approach directly targets the anthocyanin biosynthetic enzyme and could be achieved by inactivating enzyme function, demolishing the RNA transcripts, or by preventing the transcription of the encoding gene. All of these have been utilized in transgenic plants although the most widely used approach has been that of targeting of the RNA transcripts (Davies, 2009). The inhibition of anthocyanin biosynthetic gene by targeting RNA transcripts was first reported in petunia targeted CHS by using antisense RNA (van der Krol et al., 1988) and sense RNA (Napoli et al., 1990; van der Krol et al., 1990). Since then, there are numerous reports published demonstrating inhibition of anthocyanin pathway by using sense or antisense RNA, mostly targeting chalcone synthase (CHS) or dihydroflavonol reductase (DFR) in many species such as carnation (Gutterson, 1995), gerbera (Elomaa et al., 1993), and rose (Firoozabady et al., 1994). Targeting CHS by RNAi was also reported in fruit species such as strawberry (Lunkenbein et al., 2006), and tomato (Schijlen et al., 2007). However, blocking the expression of CHS resulted in unexpected biochemical phenotypes and would be detrimental to plant health (Lunkenbein et al., 2006; Suzuki et al., 2000). A list of publications reporting inhibition of flavonoid biosynthesis was reviewed in Davies and Schwinn (2006).

Another approach to inhibit anthocyanin production is through enzyme-substrate competition. The over-production of enzymes that compete for substrates used for

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anthocyanin biosynthetic enzymes could be feasible to reduce pigment level. By shifting the biosynthesis flow away from anthocyanin production, paler flower colors could be generated. For example, over-producing stilbene synthase (Fischer et al., 1997) or polyketide reductase (Joung et al., 2003) was able to compete with CHS. Another possible target is over-production of proanthocyanin as proanthocyanin biosynthesis utilizes anthocyanidin as a substrate that is also used for anthocyanin production. Introduction of excess anthocyanidin reductase to compete with F3GT successfully reduced anthocyanin production resulting in pale or white flower color in tobacco (Xie et al., 2003). Preventing production of anthocyanin commonly results in expected pale or white flower color phenotypes; however, some unexpected phenotypes have been observed. Male or female sterility was rendered after inhibiting flavonoid production (Fischer et al., 1997; Jorgensen et al., 2002). Flowers with more fragrant smells were produced by inhibition of F3H production resulting in an increase of methyl benzoate levels (Zuker at al., 2002).

1.5.2 Increasing anthocyanin production

The anthocyanin biosynthesis could be altered in several ways to increase the production of color pigmentation including increased activity in a key enzyme (e.g. DFR), redirection of pathway flux to reduce another competing pathway (e.g. reducing flavonol synthase (FLS) production), and manipulation at transcriptional levels by the use of transcription factors.

Initially, over-production of CHS is thought to be able to increase anthocyanin levels as it is a key enzyme in the early step of the flavonoid biosynthesis pathway. Unfortunately, when it was introduced under the control of the strong Cauliflower Mosaic Virus 35S (*35S:CHS*) promoter; it was revealed that CHS did not increase anthocyanin level (Napoli et al., 1990. Later, *DFR* gene was found to be a useful gene in order to enhance anthocyanin production in some species such as petunia and tobacco (Davies et al., 2003; Polashock et al., 2002). Over-expression of DFR and anthocyanin synthase (ANS) successfully increased levels of anthocyanin in forsythia (*Forsythia x intermedia*) (Rosati et al., 2003). Moreover, redirection of the pathway flux has been

proposed as an alternative approach as DFR served as a key flux point competing with FLS in many plant species. Thus, reducing FLS production could result in increasing anthocyanin biosynthesis. This approach was successfully used to increase anthocyanin content in flowers of lisianthus, petunia, and tobacco (Holton et al., 1993b; Nielsen et al., 2002; Davies et al., 2003).

Anthocyanin biosynthesis has been reported to be transcriptionally regulated by transcription factors although it is not clear what metabolic channeling may be involved in regulation of the flavonoid pathway (Grotewold, 2006). Ludwig and Wessler (1990) reported on characterization of MYB and bHLH from anthocyanin-related gene families where ectopic expression of an MYB and a BHLH was found to be necessary and sufficient to induce anthocyanin production in maize. Expression of maize Lc regulatory gene in apple enhanced production of anthocyanin in regenerated shoot (Li et al., 2007). MYB, bHLH, and WD40 factors seem to work in concert in compelling flavonoid-related gene expression (Ramsay and Glover, 2005). However, the presence of MYB TF is sufficient to activate anthocyanin biosynthesis genes, possibly through complex formation with endogenous bHLH and WD40 (Borevitz et al., 2000). Therefore, the use of TFs is a powerful gene technology tool to manipulate the anthocyanin biosynthesis temporally and spatially in plants. More detail has been described above in section 1.4 and Table1.2.

1.6 Global gene expression

DNA microarray are useful tools in molecular biology consisting of an array of DNA-oligonucleotide spots depositing on a solid surface. The solid supports could be glass microscope slides, silicon chips, or nylon membrane where DNA is printed or synthesized directly onto the support. These DNA spots have to be arranged in a specific order on a microarray so that a researcher could identify a particular gene sequence according to the location of each spot. These DNA oligonucleotides serve as probes and are designed using a small section of gene or other DNA elements, which are around 20 nucleotides long. Generally, a microarray is based on a single species although it could be applied for other related species. In the past, it took a long time for scientists to generate

small amounts of research data by tedious observation of single variable experiments. Today, microarray technology has transformed the field of biological science such that a single experiment can generate large amounts of data, leading global expression profiles.

Microarray technology is commonly used as a tool to study relative gene expression. It is used to determine the large number of genes that are simultaneously expressed in response to a biological perturbation. Basically, a set of genes is turned on or off in a certain condition depending on both environment and cell's needs, mRNA is then transcribed. Thus, gene expression between two conditions could be compared on the microarray.

In differential gene expression analysis, a microarray requires many steps including reverse transcribing mRNA to cDNA, labeling cDNA, hybridizing cDNA to the probes, visualizing the microarray spots, and analyzing differential gene expression. A general purpose DNA microarray experiment is shown in Figure1.2. Complimentary DNA is reversed transcribed from mRNA and fluorescently labeled. Each cDNA sample is labeled with different color (red or green) so that it could be differentiated in subsequent steps. Labeled cDNA is then used to hybridize the DNA probes on the microarray slide. Once the hybridization step is complete, a laser scanner reads the microarray and a digital image of the array is generated. Different colors including red, green, yellow, or black can be seen in each spot and they have different interpretations (Figure1.3). A special software program is used to calculate the red-to-green fluorescence ratio, and this data is stored.

Currently, there are three types of microarray technologies used including spotted microarray in which oligonucleotides are presynthesized and deposited onto a solid surface, Affymetrix GeneChips consisting of relatively short oligonucleotides synthesized on a chip surface, and other in situ synthesis platforms e.g. arrays made by Agilent and NimbleGen.

Spotted microarrays were the first platform array and are commonly used for a comparative technology. They are widely used to study a relative concentration of target between two samples. The general procedure was mentioned above. Since the basic principle is somewhat simple and all the required reagents are available, many

laboratories have produced the arrays locally. However, these arrays are not uniformly produced so variability among microarray batches would need to be taken into account when planning or comparing experiments.

Affymetrix GeneChips are commercial arrays that are widely used. Unlike spotted microarrays, relatively short oligonucleotides (25 oligos) are synthesized in situ on the surface of a glass chip using a photolithography mask by which oligo sequences are synthesized one nucleotide at a time at defined locations (Fodor et al., 1991; Pease et al., 1994). Currently, a chip is composed of 6.5 million unique probes in which 22 probes represent a single gene scattered randomly on the surface to avoid local hybridization artifacts. Expression levels of a gene compared to summation of all readings of such gene probes. Instead of comparative gene expression of two samples, as in spotted microarrays, these arrays measure relative expression of every gene in a single sample. Thus, a separate chip must be performed in comparative study of two or more samples.

Unlike Affymetrix, in situ synthesis platforms employ successful steps to create probes that are synthesized one nucleotide at a time. There are two alternative in situ synthesis methods at this time, and different companies pursue different approaches to synthesize probes. NimbleGen utilizes Maskless Photolithography by which probes are created with small rotating mirrors to control light and accomplish a similar task to Affymetrix without masking (Blanchard and Friend, 1999). For Affymetrix, a unique set of masks specific for a single array design is used. Construction of masks is expensive, thus arrays are very costly. On the other hand, in the NimbleGen technology, creating a new array design is easy by changing a pattern of electrically-controlled micromirrors. The second alternative in situ synthesis approach is developed by Agilent. Array creation involved traditional oligo synthesis chemistry but employs ink-jet technology, developed by Hewlett Packard for consumer printers, to control the liquid precursors of DNA synthesis so that 60-mer oligos can be synthesized on glass slides (Hughes et al., 2001). Due to flexibility of such a technology, it enables users to customize an array design. However, it could cause a data-analysis problem when comparing experiments. As changing the probes is easy, this is highly variable in probe design from one array to another as well as the content of the array can vary from one experiment to another.

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In addition to microarray platforms, high-throughput sequencing method could be used for gene expression profiling. For example, comparative transcript profiling using massively parallel signature sequencing (MPSS) was conducted to compare differential gene expression in sweet orange (*Citrus sinensis*) and its mutant, revealing the molecular processes regulating lycopene accumulation (Xu et al., 2009). Since microarray is designed to cover only parts of transcribed genomic regions, some important sequences that are not on the array would be neglected. In contrast, the high-throughput sequencing directly produces a large number of DNA sequences at once; thus, all expressed genes ideally are covered. The high-throughput sequencing was developed due to high demand for low-cost sequencing and the cost is lowering continuously. Therefore, an increasing number of studies using such technology are expected in the near future.

The MPSS was the first high-throughput sequencing technology developed by Brenner et al. (2000). However, MPSS was a complex technology and it was susceptible to sequence-specific bias. Newer technologies with more simple approach are now available such as the 454 Genome Analyzer FLX (Roche), the HiSeq (Illumina) and the 5500xl SOLiD System (Life Technologies). They have their own advantages that differ in the total number of sequenced reads output and the length of sequenced reads. The 454 Genome Analyzer FLX produces longer reads but less number of sequenced reads than the HiSeq and the SOLID. Therefore, the 454 Genome Analyzer FLX is generally suitable for de novo genome and transcriptome assemblies whereas the HiSeq and the SOLID are suited for quantitative applications such as small RNA analysis. Although each technology may better apply for a particular experiments, all of them could be used for all the applications.

TABLES

Table 1.1 Summary of structural genes involved in flavonoid biosynthesis

Gene	Species	Citation
Central flavonoid pathway		
Chalcone synthase (CHS)	Parsley	Kreuzaler et al., 1983
Dihydroflavonol reductase (DFR)	Maize (*Zea mays*)	Martin et al., 1985
	snapdragon (*Antirrhinum majus*)	O'Reilly et al., 1985
Chalcone isomerase (CHI)	Bean	Mehdy and Lamb, 1987
Flavanone 3-hydroxylase (F3H)	Petunia	Britsch et al., 1992
Flavonoid 3',5'-hydroxylase (F3'5'H)	Petunia	Holton et al., 1993a
Flavonol synthase (FLS)	Petunia	Holton et al., 1993b
UDP rhamnose: anthocyanidin-3-glucoside rhamnosyltransferase	Petunia	Brugliera et al., 1994
Flavonoid 3'-hydroxylase (F3'H)	Petunia	Brugliera et al., 1999
Anthocyanin 5-O-glucosyltransferase	Perilla (*Perilla frutescens*)	Yamazaki et al., 1999
Proanthocyanidin branch pathway		
Leucoanthocyanidin dioxygenase (LDOX) or Anthocyanidin synthase (ANS)	Maize	Menssen et al., 1990
Leucoanthocyanidin reductase (LAR) or Banyule (BAN)	Arabidopsis	Devic et al., 1999
Aurone branch pathway		
Aureusidin synthase	Snapdragon	Nakayama et al., 2000
Flavone branch pathway		
Flavone synthase II (FSII)	Gerbera (*Gerbera hybrida*)	Akashi et al., 1999
	Torenia (Torenia hybrida)	Martens and Forkmann, 1999
Isoflavonoid branch pathway		
Chalcone reductase (CHR)	Soybean	Welle et al., 1991
Isoflavone reductase (IFR)	Alfalfa	Paiva et al., 1991
Vestitone reductase (VR)	Alfalfa	Guo and Paiva, 1995
Isoflavone-O-methyltransferase	Alfalfa	He et al., 1998
Isoflavone 2'-hydroxylase	Licorice (*Glycyrrhiza echinata*)	Akashi et al., 1998
2-Hydroxyisoflavanone synthase	Soybean, licorice	Akashi et al., 1999 ; Steele et al., 1999 ; and Jung et al., 2000
Flavonoid 6-hydroxylase	Soybean	Latunde-Dada et al., 2001
Vacuolar transport of flavonoids		
Bronze-2 (glutathione *S*-transferase)	Maize	McLaughlin and Walbot, 1987
TRANSPARENT TESTA 12 (MATE transporter)	Arabidopsis	Debeaujon et al., 2001

Table 1.2 Examples of TFs that regulate anthocyanin and proanthocyanin biosynthesis

Family	Species	Protein	Citation
MYB	*Arabidopsis*	PAP1	Borevitz et al., 2000
		PAP2	Borevitz et al., 2000
		TT2	Nesi et al., 2001
	Maize	C1	Cone et al.,1993 and Paz-Ares et al., 1986
		P	Lechelt et al., 1989
	Petunia	AN2	Quattrocchio et al., 1999
		AN4	Spelt et al., 2000
		PhMYB3	Solano et al., 1995
		PhMYB27	Spelt et al., 2000
	Antirrhinum	AmMYB305	Sablowski et al., 1994
		AmMYB340	Mol et al., 1998
		Venosa	Schwinn et al., 2006
		Rosea1	Schwinn et al., 2006
		Rosea2	Schwinn et al., 2006
	Grape	VvMYBA	Kobayashi et al., 2002
	Strawberry	FaMYB1	Aharoni et al., 2001
	Apple	MdMYB1	Takos et al., 2006
		MdMYBA	Ban et al., 2007
		MdMYB10	Espley et al., 2007
bHLH	*Arabidopsis*	TT8	Nesi et al., 2000
		GL3	Payne et al., 2000
		EGL3	Zhang et al., 2003
	Maize	Lc	Ludwig et al., 1989
		B	Goff et al., 1992
		R	Ludwig and Wessler, 1990
	Petunia	JAF13	Quattrocchio et al, 1998
		AN1	Spelt et al., 2000
	Antirrhinum	Delilah	Goodrich et al., 1992
		Mutabilis	Martin et al., 2001
WD40	*Arabidopsis*	TTGLABRA1	Walker et al., 1999
	Apple	TTG1	Brueggemann et al., 2010
	Maize	PAC1	Carey et al., 1999
	Petunia	AN11	de Vetten et al., 1997
WRKY	*Arabidopsis*	GLABRA2	Johnson et al., 2002
bZIP	Parsley	CPRF1, CPRF2	Weisshaar et al., 1991
		CPRF4	Kircher et al., 1998
		CPRF5, CPRF6, CPRF7	Rügner et al., 2001

Figure 1.1 Schematic of the major branch pathways of flavonoid biosynthesis
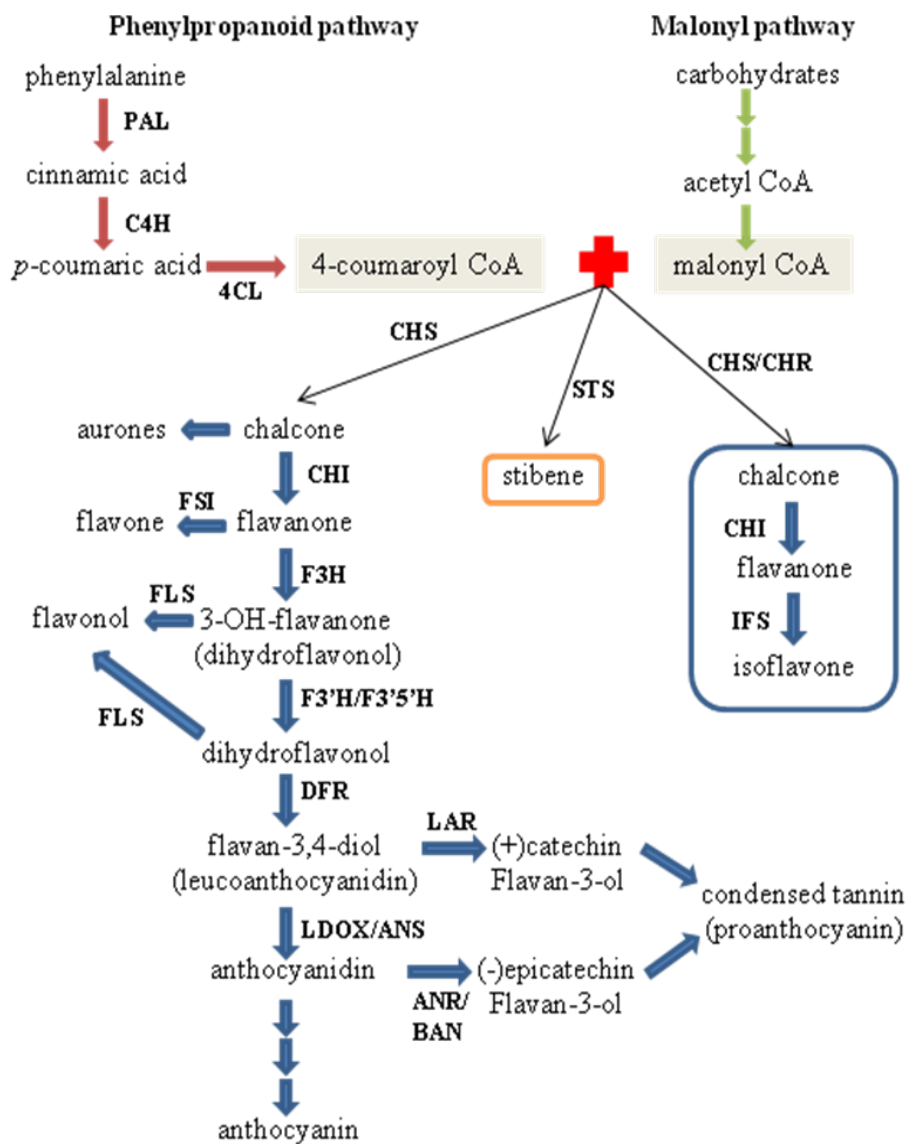
Derived from phenylpropanoid metabolism combined with malonate pathway, flavonoids can be divided into nine major subgroups: the chalcones, stilbenes, aurones, isoflavonoids, flavones, flavonols, flavandiols, anthocyanins, condensed tannins. The committed enzyme names are abbreviated as follows: Phe ammonia-lyase (PAL) cinnamate-4-hydroxylase (C4H), 4-coumaroyl:CoA-ligase (4CL), chalcone synthase (CHS), stilbene synthase (STS), chalcone reductase (CHR), chalcone isomerase (CHI), isoflavone synthase (IFS), flavone synthase (FSI and FSII), flavanone 3-hydroxylase (F3H), flavonoid 3' hydroxylase (F3'H) or flavonoid 3'5' hydroxylase (F3'5'H), (FLS), dihydroflavonol 4-reductase (DFR), leucoanthocyanidin dioxygenase (LDOX) or anthocyanin synthase (ANS), anthocyanidin reductase (ANR) or banyules (BAN), and leucoanthocyanidin reductase (LAR).

Figure 1.2 General DNA microarray experiment
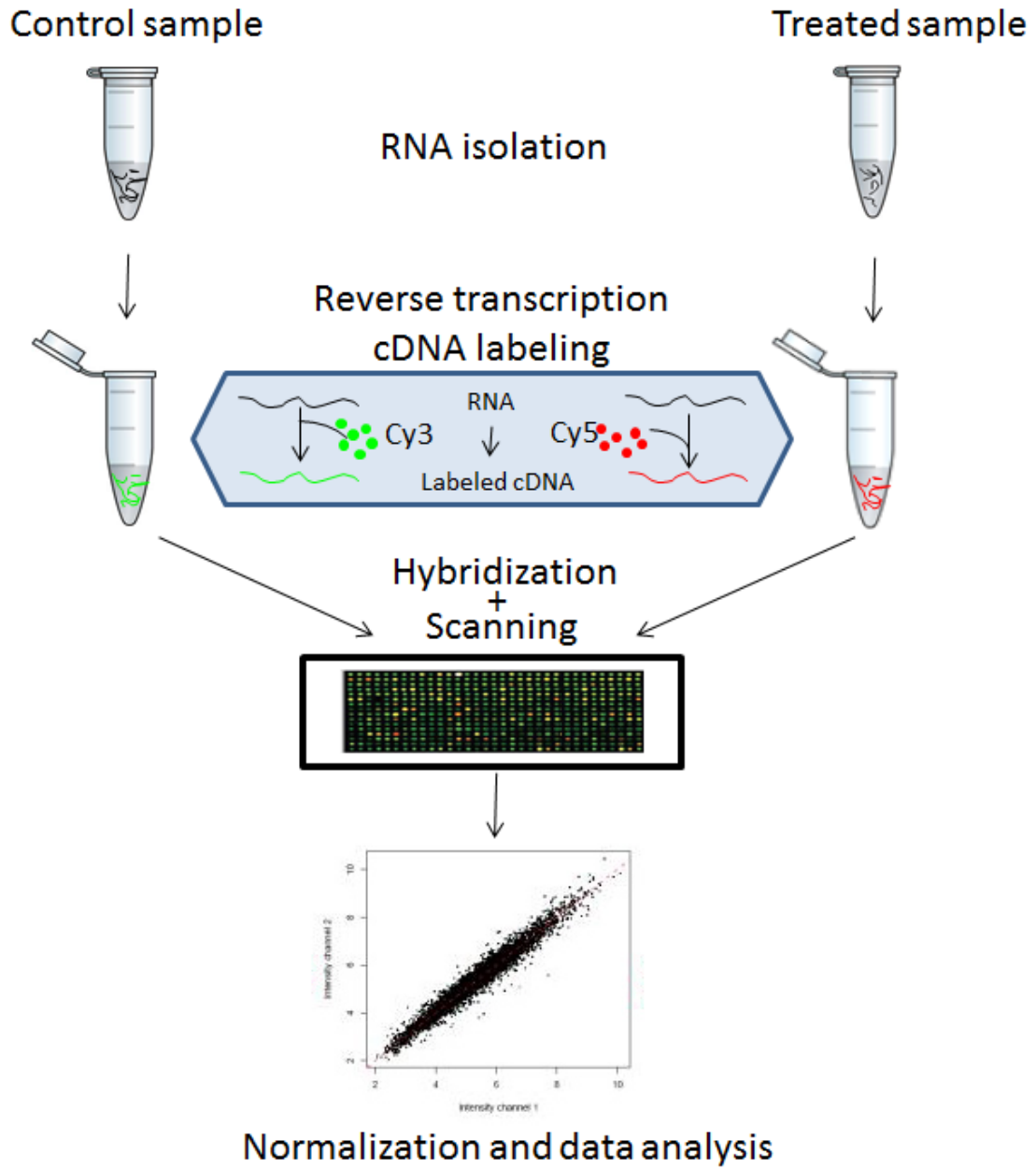
Figure 1.3 Color visualized on a microarray slide
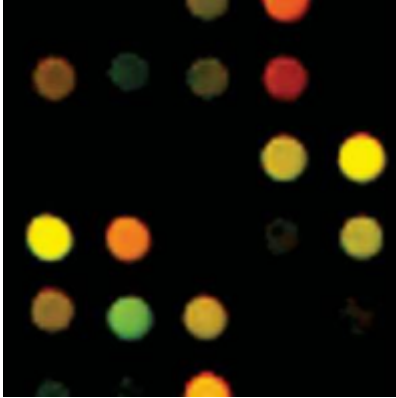
Each spot on a microarray is associated with a different gene. Hybridization of two colored dyes (e.g., red and green generates a different color on each spot. The colored spot and its intensity represent relative gene expression in each sample. The color could be interpreted as following: red with a gene highly expressed in Cy5-labeled (e.g. treated sample) sample but low expressed in Cy3-labeled sample (e.g. control sample); green with a gene highly expressed in opposite to red; yellow with no difference of gene expression in both samples; black when no hybridization happens in this spot.

CHAPTER 2

ANTHOCYANIDIN REDUCTASE (*ANR*) GENES ARE INVOLVED IN REGULATION OF SKIN COLOR DEVELOPMENT OF APPLE FRUIT

2.1 Abstract

Three genes encoding anthocyanidin reductase (ANR) in apple (*Malus × domestica* Borkh.), designated *MdANR1*, *MdANR2a*, and *MdANR2b,* have been cloned and characterized. Each of these genes is composed of six exons and five introns, containing an open reading frame of 1,332 bp encoding a putative protein of 399 amino acids. Based on their coding sequences, *MdANR1* shares 91% of nucleotide sequence identity with *MdANR2a* and *MdANR2b,* while *MdANR2a* and *MdANR2b* share 99% nucleotide sequence identities. *MdANR2a* and *MdANR2b* are allelic. *MdANR1* and *MdANR2* genes are mapped onto linkage groups 10 and 5, respectively, of the apple genome. Expression of these genes in leaves, flowers, and fruits of apple are generally higher in yellow-skinned cv. Golden Delicious than in red-skinned cv. Red Delicious. Moreover, transcript levels of all three genes gradually drop from flowering through fruit development. Ectopic expression of each of these three *MdANR* apple genes in transgenic tobacco (*Nicotiana tabacum* cv. Petite Havana SR1) lines has resulted in the recovery of plants with either white or pale pink flower colors. Moreover, a new floral pigmentation pattern is observed in *MdANR1* transgenic lines suggesting incomplete inhibition of anthocyanin production. Loss of flower color pigmentation in transgenic tobacco is likely attributed to down-regulation of tobacco *CHALCONE ISOMERASE* (*CHI*) and *DIHYDROFLAVONOL REDUCTASE* (*DFR*) genes, both important for anthocyanin production, as their transcripts were lower than those of wild-type tobacco. In addition, accumulation of cyanidin is significantly lower in all transgenic tobacco lines, while that of catechin and epicatechin are significantly higher than those in wild-type tobacco. Interestingly, levels of leucoanthocyanidin reductase (LAR) are significantly reduced, while levels of accumulated catechin, produced by the LAR enzyme, are higher in transgenic tobacco lines compared to wild-type plants. This

suggests that ANR may be capable of generating catechin via an alternative route, although this mechanism is yet to be elucidated.

Key words: apple; anthocyanidin reductase; flavonoid; anthocyanin; proanthocyanin; gene expression

2.2 Introduction

Apple (*Malus × domestica* Borkh.) is one of the most wildly cultivated fruit crops grown around the world. A major goal in apple breeding efforts is to improve fruit quality characteristics such as fruit, size, flavor, and texture. In addition, fruit skin color is an important determinant of fruit quality. The coloration of apple skin is mainly derived from the accumulation of anthocyanins, a class of secondary metabolites in the flavonoid biosynthetic pathway.

Within the diverse group of flavonoid compounds, anthocyanins, condensed tannins (CTs), and flavonols are ubiquitously found in higher plants (Harborne, 1994; Winkel-Shirley, 2001). The most predominant class of flavonoids is anthocyanins due to their role in contributing to the wide range of coloration. Flavonoids play various diverse roles in plants such as UV protection, pigmentation, and disease resistance. For fruit skin color, pigmentation attracts animals to aid in seed dispersal (Lev-Yadun et al., 2009; Lomáscolo and Schaefer, 2010) and serves to determine fruit maturation and quality. CTs, also known as proanthocyanidins (PAs), are polymers of flavonoid molecules that commonly interact with proteins, particularly saliva proteins such as α-amylase (Harborne, 1994; Gonçalves et al., 2011) and cause sensation of astringency in fresh fruit and fruit juice (Vidal et al., 2004; Obreque-Slier et al., 2010; Renard et al., 2011). Recently, there is increasing evidence of human health benefits of flavonoids, including anti-inflammatory and antioxidant properties, as well as lowering the risks of cardiovascular diseases and certain cancers (Xiao et al., 2011).

Flavonoids are synthesized via a complex network based primarily on the shikimate, phenylpropanoid, and malonate pathways (Winkel-Shirley, 2001a; Tanaka et al., 2008). Flavonoids, including anthocyanins and CTs, are synthesized from malonyl-

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CoA and from *p*-coumaroyl-CoA derived from the phenylpropanoid pathway. A number of key enzymatic steps in sequential reactions along with several other enzymes involved in flavonoid biosynthesis are illustrated in Figure 2.1. Biosynthesis of CTs diverges from the anthocyanin biosynthesis pathway, and begins with the synthesis of an initiating flavan-3-ol such as catechin or epicatechin. Catechins are synthesized from the leucoanthocyanidin reductase (LAR) (Tanner et al, 2003); whereas, epicatechins are synthesized from anthocyanidin reductase (ANR), initially isolated from *Arabidopsis* and encoded by the *BANYULS* gene (Xie et al., 2003).

In apple fruits, the molecular mechanism of coloration has been studied extensively, but has not been well elucidated. Most studies on the mechanism of coloration have generally focused on genes involved in the anthocyanin biosynthesis pathway. In fact, the CTs biosynthetic pathway is in competition with anthocyanin synthesis, especially via the *ANR* gene as it utilizes the same substrate, anthocyanidin. Therefore, this could influence anthocyanin accumulation in plant tissues. Structural genes in the CTs biosynthetic pathway, including an *MdANR* gene, have been cloned and their transcriptional activity in fruit skin from red and non-red skin colored apple cultivars have been studied (Takos et al., 2006). However, the functionality of *ANR* genes in apple has not been investigated. Understanding the function of *MdANR* genes will unravel how coloration is regulated in apple fruit, and this may also aid in our understanding of fruit coloration in other fruit crops.

Recently, large numbers of expressed sequence tag (EST) sequences from different apple genotypes, tissues, and conditions have been generated and deposited in EMBL/GenBank/DDBJ databases (Gasic et al., 2009). Together with previously constructed bacterial artificial chromosome (BAC) libraries (Xu et al., 2001; Xu et al., 2002), these EST sequences facilitate investigation of genes involved in flavonoid biosynthesis in apple. In this study, a gene family encoding ANR has been isolated in apple, positioned on the apple genetic map, and its functionality has been investigated via ectopic expression of gene members in tobacco. These findings may lead to future modification/manipulation of anthocyanin biosynthesis in apple as well as in other plants.

2.3 Materials and methods

2.3.1 Plant material

Apple (*M. × domestica*) cvs. Red Delicious and Golden Delicious, grown at the orchard at the University of Illinois Pomology Research Center, were used to represent groups of red-skinned and non-red skinned colored fruits, respectively. Leaves, flowers, and whole fruits at different stages of development were collected, and immediately frozen in liquid nitrogen, ground, and stored at -80°C until needed.

Seeds of tobacco (*N. tabacum*) cv. Petite Havana SR1 were either germinated in flats containing soil mix and grown in the greenhouse, or they were germinated in vitro for pursuing functional analysis studies as described below.

2.3.2 Identification of BAC clones containing apple *ANR* genes

The deduced amino acid sequence of an apple EST contig, accession no. Apple_0223.545.C3.Contig1220, in our apple EST database (http://titan.biotec.uiuc.edu/cgi-bin/ESTWebsite/ estima_start?seqSet=apple) was blasted against amino acid sequences in the GenBank database (http://www.ncbi.nlm.nih.gov), and found to be highly homologous to *ANR* genes from other species such as grape, strawberry, and cacao. The overall identities in amino acid sequences were > 70%. Based on this EST contig, a pair of primers was designed (forward, 5'-CACGACCAAACCTGTTCCTT-3'; reverse, 5'-GTTGCAACCCCTGTCAACTT-3') to screen an apple BAC library according to a previously described PCR-based screening protocol (Xu et al. 2001). The BAC library was previously developed from apple cv. GoldRush using *Bam*HI, and corresponded to 5× haploid genome equivalents.

2.3.3 Southern blotting

Genomic DNA (30 μg) of apple and 14 positive BAC clones were digested with *Bam*HI, electrophoresed on a 1.0% agarose gel, and then transferred to a nylon membrane (Amersham). Following DNA crosslinking using a transilluminator, the blot was pre-hybridized with the DIG Easy Hyb (Roche Diagnostics 2000) for 1 h. A specific probe

(forward, 5'-TTCTCTTATGGCCGGTCCTT-3'; reverse, 5'-CGTCCTCCACATGTGCAATG-3') was constructed from the *MdANR* cDNA template using the PCR-DIG Probe Synthesis Kit (Roche). Following overnight hybridization with the probe at 42°C, the membrane was washed using a series of SSC buffers (2 to 0.5X). The gene-specific signal was detected using a DIG Detection Kit while following the manufacturer's instructions (Roche).

2.3.4 Isolation of genomic sequences of *MdANR*

The full-length 1.332 kb cDNA of *MdANR* (GenBank accession number DQ099803) was used to design a pair of specific primers (forward, GCCTCCAAGACACTAGCTGAG; reverse, GTACCGACCAGATGCAGATTC). Based on both Southern blotting of gDNA and positive BAC clones, BAC clones representing each gene copy were selected for sequencing. The genomic DNA of each gene copy was covered by primer walking sequencing (Supplemental Table S2.1).

2.3.5 Polymerase chain reaction (PCR)

PCR was performed using *Taq* DNA polymerase (Promega, Madison, WI) and dNTPs (New England Biolab). In general, a typical PCR reaction consisted of an initial denaturation at 94°C for 3 min, 33 cycles of denaturation for 35 s, annealing at 55-58°C for 30 s, extension at 72°C for 45-90 s, followed by a final 8 min extension at 72°C. However, the PCR conditions used for annealing temperatures depended on primer sequences used; moreover, extension times also varied depending on sizes of the PCR products.

2.3.6 Mapping *MdANR* genes on the apple genetic map

Insertions and deletiosn (Indels) were used to develop molecular markers for *MdANR* genes. Based on genomic sequence data, an Indel region was used to develop a genetic marker for *MdANR1*, designated as an MdANR1-Indel. Another Indel marker for *MdANR2* was developed by aligning genomic sequences of *MdANR2a* and *MdANR2b*, and the marker was designated as an MdANR2/3-Indel. Primer sets were

designed based on these two Indel regions and then used to screen genomic DNA of the parents 'Co-op 16' and 'Co-op 17' of our apple mapping population (consisting of 142 F1 seedlings). These primer sets were as follows: MdANR1-Indel (forward 5'-CTGCCGCATGATGATCTTCAC-3' and reverse 5'-GAATCTCTTCGTGGACCACTCC-3'), and MdANR2/3-Indel (forward 5'-ATCTGCTTCTGGTCGGTACA-3' and reverse 5'-TTGGCCTTGAAGTACTCCAC-3'). PCR analysis was conducted using genomic DNA of the two parents and all 142 individuals of the F1 population of 'Co-op 16' x 'Co-op 17'. PCR products were resolved on 2% (w/v) agarose gels.

Segregation patterns of the mapping population were recorded, and mapped onto linkage groups using an EST-SSR-based integrated genetic and physical map for apple recently developed for 'Co-op 17' and 'Co-op 16' (unpublished). The linkage map was established using the JOINMAP software version 4.0.

2.3.7 Quantitative real-time PCR (qRT-PCR) analysis

Total RNA from leaves and flowers tissues were extracted using an RNAqueous Kit (Ambion) according to the manufacturer's instructions. RNA from apple fruit tissues was isolated according to the protocol of Gasic et al. (2004).

Total RNA (3 μg) from each sample was treated with DNase I (Invitrogen), and used for cDNA synthesis. The first-strand cDNA synthesis was performed with Oligo (dT) primer using the SuperScript III RT kit (Invitrogen) according to the manufacturer's instructions. The specific primers were designed to perform real-time PCR (Table 2.1). The SYBR Green real-time PCR assay was carried out in a total volume of 15 μl, containing 12.5 μl of 2× SYBR Green I Master Mix (Applied Biosystems), 0.2 μM (each) of specific primers, and 100 ng of template cDNA. The amplification program consisted of 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. The fluorescent product was detected at the last step of each cycle. Following amplification, melting temperatures of PCR products were analyzed to determine the specificity of the PCR product. Melting curves were obtained by slow heating at 0.5°C /sec, from 60°C to 90°C while continuously monitoring the fluorescence

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signal. A negative control without a cDNA template was run with each analysis to normalize the data and evaluate the overall specificity.

The qRT-PCR reactions were carried out in 96-well plates in a 7300 Real Time PCR System (Applied Biosystems). All experimental samples were run in triplicates. An apple *ACTIN* gene was used as an endogenous control gene. Differences between the cycle threshold (*Ct*) of the target gene and the *ACTIN* gene were used to determine the relative transcript level of the target gene, calculated as 2 exp-(Ct_{target} - Ct_{actin}).

2.3.8 Cloning of *MdANR* genes

The coding sequences of *MdANR1*, *MdANR2a*, and *MdANR2b* were amplified by PCR using the proofreading DNA polymerase Platinum® *Pfx* (Invitrogen) and following the manufacturer's reaction conditions. The primers used were as follows: forward 5'-GCCTCCAAGACACTAGCTGAG-3' and reverse 5'-GTACCGACCAGATGCAGATTC-3'. The blunt PCR products were ligated separately into the pCR®-Blant vector following the Zero Blunt® PCR cloning kit (Invitrogen) protocol. Several clones were sequenced at the W.M. Keck Center Core Sequencing Facility, University of Illinois Urbana-Champaign. These sequences were deposited in NCBI under the accession numbers of JN035299, JN035300, and JN035301.

2.3.9 Tobacco cultures

Seeds of tobacco (*N. tabacum* cv. Petite Havana SR1) were germinated in vitro in magenta flasks. Seeds were germinated on half-strength Murashige and Skoog (MS) (1962) medium containing 1% sucrose, pH 5.7, and solidified with 0.2% Gelrite. Seedlings were grown in a growth chamber at 26°C under a 16 h photoperiod. Ten-day old seedlings with the first pair of expanded true leaves were transferred into glass jars containing MS with vitamin, 3% sucrose, pH 5.7, and solidified with 0.2% Gelrite.

2.3.10 Tobacco transformation and analysis of transgenic lines

Each of *MdANR1* and *MdANR2a*, and *MdANR2b* were cloned separately into an expression vector, a pBI121 binary vector. Three primers, 5'-

TGACTCTAGAATGGCCACCCAACAACCCATCT-3' (forward), 5'-ATACGAGCTCCTAGTTCTGCAGCAGCCCCTTT-3' (reverse for *MdANR1*), and 5'-ATACGAGCTCTTTTATTTTCTTTTTTCTCATC-3' (reverse for *MdANR2a/b*) were designed to amplify the entire coding sequences. The forward and reverse primers contained *Xho*I/*Sac*I sites at the 5' end, respectively. PCR products digested with *Xho*I and *Sac*I were ligated into *Xho*I/*Sac*I-digested cloning vector. The binary vector containing each of the genes was sequenced to confirm sequence and orientation of the transgenes. As a result, three separate constructs carrying each of the coding sequences of *MdANR1*, *MdANR2a*, and *MdANR2b* were generated.

Each of the three constructs were immobilized into bacterial cells of *Agrobacterium tumefaciens* strain GV3101, and co-cultivated with tobacco leaf sections following the transformation and regeneration protocol as described previously by Horsch et al. (1985), but with some modification. Bacterial cells containing the binary vector from glycerol stock was inoculated into 5 ml Luria Broth (LB) medium containing appropriate concentrations of antibiotics, 100 mg/l, at 28ºC for 2 d with vigorous shaking. Cells were collected by centrifugation at 2500g for 10 min and resuspended in a liquid co-cultivation medium at an O.D.600 of 0.5 – 0.8.

Leaf sections, ~0.5 cm^2, from in vitro-grown seedlings were cut, and incubated with bacterial suspension for 30 min at 28ºC with shaking at 100 rpm. Leaf explants were transferred to a solidified co-cultivation medium consisting of MS medium with vitamins, 3% (w/v) sucrose, 1 mg/L 6-benzyladnine (BA), 0.1 mg/L α-naphthaleneacetic acid (NAA), pH 5.6, with 0.8% tissue culture (TC) agar, and containing 100 mg/L kanamycin, and co-cultivated in the dark for 4 d at 25 ºC. Co-cultivated explants were washed twice with a liquid co-cultivation medium containing 1000 mg/L cefotaxime, blotted dry onto a regeneration selection medium (co-cultivation medium with 0.8% Agar, 100 mg/L kanamycin, and 250 mg/L cefotaxime), and incubated for 2 wks in the dark at 25ºC. All cultures were transferred to a controlled environment growth chamber under 16h photoperiod, and sub-cultured biweekly onto fresh medium. Developing shoots were excised, and transferred to a rooting medium (regeneration selection

medium, but without any plant growth regulators). Kanamycin-resistant plantlets were transferred to soil mix, acclimatized, and grown in the greenhouse.

Transformed plants were subjected to PCR analysis as described above. Moreover, putative transgenic plants were subjected to Southern blot analysis. The copy number of the transgene in different lines was detected using an *nptII* gene probe (forward, 5'-TATTCGGCTATGACTGGGCA-3'; reverse, 5'-GCCAACGCTATGTCCTGAT-3') synthesized from the expression vector. The probe was then used for hybridization onto a membrane as described above.

2.3.11 Flavonoid analysis

Anthocyanins and flavonols were extracted from 50 mg of finely-ground flower tissues collected from transgenic plants and wild-type tobacco plants in 1 ml 1% HCL/methanol (v/v) at room temperature in the dark with continuous shaking for 1 h, and centrifuged at 13,000 rpm for 15 min. The 100 µl aliquot of the supernatant was transferred to a fresh tube, and acid-hydrolyzed by adding 30 µl of 3N HCl, and incubated at 70°C for 1 h in a Thermo Hybaid MBS 0.25s thermocycler (Thermo Scientific). Proanthocyanins (PAs) were extracted using 1 ml 70% (v/v) acetone containing 0.1% (w/v) ascorbate, and incubated at room temperature for 24 h in darkness, as described by Takos et al. (2006). The extract was centrifuged at 13,000 rpm for 15 min at room temperature, and the clear supernatant was transferred to a new 1.5-ml microfuge tube. An aliquot of 200-µl extract was dried out at 35 ºC and resuspended in 100 µl of 1% (v/v) HCl-methanol and 100 µl of 200 mM sodium acetate (pH 7.5).

Flavonoid contents were determined using liquid chromatography in tandem mass spectrometry (LC-MS-MS), along with the use of commercial standards for kaempferol, quercetin, cyanidin, catechin, and epicatechin (Sigma). The LC-MS-MS analysis was performed on a 5500 QTRAP mass spectrometer (AB Sciex) which was equipped with a 1200 Agilent HPLC. An Analyst (version 1.5.1, Applied Biosystems) was used for data acquisition and processing. A Phenomenex column (3u C6-Phenly 11A, 4.6×50mm) was used for separation. The HPLC flow rate was set at 0.3 mL/min. HPLC mobile phases consisted of A (0.1% formic acid in H_2O) and B (0.1% formic acid in acetonitrile). The

autosampler was kept at 5ºC. The gradient for catechin and epicatechin was as follows: 0 min, 90% A; 10 min, 50% A; 13-18 min, 0% A; and 18.1-25 min, 90% A. The injection volume was 20 μl. The gradients for cyanidin, kaempferol, and quercetin were as follows: 0 min, 70% A; 7-12.5 min, 0% A; and 13-20 min, 70% A. The injection volume was 10 μl. The mass spectrometer was operated with positive electrospray ionization.

Multiple reaction monitoring (MRM) was used to quantify catechin and epicatechin (m/z 291.0-->139.2), cyanidin (m/z 287.2-->213.2), kaempferol (m/z 287.1-->153.2), and quercetin (m/z 303.1-->153.1). The electrospray voltage was set to 5500 V, the heater was set at 600 ºC, the curtain gas was 35 psi, and both GS1 and GS2 were at 60 psi. Analysiss of each sample was repeated three times using three biological replicates. All data were subjected to analysis of variance, and means were compared using a t-test.

2.4 Results

2.4.1 Three members of a gene family encoding anthocyanidin reductase in apple

Based on the full-length cDNA of *MdANR*, specific primers were designed and used to screen the apple BAC DNA library to recover *MdANR* genomic sequences. Using a PCR-based approach, 14 individual positive BAC clones were identified. To identify all gene copies present in the apple genome, all positive clones and genomic DNA from 'GoldRush' leaves were subjected to Southern blot analysis. The presence of three positive bands in the genomic DNA suggested that there were three copies of the *MdANR* gene present in the apple genome. For the remaining 14 BAC clones, these could be merged into three groups corresponding to each of the bands present in the gDNA.   Each of BAC clones B5 and 6, B1 and 7, and B8 contained a single band corresponding to the first, second, and third copy of the gDNA, respectively (Figure 2.2). The three genes were designated *MdANR1*, *MdANR2a*, and *MdANR2b*. To recover the genomic sequence of *MdANR*, three positive BAC clones, including B6, 7, and 8, corresponding to the three copies of *MdANR* genes, were selected for sequencing.

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2.4.2 Genomic sequences of the three genes encoding ANR in apple

The genomic organization of the three genes is composed of six exons and five introns (Figure 2.3). The genomic sequence contains an open reading frame of 1,332 bp encoding a putative protein of 339 amino acids. The genomic sequence of *MdANR1* is distinct, while sequences of *MdANR2a* and *MdANR2b* are similar. Approximately, 76% and 67% nucleotide sequence identities in genomic sequence of *MdANR1* are shared with *MdANR2a* and *MdANR2b,* respectively. The coding region sequence of *MdANR1* shares 91% nucleotide sequence identity with those of *MdANR2a* and *MdANR2b*. Moreover, *MdANR2a* and *MdANR2b* share an overall identity of 91% in genomic DNA sequence, and 99% in the coding region sequence. As expected from the genomic sequence of *MdANR1*, *MdANR2a*, and *MdANR2b*, the MdANR2a deduced amino acid sequence has a slightly higher similarity to that of MdANR2b, 99% identity, than that of MdANR1, 97% identity (Figure 2.4).

The conserved region of the ANR protein was revealed when aligning the consensus sequences of MdANRs to ANRs of other plant species, including *Arabidopsis thaliana* (AtANR), *Medicago truncatula* (MtANR), and *Vitis vinifera* (VvANR). The highest similarity of the deduced amino acid sequence of MdANR is VvANR with, 84% identity; while, it shares 64% homology with AtANR and 76% with MtANR (Figure 2.5).

2.4.3 Alleles of *MdANR2*

MdANR1, *MdANR2a,* and *MdANR2b* were isolated from BAC clones B6, B7, and B8, respectively. Based on a whole-genome BAC-based physical map of the apple (Han et al. 2007), B7 and B8 were located in the same contig (ctg742) (Supplemental Fig. S2.1). *MdANR2a* and *MdANR2b* were likely to be either allelic or belonged to a cluster. To verify this finding, genomic DNA fragments of *MdANR2a* and *MdANR2b* in the 3' untranslated region were sequenced. This revealed that the two genes had 91% sequence similarity, thus suggesting that genomic fragments of *MdANR2a* and *MdANR2b* were possibly overlapping at the same locus. Furthermore, DNA blotting analysis of all BAC clones containing the *MdANR* genes from the apple BAC library developed from cv. GoldRush using *Hind*III and representing ~ 5× haploid apple genome equivalents

indicated that each of these clones contained only a single copy of the *ANR* gene, hence suggesting that the *ANR* genes were not clustered in the apple genome. Therefore, these results demonstrated that *MdANR2a* and *MdANR2b* were in fact allelic.

2.4.4 The position of the *MdANR2* gene on the apple linkage map

The *MdANR* gene was mapped onto an SSR-based linkage map developed using 'Co-op 16' × 'Co-op 17'. An insertion deletion (Indel) marker was used to map *MdANR* genes. The marker was assigned as MdANR1-Indel and MdANR2/3-Indel and then used to screen the F1 mapping population of Co-op 16' × 'Co-op 17'consisting of 142 individuals (Supplemental Fig. S2-2 and S2-3). The marker was mapped onto linkage group LG 10 for the *MdANR1* gene and LG 5 for the *MdANR2b* gene of the integrated genetic map using JoinMap software (Figure 2.6).

2.4.5 Expression profiles of the *MdANR* gene family in apple

To study expression of the three members of the *MdANR* gene family, quantitative real-time PCR (qRT-PCR) was conducted using leaves, flowers, and fruits of the red skinned fruit cultivar Red Delicious and the yellow skinned fruit cultivar Golden Delicious. *MdANR1 and MdANR2a/b* were expressed in all analyzed tissues, including leaf, flower, and fruit, and transcript levels of *MdANR1* and *MdANR2a/b* were compared in the two different apple cultivars (Figure 2.7). Transcripts of *MdANR1* and *MdANR2a/b* were most abundant at fruitlet stage Ft1 (9 DAP), but these subsequently declined, at similar levels, during fruit development, although *MdANR1* transcripts were slightly higher than those of *MdANR2a/b*. Moreover, levels of *MdANR* gene expression were different between the two cultivars. Generally, mRNA transcripts of *MdANR* in 'Golden Delicious' were higher than those in 'Red Delicious', except for late stage of flower development. Levels of transcripts decreased during flower development in 'Golden Delicious', but remained constant in 'Red Delicious'. However, transcript levels during all stages of fruit development steadily declined in both cultivars.

2.4.6 Functional analysis of *MdANR* genes in tobacco

The functionality of *MdANR* genes was investigated following ectopic expression of these genes in tobacco. Each of the three members of the *MdANR* gene family was introduced separately into a binary vector, under the control of the strong cauliflower mosaic virus (CaMV) 35S promoter, and each of these constructs was separately introduced into tobacco via *Agrobacterium*-mediated transformation. Presence of the selectable *npt*II marker gene was confmed by PCR analysis in transformed tobacco lines. A total of 10 PCR(+) lines were obtained. These plants were confirmed as transgenic following Southern blot analysis. Upon flowering, it was observed that different transgenic lines produced flowers with different pigmentation, ranging from white to pink. In addition, some T_0 transgenic tobacco plants carrying *MdANR1* produced flowers with different patterns of variegation (Figure 2.8a). Based on Southern blotting, the copy number of the transgene in each of these lines was either one or four (Fig. S2.4). Moreover, levels of *MdANR* expression were measured using qRT-PCR. Transgene copy number did not correlate with observed phenotypic differences in flower color pigmentation. For example, although the same copy number of the transgene was observed in transgenic lines ANR1-1, 1-3, and 1-13, their flower color patterns were different. Whereas, transgenic line ANR1-4 carried a high copy number of the transgene (four copies), yet flowers were pink in color and similar to wild-type flower color. Based on qRT-PCR analysis, *MdANR* levels of expression in transgenic lines ANR1-1, 1-3, and 1-13 were similar, while a high level of *MdANR* expression was detected in transgenic line ANR1-4 (Figure 2.8c). These results suggested that changes in flower color pigmentation were not influenced by the copy number of the introduced *MdANR* transgene.

When plants of different T_2 transgenic lines expressing either *MdANR1, MdANR2a,* or *MdANR2b* were evaluated, distinct flower color pigmentation was also observed in these different lines, ranging from white to pink, and with different pigmentation patterns (Figure 2.9).

2.4.7 Influence of over-expression of *MdANR* genes on expression of other flavonoid-related genes in transgenic tobacco flowers

To investigate the effects of over-expressed *MdANR* genes on other flavonoid-related genes that may contribute to observed variations in pigmentation, qRT-PCR analysis was conducted on several flavonoid-related genes. These genes include *CHS*, *CHI*, *F3H*, *F3'H*, *FLS*, *DFR*, *UFGT*, *LAR*, *ANR1*, *ANR2*, *AN2*, *TT1*, and *TT2*. mRNA transcripts isolated from flowers of transgenic tobacco lines were quantified relative to those from wild-type tobacco (Figure 2.10). It was observed that expression of all these genes involved in the flavonoid biosynthesis pathway varied among the different transgenic lines expressing one of the three *MdANR* genes. Moreover, over-expression of each of the *MdANR* genes differently altered expression of other flavonoid-related genes. For example, expression of the *NtANR* gene was up-regulated in transgenic plants carrying either *MdANR1* or *MdANR2a,* but it was down-regulated in transgenic plants carrying *MdANR2b*. Interestingly, among all genes investigated, expression of both *NtDFR* and *NtLAR* genes was down-regulated, whereas that of *NtAN2* was up-regulated in all transgenic lines analyzed. Expression of *MdANR* genes is proposed to greatly influence expression of *NtDFR* and *NtLAR* genes in tobacco flowers.

2.4.8 Accumulation of flavonol, anthocyanin, and proanthocyanin in transgenic tobacco flowers

Using LC-MS-MS analysis, flavonoid, anthocyanin, and proanthocyanin contents in flowers were determined (Figure 2.11). All transgenic lines over-expresseing *MdANR* genes showed lower anthocyanin contents than wild-type tobacco. Moreover, cyanidin contents in most of transgenic plants was too low to be detected; whereas, proanthocyanin contents were higher in transgenic plants compared to wild-type control. Unexpectedly, levels of catechin were also higher in some transgenic lines wherein *LAR* gene expression was relatively lower than that of wild-type plants.

2.5 Discussion

ANR and LAR catalyze the synthesis of flavan-3-ol, an initiating monomer of condensed tannin or proanthocyanin synthesis, from cyanidin and 3,4-*cis*-leucocyanidin, respectively. ANR differs from LAR in that ANR activity requires a double reductase in order to produce epicatechin. ANR is not only important for proanthocyanin synthesis, but it also influences the synthesis of anthocyanin by a competing reaction with UFGT activity by which it converts anthocyanidin to anthocyanin (Bog et al., 2005). Thus, ANR plays an important role in both anthocyanin and proanthocyanin synthesis in plants. Genes encoding ANR have been first isolated and characterized in Arabidopsis, and designated as 'BANYULS' (*BAN*) (Xie et al., 2003). In fruit trees, the *VvANR* gene was isolated and functionally characterized in grapevines (Bogs et al., 2005). However, it is not clear how *ANR* genes interact with other flavonoid structural genes to coordinate the biosynthesis of anthocyanin. Herein, we have isolated and conducted functional analyses of *ANR* genes from apple. This new knowledge will provide insights into the regulation of fruit coloration and proanthocyanin synthesis.

2.5.1 Incomplete inhibition of anthocyanin production resulted in novel floral pigmentation patterns

It has been well known that anthocyanin biosynthesis can be inhibited by over-production of enzymes that compete for the substrate. Although introducing an enzyme to compete with anthocyanin biosynthetic enzymes could successfully lower pigment levels, it is not sufficient to deplete flowers of all pigmentation leading to white-colored flowers (Davies et al., 1998; Joung et al., 2003). For example, the introduction of a *Medicago* chalcone reductase (CHR) into petunia altered flower color from deep purple to pale purple, but did not yield any white-colored flowers (Davies et al., 1998).

It has been reported that new floral pigmentation patterns may unexpectedly occur following modification of the anthocyanin biosynthesis pathway (Napoli et al., 1990). Inhibition of anthocyanin biosynthesis could yield new pigmentation patterns. Often, this would be detected in plant species that would naturally produce flowers with patterned coloration such as petunia, lisianthus, and torenia (Davies, 2009). It is reported that these

variations are predetermined by morphological signals within the petal that control levels of gene inhibition, and that those signals would interact with environmental signals that influence pigmentation (van der Krol et al., 1990). It is well known that tobacco plants do not produce flowers that are naturally patterned. However, variegated flower phenotypes have been observed in tobacco plants overexpressing an *Arabidopsis* transposable element, *Tag1-R* (Liu et al., 2001). Yet, it has been reported that no patterned tobacco flowers are generated following inhibition of anthocyanin biosynthesis.

The ANR enzyme is known to compete with the UFGT enzyme to convert anthocyanidin to epicatechin. The functionality of an *Arabidopsis ANR* (*BAN*) gene has been investigated in tobacco, and it is reported that over-expression of the *BAN* gene resulted in recovery of white-colored flowers (Xie et al., 2003). In this study, over-expression of an apple *ANR* gene in tobacco not only generated white flowers or pale-pink colored flowers, but also resulted in novel pigmentation of patterned flowers. This phenomenon is unlikely to be the result of the effects of either transgene copy number or levels of gene expression as all these patterned transgenic lines have been carrying a single copy of the transgene and have exhibited similar levels of gene expression. Thus, it is likely that incomplete inhibition of anthocyanin production may occur at the post-transcriptional level resulting from the positional effects of the transgene within the plant genome.

2.5.2 The apple *ANR* gene may also have a redundant function of converting anthocyanidin into catechin

It is well documented that LAR and ANR catalyze the conversion of leucocyanidin and cyanidin into catechin and epicatechin, respectively (Bogs et al., 2005). In this study, flowers of transgenic tobacco lines overexpressing an *MdANR* gene have accumulated higher levels of both catechin and epicatechin compared to those of wild-type tobacco plants. However, overexpression of an *MdANR* gene has contributed to significant suppression of the function of the *LAR* native gene, as expression levels of the *LAR* gene in most transgenic tobacco lines have been too low to be detected. Thus,

other genes in the anthocyanin biosynthesis, besides *LAR,* must be capable of promoting accumulation of catechin.

Incubation of BAN proteins of *M. truncatula* and *Arabidopsis* with anthocyanidins, cyanidin and pelargonidin, has resulted in the formation of epicatechin as the major product and catechin as a minor product (Xie et al., 2003). Similarly, findings in this study also suggest that ANR or BAN could synthesize both epicatechin and catechin. This in turn raises a question as to whether the synthesis of catechin results from epimerization of epicatechin or whether it is a product that is catalyzed by ANR? It has been reported that incubating either epicatechin or catechin with either MtANR or AtANR in the presence of NADPH cannot be epimerized into catechin or epicatechin, respectively (Xie et al., 2004). Leucoanthocyanidin dioxygenase (LDOX) is essential for PA synthesis in *Arabidopsis* (Abrahams et al., 2003), suggesting that ANR cannot efficiently use leucoanthocyanidin as a substrate to produce catechin. Thus, it is likely that BAN could directly convert anthocyanidin into catechin. In addition, it is worth noting that catechin might be formed through chemical epimerization of epicatechin (Xie et al., 2004). Further studies are needed to clarify whether the catechin detected in this study is attributed to the epimerization of epicatechin to catechin. It has been reported that high temperatures and alkalization are two main factors that induce the epimerization of epicatechin into catechin (Kofink et al., 2007). However in this study, PA extraction has been conducted under 35 °C and pH 7.5, which is not high. Moreover, the accumulated catechin, presumed to be formed through chemical epimerization, is observed as a minor product (Xie et al., 2003). In this study, the amounts of epicatechin and catechin accumulating in flowers of some transgenic tobacco lines overexpressing an *MdANR* gene are almost equal, thus, formation of catechin in these lines cannot be fully explained by chemical epimerization alone.

2.5.3 Overexpression of *MdANR* genes causes co-suppression of other structural genes involved in flavonoid biosynthesis

Overexpression of the *CHS* gene results in co-suppression of homologous genes in petunia, and this co-suppression is related to an RNA silencing mechanism

(Baulcombe, 2004).   In this study, overexpression of *MdANR* genes has resulted in co-suppression of *NtLAR*, *NtDFR*, and *NtCHI* genes in transgenic tobacco lines. Of these three genes, *NtLAR* and *NtDFR* are related to *MdANR*.   Collectively, *MdANR*, *NtLAR,* and *NtDFR* belong to the reductase-epimerase-dehydrogenase (RED) superfamily although they share less than 40% identity in their DNA coding sequences.   However, co-suppression of *NtLAR* and *NtDFR* may not be related to RNA silencing for the following reasons. First, *ANR* genes from apple and tobacco share ~ 50% identity in their DNA coding sequences, and overexpression of *MdANR* does not result in co-suppression of *NtANR* genes in tobacco. Second, *ANR* is more closely related to *DFR* than to *LAR*; however, overexpression of an *MdANR* gene suppresses expression of *NtLAR* more severely than that of *NtDFR*.   Therefore, the mechanism of co-suppression is rather complicated, and requires further investigation.

LAR converts leucoanthocyanidin into catechin and it competes with ANS/LDOX for the substrate to produce an alternative initiating unit for proanthocyanidin biosynthesis. It is known that ANR and LAR are NAPDH-dependent reductases. Thus overexpression of *MdANR* in this study will offer little opportunity for NtLAR to accept NADPH, resulting in low levels of expression of *NtLAR* in flowers of transgenic tobacco lines.   Interactions among DFR, CHS, and CHI have been previously identified in *Arabidopsis* (Burbulis and Winkel-Shirley, 1999).   Therefore, it is likely that the co-suppression of *NtLAR* and *NtDFR* may be due to interactions among enzymes involved in the flavonoid biosynthetic pathway.

2.5.4 Loss of anthocyanin in flowers of transgenic tobacco overexpressing *MdANR* is due to lower levels of expression of both *NtCHI* and *NtDFR*

In this study, expression of structural and regulatory genes involved in the flavonoid biosynthesis pathway has been investigated in flowers of transgenic tobacco lines overexpressing *MdANR* genes. Of the flavonoid structural genes, *NtCHI* and *NtDFR* have demonstrated significantly lower levels of expression in flowers of all transgenic tobacco lines compared with those of wild-type plants. It is well known that expression of *CHI* and *DFR* genes is controlled by regulatory genes such as *R2R3 MYB*

transcription factors. More recently, an R2R3 MYB regulator from tobacco (*NtAn2*) has been isolated and reported to be a key gene controlling anthocyanin production in reproductive tissues of tobacco (Pattanaik et al., 2010). Interestingly in this study, flowers of all transgenic tobacco lines have demonstrated significantly higher levels of expression of *NtAn2* than those of wild-type tobacco. Thus, it seems that overexpression of *MdANR* may affect the regulatory role of the *NtAn2* gene. It is not clear whether the ANR protein can interact with R2R3 MYB proteins, and thus leading to their enhanced transcriptional activities.

TABLES

Table 2.1 Primer sequences for qRT-PCR

Genus	Gene name	Gene identifier	Forward primer	Reverse primer
Malus	*MdANR1*		ttggagattttccgtctgagg	ttctcatcagaaagcaacacgc
	MdANR2a/b		ttgctgtgctgccaacacca	ttctcatcagaaagcaacaagaag
	*M*sp.*CHS*	X68977	tcaagcctattgggatttcg	cagctgacttcctcctcacc
	*M*sp.*CHI*	X68978	gatatcgaagccggaaatga	tgttgactcacgccaacaat
	MdF3H	AF117270	acaccaaatatggctcctgc	tttcgttgctgaagtcgttg
	MdFLS	AF119095	aatgggagtggagtctgtgg	agttggagctggcctcagta
	MdDFR	AF117268	aaggccgttacatttgttcg	gcccttgaactttgtgggta
	MdUFGT	AF117267	agctccactcggaacttcaa	aacccgccctaaatatgtcc
	MdANS	AF117269	caatttggcctcaaacacct	tcaacaccaagtgcaagctc
	MdANR	DQ099803	gttgcaacccctgtcaactt	cacgaccaaacctgttcctt
	MdLAR1	DQ139836.1	acaacacccacccttctgag	tgcagcaagggctagtaggt
	MdActin	DQ822466	ctacaaagtcatcgtccagacat	tgggatgacatggagaagatt
Nicotiana	*NbActin*	AY179605	aatgatcggaatggaagctg	tggtaccaccactgaggaca
	NtCHI	AB213651	gaaatcctccgatccagtga	caacgttgacaacatcaggc
	NbCHS	EF421432	agaaaagccttgtggaagca	acttggtccaaaattgcagg
	NtF3H	AB289450.1	acagggtgaagtggtccaag	ccttggttaaggcctccttc
	NtF3'H	AB289449.1	tccaagaatactggcccaag	ctcacaactctcggatgcaa
	NtFLS	AB289451	gaacttgaagggaaaaggggg	tccctgtaggagggaggatt
	NbDFR	EF421431	tcccatcatgcgatcatcta	atggcttctttgtcacgtcc
	NtLAR	AM827419.1	tcaaggtcctttacgccatc	acgaacctgcttctctttgg
	NtANS	AB289447	tggcgttgaagctcatactg	tttcaagggtgtccccaata
	NtUFGT	FG627024.1	gagtgcattggatgcctttt	ccagctccattaggtccttg
	NtANR1	AM791704.1	catttgactttcccaaacgc	attgggcttttgagttgtgc
	NtANR2	DW003895.1	tgttcccacttgggatgata	tgcacctatactctgttagtggc
	NtAN2	FJ472647	aggagagcaagcaaaaagca	tgaattccatcatccgtcaa
	NtTT1	FG164336	atgcaataccaaagcttgcc	caacatcaaccatccacgag
	NtTT2	EB425500	cctacccttggtacgcaaaa	tggttttgttggtcatgagg

Table 2.2 Determination of flavonoid contents in transgenic and wild-type tobacco flowers by LC-MS-MS

	Flavonoid (µg/g)		Anthocyanin (ng/g)	Proanthocyanidin (ng/g)	
	Hydroxylation of B-ring				
	4'	3',4'			
Flower	Kaempferol	Quercetin	Cyanidin	Catechin	Epicatechin
WT	60.57	71.80	1722	4.80	6.47
ANR1-1	53.73	87.60	n/a	9.07	9.93
ANR1-3	32.53	78.17	759	5.33	8.40
ANR1-13	43.57	86.57	1063	5.47	7.20
ANR2a-5	42.10	99.67	n/a	9.53	9.60
ANR2a-6	26.30	68.40	n/a	6.07	8.80
ANR2a-8	42.20	83.13	n/a	10.33	11.93
ANR2b-3	68.77	92.90	n/a	6.80	8.73
ANR2b-4	145.33	493.67	n/a	5.60	16.73

*n/a: not detected

Kaempferol, quercetin, cyanidin, catechin, and epicatechin were used as a standard. All data correspond to mean values of three biological replicates.

Supplemental Table S2.1 The list of primers used to isolate the genomic sequences of all *MdANR* genes by primer walking sequencing procedure

Primer	Sequences (5′ to 3′)
GWA1	ttctcttatggccggtcctt
GWA2	cgtcctccacatgtgcaatg
GWA3	ctgtggagtacttcaaggca
GWA4	ctgtagcagctgatgatgtc
GWA5	ggtcgctgttgttgcagaag
GWA6	cacaagtacggcgttttgaag
GWA7	cttcttgtggttgtctgcac
GWA8	ggtctctgacggtggttctg
GWA9	ttcgtggcgtctctgctggt
GWA10	cctctgaggcaaagttgacag
GWA11	gtctcgctggcttatgatcct
GWA12	ttctggagcagcagcttgacc

Figure 2.1 A general schematic representation of the flavonoid biosynthetic pathway
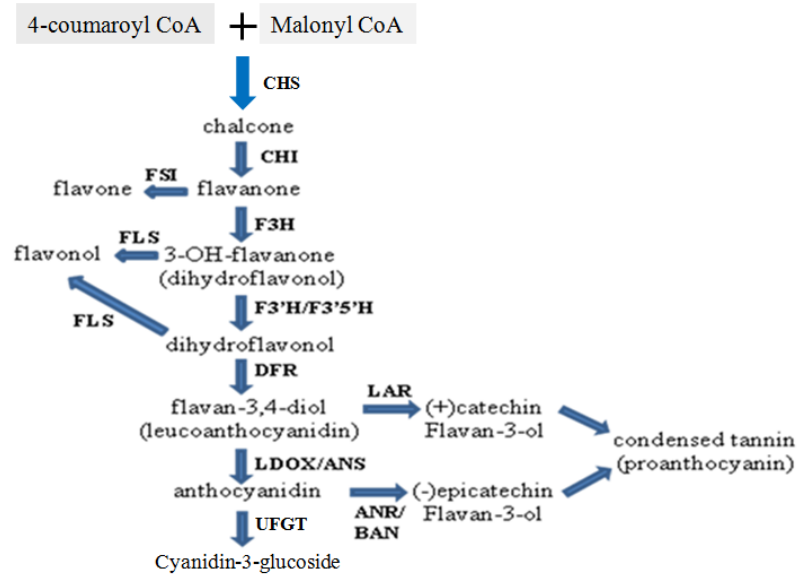
Figure 2.2 Southern blot of BAC clones containing *MdANR* genes
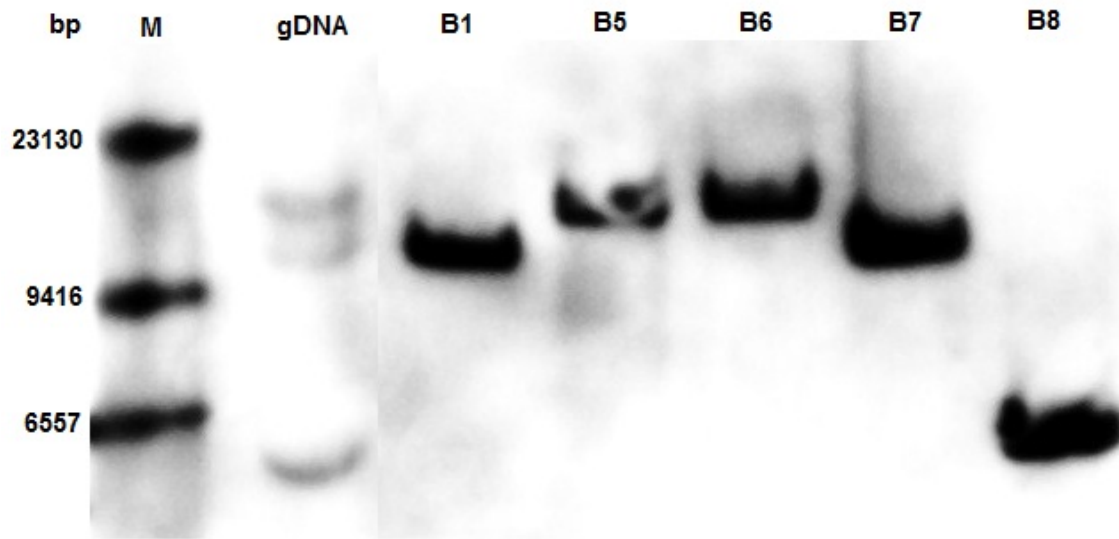
Genomic DNA from apple 'GoldRush' (gDNA) was used to determine the number of gene copies of *MdANR* genes present in the apple genome. The five selected BAC clones were B1, B5, B6, B7, and B8.

Figure 2.3 Genomic organization of three members of *MdANR* gene family
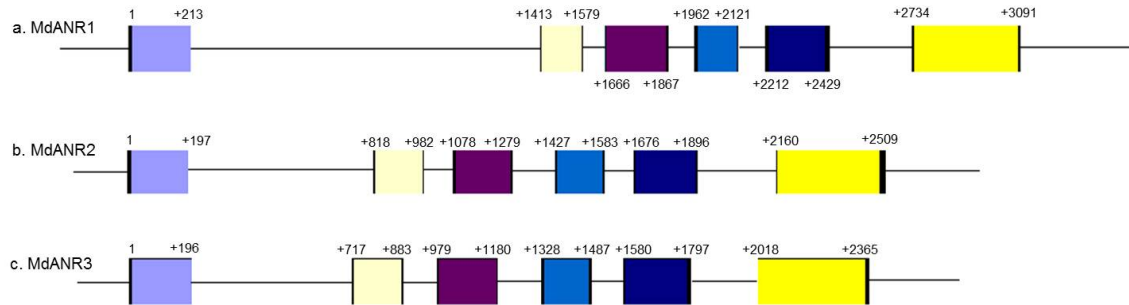
Exon is shown in a box and intron is shown as a line. The nucleotide positions of coding sequences are labeled.

Figure 2.4 Alignment of three apple ANR deduced amino acid sequences

```
MdANR1    MATQQPISKKTACVVGGTGFVASLLVKLLLQKGYAVRTTVRDPDNHKKVSHLTALQELGE 60
MdANR2a   MATQQPISKKTACVVGGTGFVASLLVKLLLQKGYAVRTTVRDPDNHKKVSHLTSLQELGE 60
MdANR2b   MATQQPISKKTACVVGGTGFVASLLVKLLLQKGYAVRTTVRDPDNHKKVSHLTALQELGE 60
          ************************************************** ** ******

MdANR1    LEILAGDLTDEGSFDAPIAGCDLVFHVATPVNFASQDPENDMIKPAIQGVLNVLKSCVKA 120
MdANR2a   LEILAGDLTDEGSFDAPIAGCDLVFHVATPVNFASEDPENDMIKPAIQGVLNVLKSCVKA 120
MdANR2b   LEILAGDLTDEGSFDAPIAGCDLVFHVATPVNFASEDPENDMIKPAIQGVLNVLKSCVKA 120
          ***********************************.************************

MdANR1    KTVKRVVLTSSAATVSINTLEGTGLVVDEKDWSDLEFLTNVKPPTWGYPASKTLAEKTAW 180
MdANR2a   KTVKRVVLTSSAATVSINTLEGTGLVMDEKDWSDLEFLTTVKPPTWGYPASKTLAEKTAW 180
MdANR2b   KTVKRVVLTSSAATVSINTLEGTGLVMDEKDWSDLEFLTTVKPPTWGYPASKTLAEKTAW 180
          *************************.************* .********************

MdANR1    KFAEENNIDLITVIPSLMAGPSLTPDVPSSIGLAMALITGDDFLINMALKGMQMLSGSIS 240
MdANR2a   KFAEENNIDLITVIPSLMAGPSLTPDVPSSIGLAMSLITGNDFLINMALKGMQMLSGSIS 240
MdANR2b   KFAEENNIDLITVIPSLMAGPSLTPDVPSSIGLAMSLITGNDFLINMALKGMQMLSGSIS 240
          ***********************************.****.*******************

MdANR1    IAHVEDVCRAHIFLAEKESASGRYICCAANTGVPELAKFLNKRYPQYKVPTEFGDFPSEA 300
MdANR2a   ISHVEDVCRAHIFLAEKESASGRYICCAANTSVPELARFLNKRYPQYKVPTEFGDFPSKA 300
MdANR2b   ISHVEDVCRAHIFLAEKESASGRYICCAANTSVPELARFLNKRYPQYKVPTEFGDFPSKA 300
          *.**************************** ***** ***************** *

MdANR1    KLIISSEKLIKEGFDFKYGIEEIYDQTVEYFKAKGLLQK 339
MdANR2a   KLIISSEKLIKEGFDFKYGIEEIYDQTVEYFKAKGLLQN 339
MdANR2b   KLIISSEKLIQEGFDFKYGIEEIYDQTVEYFKAKGLLQN 339
          **********.*************************** 
```

The CLUSTALW2 program was used to align peptide sequences.

Figure 2.5 Alignment of MdANR, *V. vinifera* VvANR, *M. truncatula* MtANR, and *A. thaliana* AtANR deduced amino acid sequences

```
MdANR   -MATQQPISKKTACVVGGTGFVASLLVKLLLQKGYAVRTTVRDPDNHKKVSHLTALQELG 59
VvANR   -MATQHPIGKKTACVVGGTGFVASLLVKLLLQKGYAVNTTVRDPDNQKKVSHLLELQELG 59
MtANR   -MATQHPIGKKTACVVGGTGFVASLLVKLLLQKGYAVNTTVRDPDNQKKVSHLLELQELG 59
AtANR   MDQTLTHTGSKKACVIGGTGNLASILIKHLLQSGYKVNTTVRDPENEKKIAHLRKLQELG 60
          *     ..*.***:**** :**:*:* ***.** *.******:*.**::**  *****

MdANR   ELEILAGDLTDEGSFDAPIAGCDLVFHVATPVNFASEDPENDMIKPAIQGVLNVLKSCVK 119
VvANR   DLKIFRADLTDELSFEAPIAGCDFVFHVATPVHFASEDPENDMIKPAIQGVVNVMKACTR 119
MtANR   DLKIFRADLTDELSFEAPIAGCDFVFHVATPVHFASEDPENDMIKPAIQGVVNVMKACTR 119
AtANR   DLKIFKADLTDEDSFESSFSGCEYIFHVATPINFKSEDPEKDMIKPAIQGVINVLKSCLK 120
        :*:*: .***** **::.::**: :*******:.* *****.***********:**.:*:* :

MdANR   AKTVKRVVLTSSAATVSINTLEGTGLVMDEKDWSDLEFLTTVKPPTWGYPASKTLAEKTA 179
VvANR   AKSVKRVILTSSAAAVTINQLDGTGLVVDEKNWTDIEFLTSAKPPTWGYPASKTLAEKAA 179
MtANR   AKSVKRVILTSSAAAVTINQLDGTGLVVDEKNWTDIEFLTSAKPPTWGYPASKTLAEKAA 179
AtANR   SKSVKRVIYTSSAAAVSINNLSGTGIVMNEENWTDVEFLTEEKPFNWGYPISKVLAEKTA 180
        :*:*****: *****:*:** *.***:*::*::*:*:**** ** .**** **.****:*

MdANR   WKFAEENNIDLITVIPSLMAGPSLTPDVPSSIGLAMSLITGNDFLINMALKGMQMLSGSI 239
VvANR   WKFAEENNIDLITVIPTLMAGSSLTSDVPSSIGLAMSLITGNEFLIN-GMKGMQMLSGSV 238
MtANR   WKFAEENNIDLITVIPTLMAGSSLTSDVPSSIGLAMSLITGNEFLIN-GMKGMQMLSGSV 238
AtANR   WEFAKENKINLVTVIPALIAGNSLLSDPPSSLSLSMSFITGKEMHVT-GLKEMQKLSGSI 239
        *:**:**:*:*:****:*.** .* ***:.*:**:***:: .. ..:* ** ****:

MdANR   SISHVEDVCRAHIFLAEKESASGRYICCAANTSVPELARFLNKRYPQYKVPTEFGDFPSK 299
VvANR   SIAHVEDVCQAHIFVAEKESASGRYICCAANTSVPELAKFLSKRYPQYKVPTDFGDFPPK 298
MtANR   SIAHVEDVCQAHIFVAEKESASGRYICCAANTSVPELAKFLSKRYPQYKVPTDFGDFPPK 298
AtANR   SFVHVDDLARAHLFLAEKETASGRYICCAYNTSVPEIADFLIQRYPKYNVLSEFEEGLSI 299
        *: **:*:..:**:*.*:**** :.****** ******:* ** :***:*:* ::* :  .

MdANR   AKLIISSEKLIKEGFDFKYGIEEIYDQTVEYFKAKGLLQN- 339
VvANR   SKLIISSEKLVKEGFSFKYGIEEIYDESVEYFKAKGLLQN- 338
MtANR   SKLIISSEKLVKEGFSFKYGIEEIYDESVEYFKAKGLLQN- 338
AtANR   PKLTLSSQKLINEGFRFEYGINEMYDQMIEYFESKGLIKAK 340
        .** :**:**::*** *:***:*:**: :***::***::
```

The CLUSTALW2 program was used to align peptide sequences.

Figure 2.6 The genetic mapping of the *MdANR1* and *MdANR2a/b* genes on the apple genome

LG10

0.0	CN884552
4.6	CTG1063360
11.1	CTG1062704
13.4	CTG1062183
16.1	AF057134-SSR
20.0	CTG1070830
23.2	CH01e09b
25.5	CTG1065993
27.1	CH02a10
29.6	CH04f03
32.5	CN913979
35.9	CH02c11
39.5	AU223548-SSR
41.9	CTG1075992
51.0	BACSSR18
54.4	CN857316
70.6	AT000347
77.4	CTG1063413
86.6	MdANR1-Indel

LG5

0.0	CTG1063512
2.3	MdANR2/3-Indel
3.7	CN943151
5.0	CTG1076729
6.4	Hi09b04
9.1	CTG1067456
10.1	CTG1065871
15.2	CTG1059163
17.6	CTG1058943
18.8	CTG1063619
25.4	BACSSR164
27.2	CH05e06
35.4	CN870202
36.5	CTG1072784
37.7	CN911587
39.6	CTG1061931
47.2	KB01-A7
50.7	CTG1062470
52.2	CTG1061792
54.8	CTG1076879
64.5	CTG1065813
67.4	CTG1064036
76.9	Hi08h08
81.3	CH04c03

The MdANR1-Indel and MdANR2/3-Indel markers were anchored onto linkage groups LG 10 and LG 5 of the apple genetic map, respectively.

Figure 2.7 Real-time (RT)-PCR analysis of *MdANR1* and *MdANR2a/b* gene expression in various floral and fruit tissues of 'Red Delicious' and 'Golden Delicious'
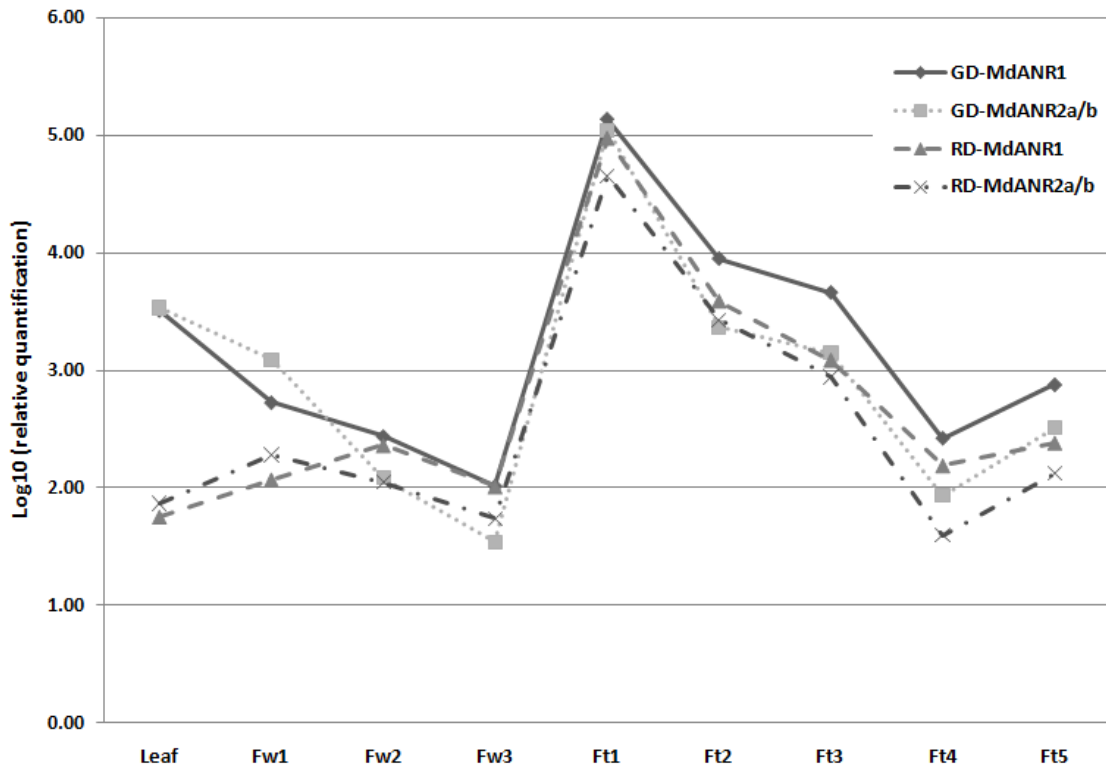
Differential expression of *MdANR1* and *MdANR2a/b* between the two apple cultivars Red Delicious and Golden Delicious was compared. Abbreviations for stages of development are listed as follows: Fw1: Flower buds at the pink stage; Fw2: flower buds at the balloon stage; Fw3: flowers at full bloom; Ft1: fruitlets 9 DAP; Ft2: fruitlets 16 DAP; Ft3: fruitlets 44 DAP; Ft4: fruits at 104 DAP; Ft5: fruits at 145 DAP; RD: 'Red Delicious'; GD: 'Golden Delicious'. All expression data were normalized the apple actin gene, and values are means of three technical replicates.

Figure 2.8 Ectopic expression of *MdANR1* gene in tobacco
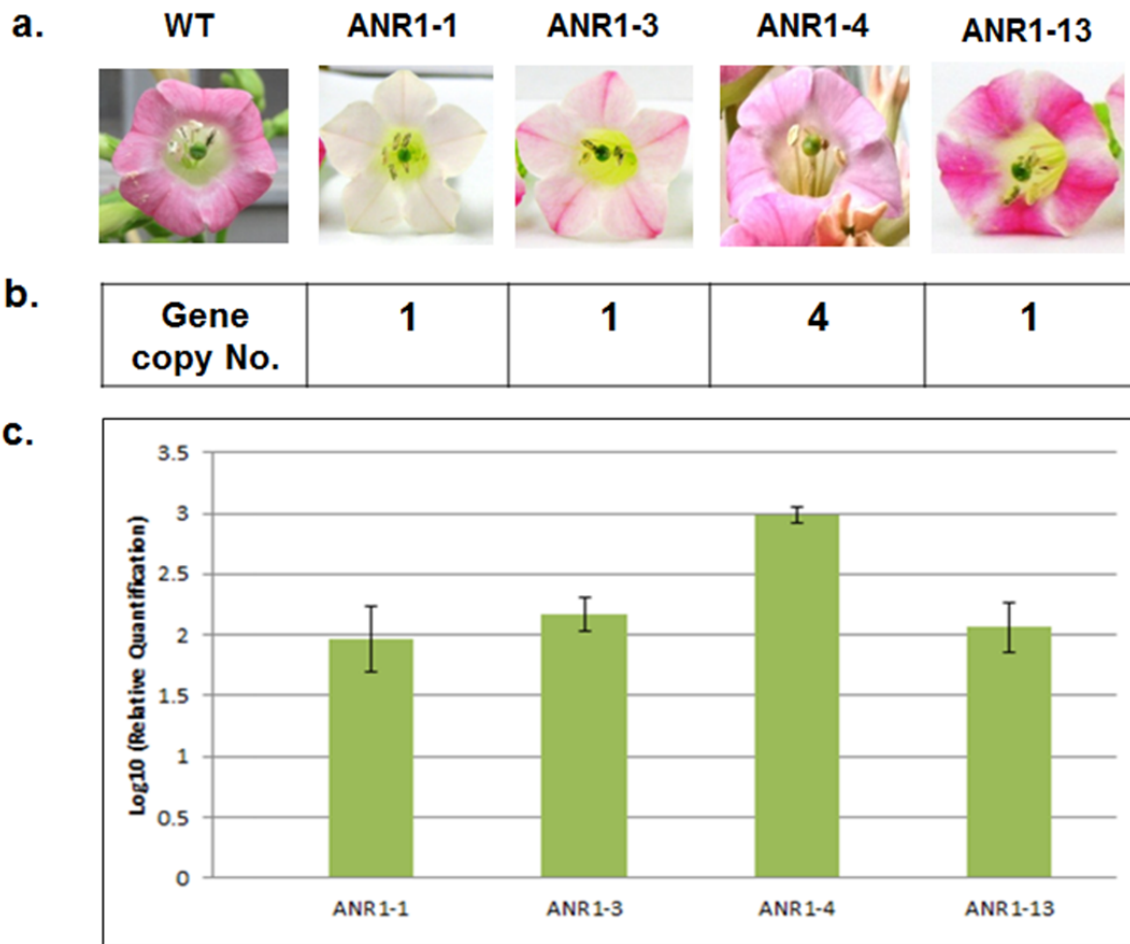
a. Tobacco flowers of wild-type and four T_0 transgenic lines ANR1-1, 1-3, 1-4, and 1-13. **b.** The transgene copy number present in each of the T_0 transgenic lines was determined by Southern blotting. **c.** Expression profiles of *MdANR* genes in transgenic tobacco flowers quantified (using qRT-PCR) relative to wild-type tobacco flowers.

Figure 2.9 Phenotypic variation in tobacco flowers detected in several T$_2$ transgenic plants expressing *MdANR1*, *MdANR2a*, or *MdANR2b*

Wild Type	**ANR2b-3**	**ANR2b-4**
ANR1-1	**ANR1-3**	**ANR1-13**
ANR2a-5	**ANR2a-6**	**ANR2a-8**
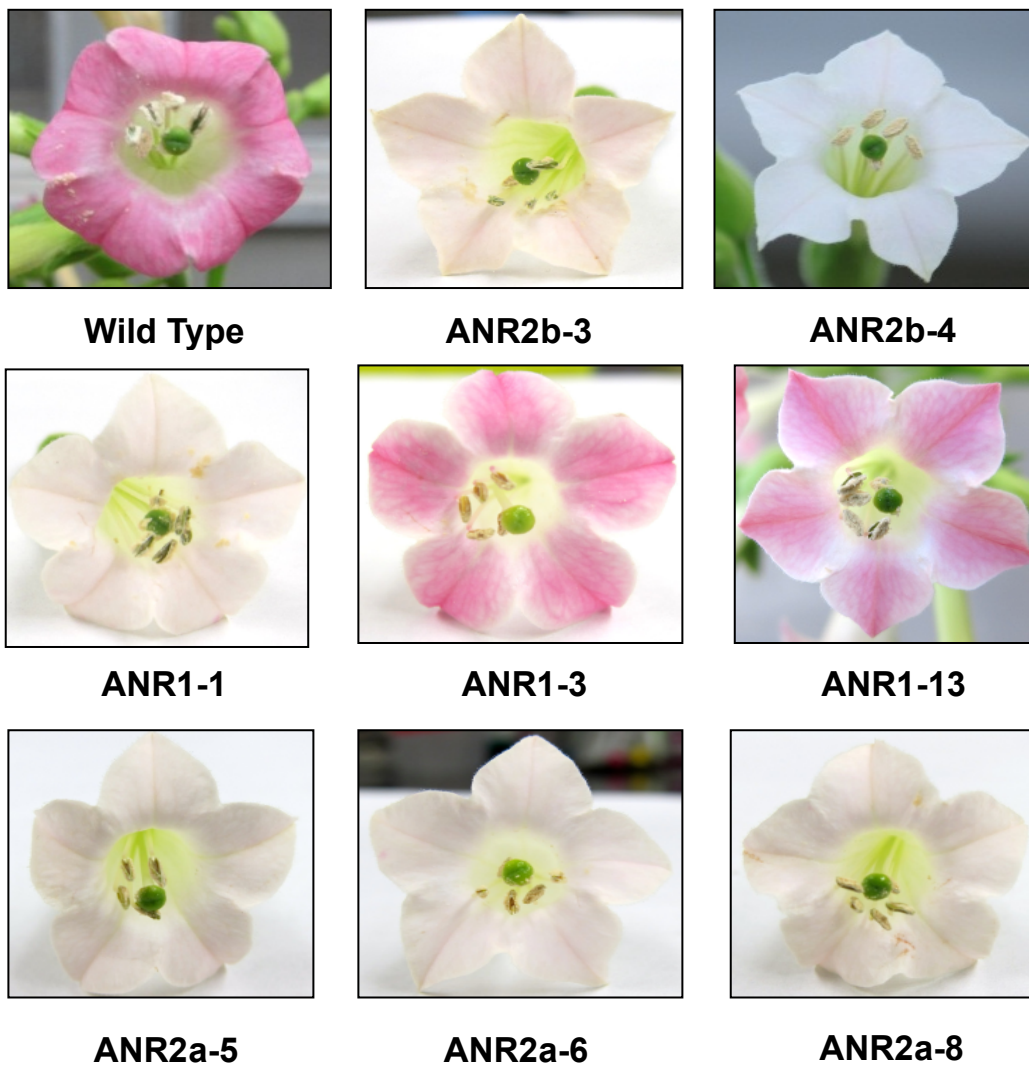
Figure 2.10 Expression profiles of flavonoid-related structural biosynthetic genes in flowers of transgenic tobacco lines carrying *MdANR* genes
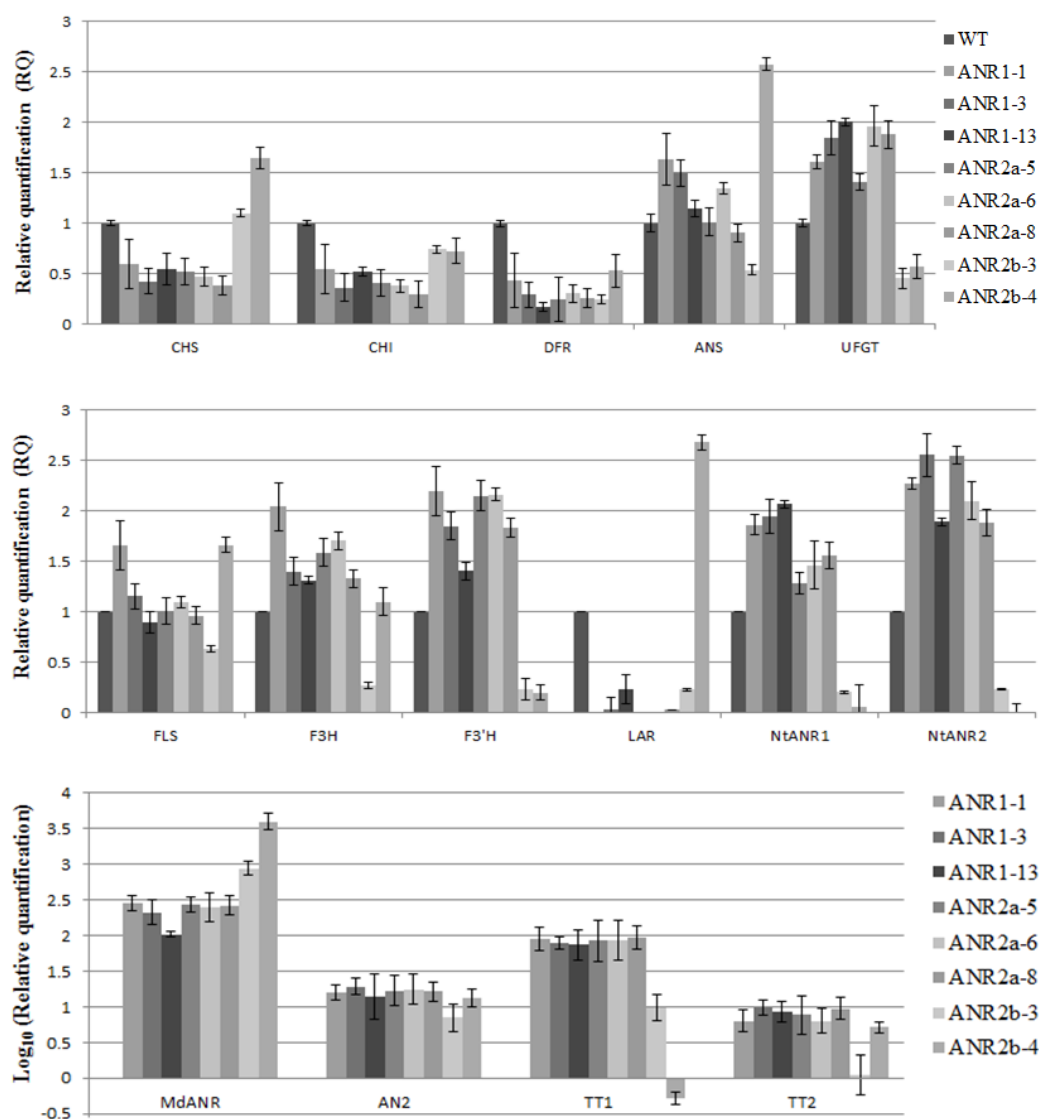
The mRNA transcripts expressed in transgenic flowers were relatively quantified based on those expressed in wild-type tobacco flowers. Abbreviations are listed as followings: WT: wild type; CHI: chalcone isomerase; CHS: chalcone synthase; F3H: flavonoid 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol reductase; LAR: leucoanthocyanidin reductase; ANS: anthocyanidin synthase; UFGT: UDP-glucose: flavonoid 3-o-glucosyltransferase; MdANR: apple anthocyanidin reductase; AN2: anthocyanin2; TT1: transparent testa1; TT2: transparent testa2. Normalization was made to the expression of actin gene and values are the average of three technical replicates.

Supplemental Figure S2.1 Physical map of BAC clones B7 and B8 located are on the same contig
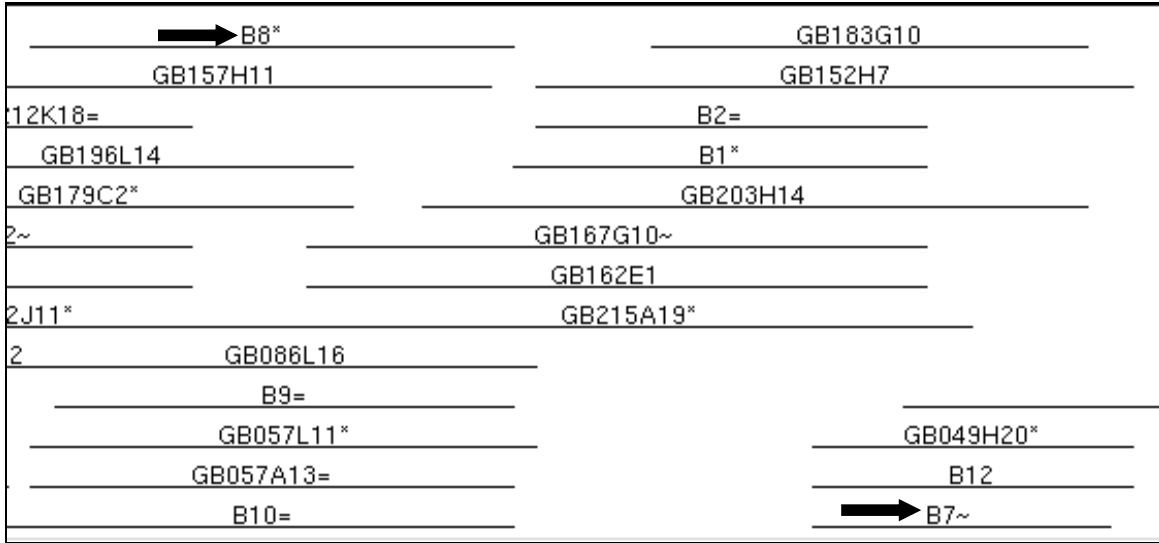
B7 and B8 clones are highlighted by arrows.

Supplemental Figure S2.2 Segregation of DNA bands of the MdANR1-Indel marker for *MdANR1* gene
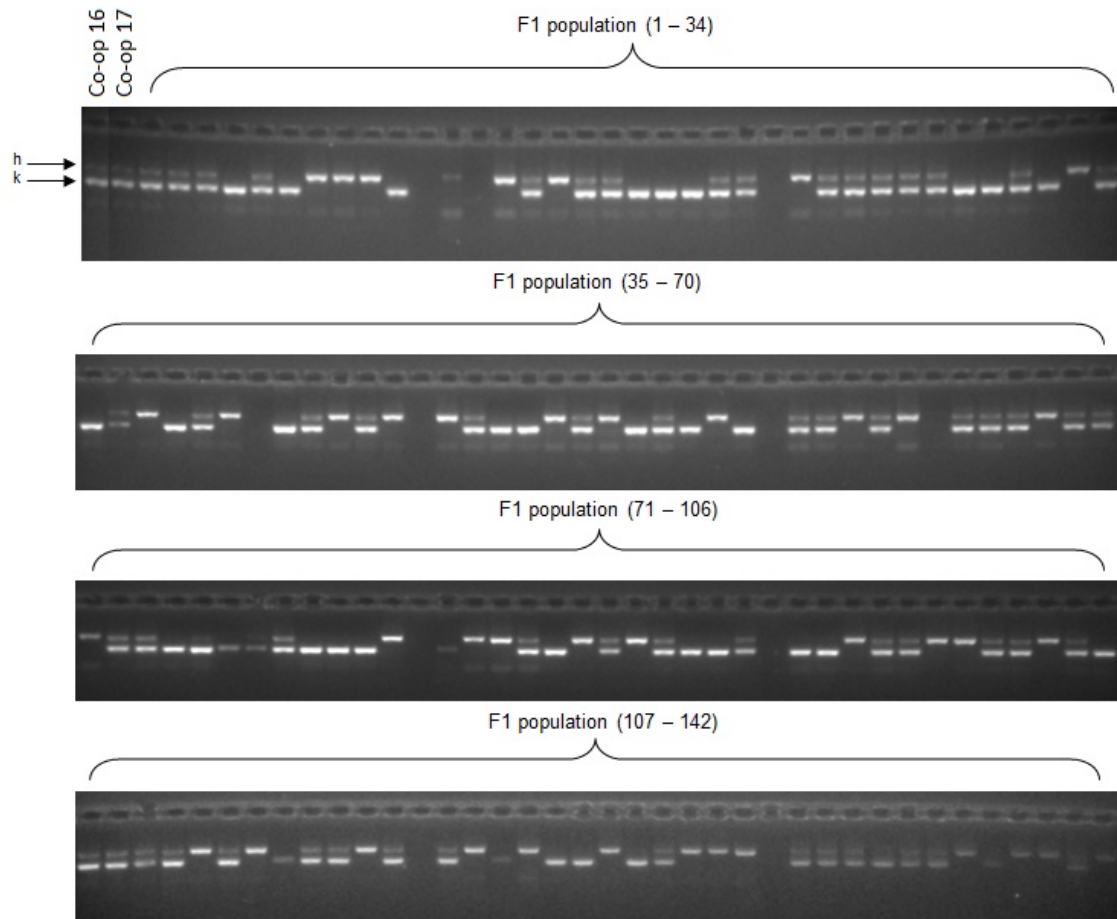
Parents (Co-op 16 and Co-op 17) and 142 individuals of 'Co-op 16' x 'Co-op 17' F1 population were tested. The arrows indicated the segregated DNA bands where 'h' represented the upper band and 'k' represented the lower band.

Supplemental Figure S2.3 Segregation of DNA bands of the MdANR2/3-Indel marker for *MdANR2a* and *MdANR2b* genes
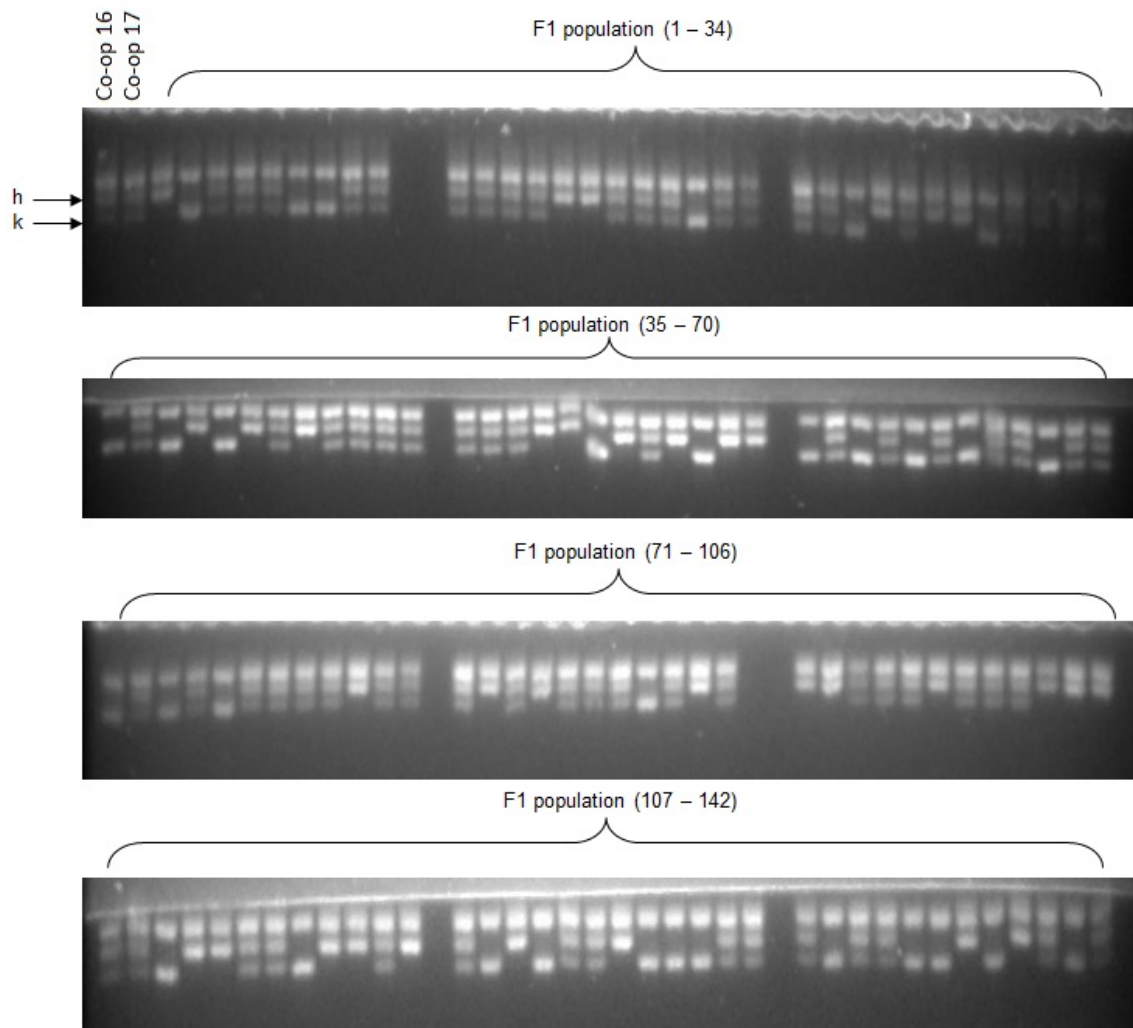
Parents (Co-op 16 and Co-op 17) and 142 individuals of 'Co-op 16' x 'Co-op 17' F1 population were tested. The arrows indicated the segregated DNA bands where 'h' represented the upper band and 'k' represented the lower band.

Supplemental Figure S2.4 The copy number of *nptII* gene in transgenic plant determined by Southern blotting
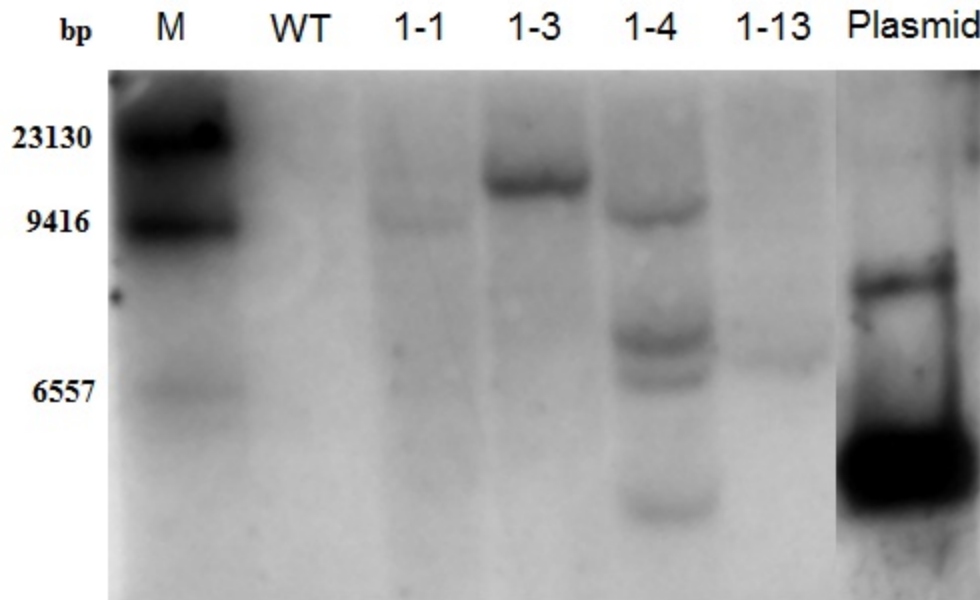
DIG marker II was used as a DNA marker (M). Wild type tobacco gDNA (WT) and MdANR-pBI121 (plasmid) served as negative and positive control, respectively.

CHAPTER 3

ECTOPIC EXPRESSION OF A GENE ENCODING AN APPLE MYB TRANSCRIPTION FACTOR, MDMYB11, ENHANCES PETAL COLORATION AND MODIFIES STYLE LENGTH IN TOBACCO FLOWERS

3.1 Abstract

Anthocyanins contribute to red coloration of apple fruit. Anthocyanin production in plants is transcriptionally controlled via the R2R3 MYB transcription factor (TF). In apple, red color pigmentation of fruit is regulated by three MYB TFs including *MYBA*, *MYB1*, and *MYB10*. However, these three *MYB* TF genes are likely to be allelic. In this study, we have isolated and characterized a new gene encoding MYB TF, designated as *MdMYB11*. The *MdMYB11* genomic sequence is comprised of three exons and two introns, and it contains an open reading frame of 1,193 bp encoding a putative protein of 310 amino acids. A simple sequence repeat (SSR) marker found in the promoter region of this sequence has anchored the *MdMYB11* onto linkage group (LG) 15 of the apple genome. Characterization of the *MdMYB11* gene has demonstrated that *MdMYB11* expression in leaves, flowers, and fruits, is higher in red-skinned cv. Red Delicious than in yellow-skinned cv. Golden Delicious. When the *MdMYB11* gene is introduced into tobacco, it has resulted in higher pigmented pink flowers compared to wild-type flowers. Moreover, *MdMYB11* specifically enhances expression of other anthocyanin-related genes including *CHALCONE SYNTHASE* (*CHS*), *CHALCONE ISOMERASE* (*CHI*), and *UDP-GLUCOSE: FLAVONOID 3-O-GLUCOSYLTRANSFERASE* (*UFGT*). These findings suggest that *MdMYB11* is a regulatory gene that is responsible for red color pigmentation of apple fruit.

Keywords: Anthocyanin; apple; MYB transcription factors; *Malus × domestica,* flavonoids; floral tissues

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3.2 Introduction

Color of the skin of apple fruits is an important determinant of fruit quality. Generally, consumers prefer red-skinned apples as they are perceived to be associated with better taste and flavor (King and Cliff, 2002). Coloration of apple fruit is attributed to accumulation of anthocyanin pigments belonging to flavonoids, a class of plant secondary metabolites. Flavonoids are ubiquitous in plants, and they are involved in different roles, including UV protection, pigmentation, disease resistance, and plant/animal interactions (Kevan et al, 1996; Dewick, 2002; Schaefer et al., 2004). More importantly, there is increasing evidence that flavonoids are important in human health, such as lowering the incidence of cardiovascular disease, obesity, diabetes, pulmonary disease, and cancer (Nijveldt et al., 2001; Boyer and Liu, 2004; Ruxton et al., 2006; Gerhauser, 2008; Prasad et al., 2010).

Color pigmentation of floral and fruit tissues is genetically determined by several structural genes. The biosynthetic pathway of anthocyanins has been well established and several genes coding for enzymes in the biosynthetic pathway of anthocyanin have been isolated and characterized (Winkel-Shirley, 2001a; Tanaka et al., 2008). In recent years, expression profiles of these enzymes during fruit development have been investigated. Honda et al. (2002) have reported that induction of most genes in the anthocyanin biosynthetic pathway would increase accumulation of anthocyanin in the skin of apple fruits. Moreover, it is known that anthocyanin biosynthesis encoding genes are largely regulated at the transcriptional level, and expression patterns of regulatory genes consequently determine pigmentation patterns (Holton and Cornish, 1995; Grotewold, 2006).

Among several families of transcription factors, the basic helix-loop-helix (bHLH), R2R3 MYB transcription factors (TFs), and the WD40 repeat protein predominantly regulate genes in the anthocyanin biosynthesis pathway across all plant species reported to date, including apple (Stracke et al., 2001; Allan et al., 2008). MYB TFs have been reported to play diverse functions in controlling pathways such as secondary metabolism, development, signal transduction, and disease resistance in plants (Jin and Martin, 1999). They are classified by the numbers of highly conserved imperfect

repeats in the DNA-binding domain, and consisting of either single or multiple repeats. Among these MYB TFs, the class of two-repeats (R2R3) is deemed the largest, with 339 TFs reported in Arabidopsis (Feller et al., 2011), and is associated with the anthocyanin biosynthesis pathway.

The regulation of genes involved in the anthocyanin biosynthesis pathway could occur in various ways. For example, some TFs transcriptionally regulate virtually all genes involved in anthocyanin biosynthesis, as reported in perilla (*Perilla frutescens*), (Saito and Yamazaki, 2002). Regulation has also been found to act specifically on discrete subsets of structural genes, those found either early or late in the anthocyanin biosynthetic pathway (Davies and Schwinn, 2003). For instance, MYBA in grape (*Vitis vinifera*) specifically regulates genes down-stream of anthocyanin production, but not those of earlier steps (Kobayashi et al., 2002). Like other transcription factors, regulation could serve to either activate or repress expression of these genes. For example, accumulation of anthocyanin is increased by overexpression of such TFs as PAP1 (Borevitz et al., 2000), AtPAP2, AtMYB113, and AtMYB114 (Gonzales et al., 2008) of *Arabidopsis thaliana*, VvMYB1a of *V. vinifera* (Kobayashi et al., 2004), GhMYB10 of *Gerbera* hybrid (Elomaa et al., 2003), and PyMYB10 of *Pyrus pyrifolia* (Feng et al., 2010; Zhang et al., 2011). Repression of the phenylpropanoid pathway has been observed by expression of a strawberry FaMYB1 (Aharoni et al., 2001), *Antirrhinum* AmMYB308 (Tamagnone et al., 1998), *Arabidopsis* AtMYB6, 4, and 3 (Jin et al., 2000), a single repeat MYB of an *Arabidopsis* AtMYBL2 (Dubos et al., 2008; Matsui et al., 2008), and an apple MdMYB6 (Gao et al., 2011).

In apple (*Malus × domestica* Borkh.), there are a few transcription factors that are reported to regulate genes in the anthocyanin biosynthesis pathway, including MYB1, MYBA, and MYB10 (Takos et al., 2006; Ban et al., 2007; Espley et al., 2007). However, *MYB1* and *MYBA* sequences are identical (Ban et al., 2007); whereas, *MYB10* and *MYB1* genes reside in the same linkage group, LG9, of the apple genetic map and at similar locations (Chagné et al., 2008). Recently, an allele-specific PCR primer approach has been used to demonstrate that these three MYB TFs are allelic in the apple genome (Lin-Wang et al., 2010).

Isolation of TFs associated with fruit color is an important key to understanding and manipulating color pigmentation of the fruit, and in providing useful tools for developing new cultivars. In this study, Arabidopsis MYB TFs involved in anthocyanin pathway has been accessed from Genbank and used to isolate a new MYB TF in apple. Mining an apple expressed sequence tag (EST) database (Gasic et al., 2009), a candidate gene is isolated and designated as *MdMYB11*. The *MdMYB11* gene is highly expressed in fruit peel of the red-skinned cultivar Red Delicious when compared to that of the yellow-skinned cultivar Golden Delicious. Overexpression of *MdMYB11* in tobacco plants has resulted in recovery of highly pigmented flowers with longer styles in transgenic lines than those of wild-type plants. These results strongly suggest that *MdMYB11* regulates accumulation of anthocyanins, and pistil morphology in tobacco flowers.

3.3 Materials and Methods

3.3.1 Isolation of a genomic sequence of an MYB transcription factor

Using sequence-based homology, an Arabidopsis MYB TF gene, *AtMYB11* (NM_116126), involved in the flavonoid biosynthesis pathway was BLASTed against an apple EST database (http://titan.biotec.uiuc.edu/cgi-bin/ESTWebsite/estima_start?seqSet=apple). An EST contig (Mddb5012a14.y1) with high significant alignment was detected. A pair of primers (Forward, 5'-GGGAGAGCACCTTGTTGTGAG-3'; reverse, 5'-GATCTCGTTGTCGGTTCTTCC-3') was designed based on the EST contig, and used to screen an apple BAC library developed from cv. GoldRush using *Bam*HI, representing 5× haploid genome equivalents. A PCR-based screening method was performed as previously described by Xu et al. (2002), and positive BAC clones were picked, and fingerprinted. Selected positive clones representing different contigs were subjected to sequencing. To cover the full-length genomic sequence of the TF, primer walking was performed (Supplemental Table S2.1).

3.3.2 Isolation of an MYB transcription factor

Based on genomic sequences of candidate MYB TFs, the open reading frame (ORF) was predicted using FGENESH-M, a gene finding tool in Eukaryote. Forward and reverse primers were designed based on the predicted gene (Forward, 5'-GGAGAGCACCTTGTTGTGAG-3'; reverse, 5'-ACTGACAATTGCTGCATGCC-3'), and used to PCR-amplify the gene from cDNA pools. The PCR product was run on 1% agarose gel, and the amplified product of the expected size of 872 bp was extracted, purified, and subjected to sequencing. The obtained sequence was then compared to the predicted ORF sequence to verify the coding sequence.

To obtain a full-length cDNA, the coding sequence was BLASTed against the EST database (NCBI BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi). The full-length cDNA was assembled based on EST database. Forward and reverse primers (Forward, 5'-CTGATCCAGAAGAAGAAACAGATG-3'; reverse, 5'-TGGATTCAAAGCAGGTCTGTG-3') were then designed to cover the coding sequence of the TF, and to amplify the sequence for functional analysis.

3.3.3 Cloning of the *MdMYB11* gene in the expression vector

The coding sequence of *MdMYB11* was amplified by PCR using the proofreading DNA polymerase Platinum® *Pfx* (Invitrogen) following the manufacturer's reaction conditions. Forward 5'-TGAC<u>TCTAGA</u>CTGATCCAGAAGAAGAAAC-3' and reverse 5'-ATAC<u>GAGCTC</u>TGGATTCAAAGCAG-3' primers amplified the PCR product carrying 5'- and 3'- end restriction sites of *Xba*I and *Sac*I designed for the pBI121 binary cloning vector. The blunt-end PCR product was ligated into the pCR®-Blunt vector using the Zero Blunt® PCR cloning kit (Invitrogen), according to the manufacturer's protocol. Several clones were sequenced.

3.3.4 Polymerase chain reaction (PCR) and Southern blotting

The PCR was performed using *Taq* DNA polymerase (Promega, Madison, WI; or Invitrogen) and dNTPs (Biolab) unless otherwise specified. The reaction consisted of an initial denaturation at 94°C for 3 min, followed by 33 cycles of denaturation for 35 s,

annealing at 55°C for 30 s, extension at 72°C for 60 s, and followed by a final 8 min extension at 72°C.

Genomic DNA (10 μg) of tobacco were digested with *EcoR*I, electrophoresed on a 1.0% agarose gel, and then transferred to a nylon membrane (Amersham). Following DNA crosslinking using a transilluminator, the blot was pre-hybridized with the DIG Easy Hyb (Roche Diagnostics 2000) for 1 h. An *nptII* gene probe (forward, 5'-TATTCGGCTATGACTGGGCA-3'; reverse, 5'-GCCAACGCTATGTCCTGAT-3') was synthesized from the expression vector using the PCR-DIG Probe Synthesis Kit (Roche). The membrane was hybridized with the probe at 42°C, and washed using a series of SSC buffers (2 to 0.5X). The detection of gene-specific signal was carried out using a DIG Detection Kit while following the manufacturer's instructions (Roche).

3.3.5 Mapping of the *MdMYB11* gene onto the apple linkage map

Simple sequence repeats (SSRs) were used to develop a gene marker for *MdMYB11*. According to the genomic sequence, SSRs were searched by using the SSRIT tool (http://www.gramene.org/db/markers/ssrtool) developed by Temnykh et al. (2001). The primer sets were designed based on two identified SSRs, and these were used to screen the parents 'Co-op 16' and 'Co-op 17' of a mapping population. The primer sets were as follows: SSR1 (forward 5'-TCACCTCTTCAAACAACACACC-3' and reverse 5'-TGCTCTCCCCATCTGTTTCT-3'), and SSR2 (forward 5'-CAAGACTGCAGCGGATCATA-3' and reverse 5'-GCCTCACTGCTTTGAAATCC-3'). PCR analysis was conducted using the two parents and all 142 individuals of the F1 mapping population 'Co-op 16' x 'Co-op 17'. The PCR product was resolved on 2% (w/v) metaphor gel. Segregation patterns of the F1 population were recorded, and mapped onto an SSR-based apple genetic map (manuscript in submission). The linkage map was constructed using JOINMAP software version 4.0.

3.3.6 Plant material

Leaves, flowers, and fruits at different stages of development were collected from trees of apple *(M. × domestica* Borkh.) cvs. Red Delicious and Golden Delicious grown

at the Pomology Research Center at the University of Illinois.   All tissues were immediately frozen, ground, and stored at -80 °C.

Seeds of *Nicotiana tabacum* cv. Petite Havana SR1 were placed in a 1.5 ml eppendorf tube, sterilized in 1 ml 20% bleach (Clorox®) containing a drop of tween-20, vigorously vortexed for 10-15 min, and washed at least 5 times with sterilized-distilled water. Seeds were germinated on half- strength Murashige and Skoog (MS) (1962) basal salt medium containing 10 g/l sucrose, pH adjusted to 5.7, and solidified with 0.2% gelrite.   Seeds were maintained in a controlled temperature growth chamber under 16 photoperiod, providing 25 $\mu mol.m^{-2}.s^{-1}$ light intensity, and at 25 °C. Ten-day-old seedlings with the first pair of true expanded leaves were transferred into glass jars containing 30 ml of MS medium along with MS vitamins, 30 g/l sucrose, pH 5.7, and solidified with 0.2% gelrite.

3.3.7 Tobacco transformation

The coding sequence of *MdMYB11* was introduced into the pBI121 cloning vector, and used to transform tobacco by *Agrobacterium*-mediated transformation. A schematic diagram of the construct is presented in Figure 3.1. The construct was introduced into the binary vector pBI121 using *Xba*I and *Sac*I restriction enzymes, and immobilized into bacterial cells of *Agrobacterium tumefaciens* strain GV3101 using electroporation.

Leaf sections of tobacco were used for transformation as described by Horsch et al. (1985), but with some modification. Bacterial cells containing the binary vector pBI121 from a glycerol stock were inoculated into 5 ml Luria Broth (LB) medium containing 100mg/l kanamycin at 28 ºC for 2 d with vigorous shaking. Cells were collected by centrifugation at 2500g for 10 min and resuspended in a liquid co-cultivation medium to an OD_{600} of 0.5 to 0.8.

Leaves from in vitro-grown seedlings were cut into ~0.5 cm^2 segments, and incubated with *Agrobacterium* cell suspension for 30 min at 28 ºC with shaking at 100 rpm. Explants were transferred to a solid co-cultivation medium, consisting of MS medium with MS vitamins, 30 g/l sucrose, 1 mg/L 6-benzyladenine (BA), 0.1 mg/L α-

naphthaleneacetic acid (NAA), pH 5.6, with 0.8% tissue culture agar (Phytotechnology), and 100 mg/L kanamycin, and incubated in the dark for 4 d at 25 ºC. Subsequently, explants were washed twice with liquid co-cultivation medium containing 1,000 mg/L cefotaxime, blotted dry onto a filer paper, transferred to 150 x 100 mm petri plates containing a selection medium (co-cultivation medium with 0.8% Agar, 100 mg/L kanamycin, and 250 mg/L cefotaxime), and incubated for 2 wks in the dark at 25 ºC. All petri plates were transferred to a controlled environment growth chamber under 16 h photoperiod, 25µmol. m^{-2}.s^{-1} light intensity, and sub-cultured biweekly.

Developing shoots from explants were excised, and transferred to a rooting medium, consisting of the selection medium, but without any plant growth regulators (PGRs). Rooted kanamycin-resistant shoots were transferred to magenta boxes containing Sunshine professional pert-lite mix LC1, acclimatized, and then transferred to the greenhouse.

Molecular analysis of transformed plants was conducted using PCR and Southern blot analysis as described above to determine the presence of the transgene and the transgene copy number in different transgenic lines.

3.3.8 Expression analysis

Total RNA from leaf and flower tissues were extracted using an RNAqueous Kit (Ambion) according to the manufacturer's instructions. RNA from fruit tissues was isolated according to the protocol of Gasic et al., (2004).

Total RNA (2 µg) from each tissue was treated with *DNase*I (Invitrogen), and used for cDNA synthesis. The first-strand cDNA synthesis was performed with Oligo (dT) primer using the SuperScript III RT kit (Invitrogen), according to the manufacturer's instructions. Specific primers for the *MdMYB11* and each flavonoid-related gene were designed by using Biology Workbench version 3.2 (http://workbench.sdsc.edu) to perform real-time PCR. Specific primer sequences and accession numbers of genes used to design primers have been listed in Table 3.1. The SYBR Green real-time PCR assay was carried out in a total volume of 25 µl, and consisting of 12.5 µl of 2× SYBR Green I Master Mix (Applied Biosystems), 0.2 µM (each) specific primers, and 100 ng of

template cDNA. The amplification program consisted of 1 cycle of 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The fluorescent product was detected at the last step of each cycle. Following amplification, melting temperatures of PCR products were analyzed to determine the specificity of the PCR product. Melting curves were obtained by slow-heating at 0.5°C /sec, from 60°C to 90°C, while continuously monitoring the fluorescence signal. A negative control without a cDNA template was run with each analysis to evaluate the overall specificity.

Amplifications were carried out in a 96-well plate in a 7300 Real Time PCR System (Applied Biosystems). All experimental samples were run in triplicates. The *Actin* gene was used as the constitutive control gene. Differences between the cycle threshold (*Ct*) of the target gene and the *Actin* gene were used to obtain the relative transcript level of the target gene, and calculated as 2 exp-(Ct_{target} - Ct_{actin}).

3.3.9 Flavonoid analysis

Anthocyanins and flavonols were extracted from 50 mg of finely ground tissues in 1 ml 1% HCL/methanol (v/v), at room temperature in the dark, with continuous shaking for 1 h, and centrifuged at 13,000 rpm for 15 min. An aliquot of 100 μl of the supernatant was transferred to a fresh tube, and acid-hydrolyzed by adding 30 μl of 3N HCl, and incubated at 70°C for 1 h in a thermal cycler (Thermo Hybaid MBS 0.25s, Thermo Scientific). Proanthocyanins (PAs) were extracted using 1 ml 70% (v/v) acetone containing 0.1% (w/v) ascorbate, and incubated at room temperature for 24 h in darkness as described in Takos et al. (2006). The extract was centrifuged at 13,000 rpm for 15 min at room temperature, and the clear supernatant was transferred to a new tube. An aliquot of 200-μl extract was dried at 35 ºC, and resuspended in 100 μl of 1% (v/v) HCl-methanol and 100 μl of 200mM sodium acetate (pH 7.5).

Flavonoid contents were determined using LC/MS/MS along with the use of commercial standards for kaempferol, quercetin, cyanidin, catechin, and epicatechin (Sigma). The LC/MS/MS analysis was performed on a 5500 QTRAP mass spectrometer (AB Sciex) which was equipped with a 1200 Agilent HPLC Analyst (version 1.5.1, Applied Biosytems) was used for data acquisition and processing. A Phenomenex

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column (3 μ C6-Phenly 11A, 4.6 × 50 mm) was used for separation. The HPLC flow rate was set at 0.3 mL/min, and HPLC mobile phases consisted of A (0.1% formic acid in H_2O) and B (0.1% formic acid in acetonitrile). The autosampler was maintained at 5ºC. The gradient for catechin and epicatechin was as follows: 0 min, 90% A; 10 min, 50%A; 13-18min, 0% A; and 18.1-25 min, 90% A. The injection volume was 20 μl. The gradient for cyanidin, kaempferol, and quercetin was as follows: 0 min, 70% A; 7-12.5 min, 0% A; and 13-20 min, 70% A. The injection volume was 10 μl. The mass spectrometer was operated with positive electrospray ionization. Multiple reaction monitoring (MRM) was used to quantify catechin and epicatechin (m/z 291.0-->139.2), cyanidin (m/z 287.2-->213.2), kaempferol (m/z 287.1-->153.2), quercetin (m/z 303.1-->153.1). The electrospray voltage was set to 5500 V, the heater was set at 600 ºC, the curtain gas was 35 psi, and GS1 and GS2 were both 60 psi. Analyses of each sample were repeated three times using three biological replicates.

3.4 Results

3.4.1 Isolation of *MdMYB11* transcription factor

It has been reported that the AtMYB11 transcription factor controls flavonoid accumulation in Arabidopsis (Stracke et al, 2007). Based on *AtMYB11* gene sequence, a putative MYB transcription factor is identified in an apple EST database, and this has been designated as *MdMYB11*. Following PCR-based screening of an apple BAC library, seven positive BAC clones have been identified of two different band sizes. Based on BAC fingerprinting profiles, two BAC clones, BAC66K1 (lower band) and BAC159H19 (upper band), from different contigs have been selected for sequencing.

Using primer walking, the genomic sequence of *MdMYB11* has been identified and found to consist of three exons and two introns (Figure 3.2). The full-length cDNA of *MdMYB11* is 1,193 bp encoding a putative protein of 310 amino acids with the start codon at nucleotide position 162 and the stop codon at position 1094 (Figure 3.3). Alignment of the deduced amino acid sequence of MdMYB11 with those of several previously characterized MYB transcription factors in apple (MdMYB1, MdMYBA, and MdMYB10), as well as those identified in other plant species (AtMYB3, AtMYB4,

AtMYB6, AtMYB32, AtMYB7, AtMYB11, AtPAP1, FaMYB1, FaMYB10, VlMYBA1-1, GMYB10, PhMYBAN2, ZmMYB31, ZmMYBC1, and VvMYBCs1) has revealed highly similar sequences in R2 and R3 DNA-binding domains (Figure 3.4). An R/B-like bHLH binding motif ([D/E]Lx$_2$[R/K]x$_3$Lx$_6$Lx$_3$R; Zimmermann et al. 2004) was found in the R3-DNA binding domain of MdMYB11. Moreover, MdMYB11 contains conserved motif L1srGIDPx^T/$_S$HRx^I/$_L$ (C1-motif) and pdLNL^D/$_E$Lxi^G/$_S$ (C2-motif) at the C-terminus that is similar to those of MYB subgroup 4 (Kranz et al., 1998) encoding transcription repressors such as AtMYB3, AtMYB4, AtMYB6, AtMYB32, AtMYB7, FaMYB1, and ZmMYB31 (Figure3.5). However, outside of the conserved domain, their C-termini are highly divergent. MdMYB11 has the highest similarity of the deduced amino acid sequence to AtMYB6 with 56% identity; whereas, it shares about 30% homology with MdMYB1, MdMYBA, and MdMYB10, and 34% with AtMYB11 from which it was used to disclose MdMYB11 sequence.

3.4.2 Mapping of the *MdMYB11* gene on the apple genetic map

To map the *MdMYB11* gene, an available genetic map of the mapping population 'Co-op 16' x 'Co-op 17', previously established in our laboratory, was used. Based on the genomic sequence of *MdMYB11*, three simple sequence repeats (SSRs) were found. Of these, two SSRs were found within the promoter region, while the third SSR was found within the last exon. Two pairs of primers, SSR1 and SSR2, were designed. As the two SSRs that were found in the promoter region of *MdMYB11*, were tandemly arranged, the SSR1 primer set was used to cover both repeats.

PCR-based screening of the parents 'Co-op 16' and 'Co-op 17' was carried out using SSR1 and SSR2 primer sets. As the two repeat domains were detected with SSR1 but not with SSR2 (Supplemental Figure S3.1), the SSR1 marker was further used to screen both parents and their F1 progeny, consisting of 142 individuals. The two DNA bands were amplified in both parents, indicating that both have heterozygous alleles for the SSR1 marker, and these segregated in the F1 mapping population (Supplemental Figure S3.2). The genotypes of the parents were asssinged as 'hk' and 'hk', wherein 'h' and 'k' corresponded to the upper and lower bands, respectively. Genotypes of seedlings

in the mapping population were assigned as either 'hh' (upper band), 'hk' (upper and lower bands), or 'kk' (lower band). Based on segregation of the mapping population, the *MdMYB11* was mapped onto linkage group (LG) 15 on the apple genetic map (Figure 3.6).

3.4.3 Expression profiles of *MdMYB11* in apple

Expression of *MdMYB11* in apple cvs. Red Delicious (red-skinned fruit) and Golden Delicious (non-red skinned fruit) was analyzed by real-time (RT) PCR. Transcripts of *MdMYB11* were expressed in all analyzed tissues including leaves, flowers, and fruits (Figure 3.7). Overall, mRNA transcripts of *MdMYB11* in all analyzed tissues were higher in cv. Red Delicious than those in cv. Golden Delicious. Levels of transcripts increased during flower development and peaked at the full-bloom stage in cv. Red Delicious, while these remained constant in cv. Golden Delicious. Moreover, transcripts of *MdMYB11* in developing fruitlets were high at fruitlet stage I in both cultivars, but then these steadily dropped for a period of time during fruit development and then increased in Golden Delicious. In Red Delicious, transcripts of *MdMYB11* dropped slightly and then reached peak levels at fruitlet stage IV before undergoing another drop followed by increased levels at fruit maturity (Figure 3.7).

3.4.4 Functional analysis of *MdMYB11* in tobacco

The functionality of *MdMYB11* was investigated following ectopic expression of the introduced gene in tobacco. The coding sequence of *MdMYB11* was under the control of the cauliflower mosaic virus (CaMV) 35S promoter, and this was introduced into tobacco via *Agrobacterium*-mediated transformation. Presence of the *MdMYB11* transgene in putative transgenic tobacco lines T1, T5, and T8 was confirmed by PCR. Moreover, integration and copy number of the transgene was confirmed following Southern blot hybridization (Figure 3.8).

Among the morphological observations detected in reproductive tissues of transgenic tobacco lines, flower color pigmentation was markedly different with increased pink coloration in different transgenic lines when compared to those of wild-

type tobacco (Figure 3.9).   Early on during the first stages of flower development, corolla had increased color pigmentation, and this continued until full bloom wherein differences in pigmentation were clearly noted. In addition, the size of transgenic flowers was larger than that of wild-type flower (Figure 3.9b).

Flowers of the T2 generation of three transgenic lines T1, T5, and T8 were evaluated for levels of expression of flavonoid-related genes involved in the anthocyanin biosynthesis pathway.   It was observed that *CHS*, *CHI*, and *UFGT* genes were significantly up-regulated; whereas, *DFR*, *C4H*, and *4CL2* genes were down-regulated (Figure 3.10). *ANS* gene was also up-regulated in transgenic line, particularly in line T5. Moreover, morphological changes in the reproductive floral structure were readily observed, wherein styles of flowers of transgenic lines were longer, on average of 8 to 10 mm, than those of wild-type flowers leaving stigmas positioned above anthers in these transgenic lines, making self-pollination difficult (Figure 3.11).

Upon LC-MS-MS analysis of chemical profiles of flavonoid, anthocyanin, and proanthocyanin, it was observed that these contents were modified in flowers of all transgenic lines carrying the *MdMYB11* transgene compared to those of wild-type tobacco (Table 3.2). In general, both of the flavonoid compounds including kaempferol and quercetin were higher in flowers of transgenic lines T1, T5 and T8, except for level of kaempferol in T1 than those in wild-type tobacco. Levels of cyanidin were 2- to 4-fold higher in flowers of transgenic lines than those of wild-type tobacco, whereas, levels of the proanthocyanins catechin and epicatechin were 1.1- to 1.4-fold and 1.3 to 4.5-fold higher, respectively, in flowers of transgenic lines than those of wild-type tobacco (Table 3.2). These findings suggested that *MdMYB11* is indeed involved in the regulation of flower color pigmentation as well as in modifying reproductive floral structures.

3.5 Discussion

Apple fruits vary considerably in color, ranging from yellow, green, or red, along with varied differences in red color pigmentation patterns. To date, three R2R3 transcription factors (TFs) responsible for red color pigmentation, including *MdMYB1*, *MdMYB10,* and *MdMYBA,* have been reported (Ban et al., 2007; Espley et al., 2007;

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Takos et al., 2006). Of these three TFs, *MdMYB1* and *MdMYBA* contribute to red skin coloration, while *MdMYB10* controls red flesh coloration. Recently, it has been reported that these three TFs are in fact allelic (Lin-Wang et al., 2010).

Multiple R2R3 TFs involved in anthocyanin biosynthesis have been identified in many plants (Allan et al., 2008). However, it is not clear whether there are additional loci, other than the *MdMYB10* locus, that are also involved in the regulation of anthocyanin in apple. In this study, a novel R2R3 MYB transcription factor, designated as *MdMYB11*, has been identified and characterized in apple. This TF is clearly involved in red color pigmentation of apple reproductive tissues. The BC226-STS (a1) marker for the red skin color locus (Rf) in apple was developed, and mapped onto linkage group (LG) 9 of the 'Delicious' map (Cheng et al., 1996). Previously, *MdMYBA* was also mapped to the same linkage group nearby the BC226-STS marker (Ban et al., 2007). However, *MdMYB11* was mapped onto LG15 of the apple genetic map in our study, suggesting that anthocyanin-regulating genes are not restricted to one LG.

It has been reported that *MdMYB10* and *MdMYB1* regulate expression of most flavonoid structural genes in apple (Espley et al., 2007; Takos et al., 2006). In this study, expression levels of *CHS* and *UFGT* in flowers of transgenic tobacco lines were 1.9- and 3.5-fold higher, respectively, than those of wild-type plants. Whereas, levels of expression of the *DFR* gene were lower in flowers of transgenic tobacco lines than those of wild-type plants. Interestingly, expression levels of *F3H*, *F3'H*, *FLS*, and *LDOX* in flowers of transgenic lines and those of wild-type tobacco were not different, thus suggesting that *MdMYB11* was not involved in the regulation of these flavonoid genes. Whereas, *MdMYB11* played a regulatory role in several others target genes in the anthocyanin biosynthesis pathway, including *C4H*, *4CL2*, *CHS*, *DFR,* and *UFGT* contributing to upregulation and/or downregulation of these genes. These findings suggested that the functional regulation of *MdMYB11* was different to those of either *MdMYB10* or *MdMYB1*. Moreover, the role of *MdMYB11* in the positive regulation of flavonoid biosynthesis was attributed to activation of both *CHS* and *UFGT* transcription, but primarily to that of *UFGT*.

Ectopic expression of *MdMYB11* in tobacco was contributed to anthocyanin accumulation, restricted to the reproductive tissues. Similar result was observed in tobacco transformed with *MdMYBA* (Ban et al., 2007), and Arabidopsis with *MdMYB1* (Takos et al., 2006). In contrast, several other anthocyanin-related TFs were able to induce anthocyanin accumulation in the whole plant, as reported in grape VlMybA1-1, Gerbera GMYB10, tomato LeANT1, and Arabidopsis AtPAP1 (Borevitz et al. 2000, Elomaa et al. 2003, Kobayashi et al., 2002; Mathews et al. 2003). These TF genes were previously indicated that they have an ability to activate bHLH transcription; therefore, they could form a complex with bHLH, resulting in induction of anthocyanin biosynthesis (Koes et al., 2005). However, some MYB TFs such as maize ZmP, Arabidopsis AtMYB12, and grape VvMYBF1, do not contain the R/B-like bHLH binding motif; thus, they function independently of a bHLH cofactor (Grotewold et al., 2000; Mehrtens et al., 2005; Czemmel et al., 2009). In our study, MdMYB11 may not function as a bHLH activator but require bHLH to successfully function, like MdMYBA. Since MdMYB11 contains the R/B-like bHLH binding motif, it presumably induced anthocyanin biosynthesis by forming a complex with endogenous bHLH in reproductive tissues such as flower in which it was present earlier; therefore, anthocyanin accumulation was not observed in other tissues in which bHLH should be activated by MYB TFs.

When the coding sequence of *MdMYB11* was BLASTed against the Arabidopsis genome sequence database (http://www.arabidopsis.org/Blast/index.jsp), the sequence was hit to several transcription repressor genes such as *Arabidopsis AtMYB3*, *AtMYB7*, *AtMYB4*, *AtMYB32*, and *AtMYB6* with E-value of 3e-25, 2e-23, 1e-21, 2e-10, and 3e-09, respectively. These genes function as a repressor in lignin biosynthesis as well as affected other genes in flavonoid biosynthesis pathway (Jin et al., 2000; Preston et al., 2004; Zhao et al., 2007). Thus, *MdMYB11* was expected to have similar function to those genes. *AtMYB4* and *AtMYB32* have been previously reported to be a negative regulator in the sinapate ester biosynthesis pathway (Jin et al., 2000; Preston et al., 2004). Overexpression of *MYB4* resulted in lower levels of expression of both *C4H* and *CHS* genes (Zhao et al., 2007); whereas, that of *MYB32* lowered expression level of *C4H* but

has not altered *CHS* expression level (Preston et al., 2004). In our study, when *MdMYB11* was introduced into tobacco, flowers of transgenic lines overexpressing this transgene had lower levels of *C4H* and *4CL2* than those of wild-type plants, as expected. However, ectopic expression of *MdMYB11* in tobacco significantly promoted transcription of both *CHS* and *UFGT* genes, and contributing to increased pink color pigmentation of corolla of transgenic lines, which was readily observed during early stages of flowering. This scenario was also found in overexpression of maize *ZmMYB31* in Arabiodopsis. Fornale et al. (2010) reported that ZmMYB31 down-regulated several monolignol genes such as *4CL1*, and up-regulated anthocyanin biosynthesis genes including *CHI*, *F3H*, *F3'H*, and *DFR*, resulting in anthocyanin accumulation. In addition, levels of expression of *MdMYB11* were higher in the red-skinned apple cultivar Red Delicious than in the yellow-skinned apple cultivar Golden Delicious. These findings suggest that *MdMYB11* is a positive regulator of genes involved in the anthocyanin biosynthesis pathway and may function as a negative regulator of lignin biosynthesis. Further studies must include expression analysis of genes involved in monolignol biosynthesis as well as chemical analysis of lignin accumulation.

It has been reported that AtMYB4 contains a conserved motif $\text{pdLNL}^{D}/_{E}\text{Lxi}^{G}/_{S}$ (C2-motif) at the C-terminal, which is partly responsible for repression of transcription (Jin et al., 2000). Similar to AtMYB4, MdMYB11 also contains this conserved motif, which may contribute to the observed repression of expression of both *C4H* and *DFR* in transgenic tobacco transgenic lines carrying *MdMYB11*. As MdMYB11 consists of a putative zinc-finger domain CFSCSLGIQDAKNCSC at its C-terminal (Kranz et al., 1998), this may explain its interaction with other regulatory proteins and its involvement in controlling transcription of target genes. In addition, Zhao et al. (2007) have reported that the MYB4 contains a MYB binding site motif A(A/C)C(A/T)A(A/C)C that may involve in autoregulatory and interaction with other MYB TFs. Thus, future studies must be conducted to determine whether such a MYB binding site motif in promoter regions of such flavonoid structural genes as *UFGT* may interact with MdMYB11 and activate transcription of these genes in apple.

AtMYB32 is a member of the R2R3 *MYB* gene family in Arabidopsis, and it is closely related to *AtMYB4* (Preston et al., 2004). Like *AtMYB4, AtMYB32* also represses transcription of the *C4H* gene (Preston et al., 2004). Changes in levels of expression of *AtMYB32* and *AtMYB4* can influence pollen development by changing the flux along the phenylpropanoid pathway, and influencing the composition of the pollen wall (Preston et al., 2004). For example, overexpression of *AtMYB4* in Arabidopsis transgenic lines has contributed to the recovery of lines with abnormal pollen grains, that are either partially or completely devoid of cellular contents (Preston et al., 2004). In this study, transgenic tobacco lines expressing *MdMYB11* have developed flowers with long styles, thus resulting in stigmas positioned above anthers. This suggests that *MdMYB11* is also involved in the regulation of structural genes involved in the development of pistils. Similar phenomenon was observed in MdMYB11 homolog, AmMYB308. Stigma protruded above the corolla when overexpression of *AmMYB308* in tobacco; however, flowers of *AmMYB308*-tobacco lines were reduced in size and in anthocyanin accumulation, unlike *MdMYB11*.

Taken together, this study clearly demonstrates that *MdMYB11* plays multiple important roles in plant growth and development. More specifically, overexpression of *MdMYB11* enhances accumulation of anthocyanins in apple.

TABLES

Table 3.1 List of primer sequences for real-time PCR

Genus	Gene name	Gene identifier	Forward primer	Reverse primer
Malus	*MdMYB11*		ggcaagagttgcaggttgag	gtcgcttgatgtgtgtgttcc
	*M*sp.*CHS[a]*	X68977	tcaagcctattgggatttcg	cagctgacttcctcctcacc
	*M*sp.*CHI[a]*	X68978	gatatcgaagccggaaatga	tgttgactcacgccaacaat
	MdF3H	AF117270	acaccaaatatggctcctgc	tttcgttgctgaagtcgttg
	MdFLS	AF119095	aatgggagtggagtctgtgg	agttggagctggcctcagta
	MdDFR	AF117268	aaggccgttacatttgttcg	gcccttgaactttgtgggta
	MdUFGT	AF117267	agctccactcggaacttcaa	aacccgccctaaatatgtcc
	MdANS	AF117269	caatttggcctcaaacacct	tcaacaccaagtgcaagctc
	MdANR	DQ099803	gttgcaacccctgtcaactt	cacgaccaaacctgttcctt
	MdLAR1	DQ139836	acaacacccacccttctgag	tgcagcaagggctagtaggt
	MdActin	DQ822466	ctacaaagtcatcgtccagacat	tgggatgacatggagaagatt
Nicotiana	*NbActin*	AY179605	aatgatcggaatggaagctg	tggtaccaccactgaggaca
	NtCHI	AB213651	gaaatcctccgatccagtga	caacgttgacaacatcaggc
	NbCHS	EF421432	agaaaagccttgtggaagca	acttggtccaaaattgcagg
	NtF3H	AB289450	acagggtgaagtggtccaag	ccttggttaaggcctccttc
	NtF3'H	AB289449	tccaagaatactggcccaag	ctcacaactctcggatgcaa
	NtFLS	AB289451	gaacttgaagggaaaagggg	tccctgtaggagggaggatt
	NbDFR1	EF421431	tcccatcatgcgatcatcta	atggcttctttgtcacgtcc
	NtLAR	AM827419	tcaaggtcctttacgccatc	acgaacctgcttctctttgg
	NtANS	AB289447	tggcgttgaagctcatactg	tttcaagggtgtccccaata
	NtUFGT	FG627024	gagtgcattggatgcctttt	ccagctccattaggtccttg
	NtANR1	AM791704	catttgactttcccaaacgc	attgggcttttgagttgtgc
	NtANR2	DW003895	tgttcccacttgggatgata	tgcacctatactctgttagtggc
	NtC4H	AB236952	ccaggagtgcaagtgactga	accaccaagcgttaaccaag

Table 3.2 Determination of flavonoid contents in transgenic and wild-type tobacco flowers by LC-MS-MS

| | Flavonoid (μg/g) Hydroxylation of B-ring | | Anthocyanidin (ng/g) | Proanthocyanidin (ng/g) | |
| | 4' | 3',4' | | | |
Flower	Kaempferol	Quercetin	Cyanidin	Catechin	Epicatechin
WT	60.57	71.80	1722	4.80	6.47
T1	53.93	88.83	3060	5.60	8.60
T5	73.50	136.67	7707	6.20	29.13
T8	79.27	114.67	4513	6.93	18.93

Kaempferol, quercetin, cyanidin, catechin, and epicatechin were used as standards. All data correspond to mean values of the three biological replicates.

Supplemental Table S3.1 The list of primers for primer walking sequencing procedure to cover genomic sequence of *MdMYB11*

Primer	Sequences (5′ to 3′)
GWA1	agcgcctcatcgaccacatc
GWA2	atcgttcttcgccgtcgtcc
GWA3	ggatgaatatacgtaccggc

Figure 3.1 Schematic diagram of pBI121 carrying *MdMYB11*
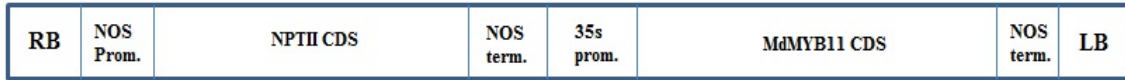
The coding sequence (CDS) of MdMYB11 was placed after CaMV35s promoter. Kanamycin-resistant gene (*NptII*) was used as a plant selection gene. The diagram was not to actual scale.

Figure 3.2 Genomic organization of *MdMYB11*
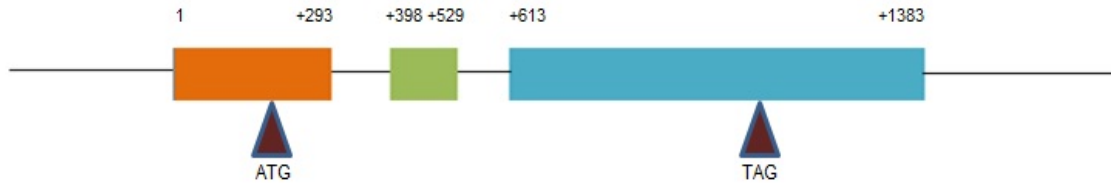
The exons are shown in colored boxes and introns are shown as lines. Nucleotide positions of the coding sequence are labeled above the boxes. The start and stop codons of the coding sequence are indicated with triangles.

Figure 3.3 Nucleotide sequence of the *MdMYB11* gene encoding 310 amino acids

```
                        tcttgcttcttatctctttcacctcttcaaacaacacacca
                        -----------------------------------------
caaactctatctctctctctctctctctctctctctctctctctctatatatatatatatat
------------------------------------------------------------
atatacctatacacacagagagttactgttgtataatatctgatccagaagaagaaacag
------------------------------------------------------------

ATGGGGAGAGCACCTTGTTGTGAGAAATCTCATACCAACAAAGGGGCATGGACGAAGGAG
 M  G  R  A  P  C  C  E  K  S  H  T  N  K  G  A  W  T  K  E

GAGGACCAGCGCCTCATCGACCACATCCGCCAGCACGGCGAGGGCTGCTGGCGCTCCCTC
 E  D  Q  R  L  I  D  H  I  R  Q  H  G  E  G  C  W  R  S  L

CCTAAAGCCGCCGGGTTGCTCAGGTGCGGCAAGAGTTGCAGGTTGAGATGGATAAACTAC
 P  K  A  A  G  L  L  R  C  G  K  S  C  R  L  R  W  I  N  Y

CTCCGCCCTGACCTCAAGCGCGGCAATTTCACACAAGAAGAAGATGAACTCATCATCAAG
 L  R  P  D  L  K  R  G  N  F  T  Q  E  E  D  E  L  I  I  K

CTTCATAGCTTGCTTGGAAACAAATGGTCATTGATTGCGGGCCGATTGCCGGGAAGAACC
 L  H  S  L  L  G  N  K  W  S  L  I  A  G  R  L  P  G  R  T

GACAACGAGATCAAAAACTACTGGAACACACACATCAAGCGAAAGCTCATCAGCCGCGGC
 D  N  E  I  K  N  Y  W  N  T  H  I  K  R  K  L  I  S  R  G

CTCGACCCTCAAACCCACCGCCCGCTTAACCAAACCACAACCGCCGCTGCTGCCGCCACG
 L  D  P  Q  T  H  R  P  L  N  Q  T  T  T  A  A  A  A  T

CCCGCCTCTCGCTTGGACCTGAGAAATCGTTCTTCGCCGTCGTCCGCGGTATTTGATCAC
 P  A  S  R  L  D  L  R  N  R  S  S  P  S  S  A  V  F  D  H

AAAACCATCAAAAACAACAAATTTGAATTGTTGAAGCACCCCAAGATGGAGCATGAGTAC
 K  T  I  K  N  N  K  F  E  L  L  K  H  P  K  M  E  H  E  Y

TACAATTACAATATAGAATCGGAGGCCAATTGCAGCACCACTACCGGCAGCGGCACCACA
 Y  N  Y  N  I  E  S  E  A  N  C  S  T  T  T  G  S  G  T  T

ACCGATGAAGACCAAAAACAACAGAACAAGTACAAGTGTTCTGATCTCAACTTGGATCTT
 T  D  E  D  Q  K  Q  Q  N  K  Y  K  C  S  D  L  N  L  D  L

TCAATTGGGCTAGAGCCGTTTCAGTCCGAGCAAACTCGGGCATCGTCTGGGAACTCGGCC
 S  I  G  L  E  P  F  Q  S  E  Q  T  R  A  S  S  G  N  S  A

GAATCAAGACTGCAGCGGATCATAGCTCCTTCTAATAGTAATAATAATCATCAGTTTTTT
 E  S  R  L  Q  R  I  I  A  P  S  N  S  N  N  N  H  Q  F  F

GGGAAAGTGCAGTCGACTCAGGCGGTGGCGGTGGCTCAGGCGGTGTGTGTGTGCTGCCAG
 G  K  V  Q  S  T  Q  A  V  A  V  A  Q  A  V  C  V  C  C  Q

GTTGGATTTCAAAGCAGTGAGGCATGCAGCAATTGTCAGTGCACCAGTTACAATGGCTTC
 V  G  F  Q  S  S  E  A  C  S  N  C  Q  C  T  S  Y  N  G  F

TACAGATTTCACAGACCTGCTTTGAATCCATAGcaccattttattataattattttcttt
 Y  R  F  H  R  P  A  L  N  P  *  ---------------------------

tttctcttcttaatacacataagttaataccttcactttcgaatttattactttctggat
------------------------------------------------------------
cttgtattgtacg
-------------
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Untranslated regions are indicated with lowercase letters.

Figure 3.4 Alignment of deduced amino acid sequences of MdMYB11 with other R2R3 MYB transcription factors

```
                                    R2 repeat                                                    R3 repeat

MdMYB11   KGAWTKEEDQRLIDHIRQHGEGCWRSLPKAAGLLRCGKSCRLRWINYLRPDLKRGNFTQEEDELIIKLHSLLGNKWSLIAGRLPGRTDNEIKNYWN
AtMYB6    KGAWTKEEDQRLVDYIRNHGEGCWRSLPKSAGLLRCGKSCRLRWINYLRPDLKRGNFTDDEDQIIIKLHSLLGNKWSLIAGRLPGRTDNEIKNYWN
AtMYB32   KGAWTKEEDDKLISYIKAHGEGCWRSLPRSAGLQRCGKSCRLRWINYLRPDLKRGNFTLEEDDLIIKLHSLLGNKWSLIATRLPGRTDNEIKNYWN
AtMYB7    KGAWTKEEDERLVSYIKSHGEGCWRSLPRAAGLLRCGKSCRLRWINYLRPDLKRGNFTHDEDELIIKLHSLLGNKWSLIAARLPGRTDNEIKNYWN
ZmMYB31   KGAWTKEEDERLVAHIRAHGEGCWRSLPKAAGLLRCGKSCRLRWINYLRPDLKRGNFTEEEDELIVKLHSVLGNKWSLIAGRLPGRTDNEIKNYWN
FaMYB1    KGAWSIQEDQKLIDYIQKHGEGCWNSLPKAAGLRRCGKSCRLRWINYLRPDLKRGSFGEDEEDLIIRLHKLLGNRWSLIAGRLPGRTDNEVKNYWN
AtMYB11   KGRWTAEEDRTLSDYIQSNGEGSWRSLPKNAGLKRCGKSCRLRWINYLRSDIKRGNITPEEEDVIVKLHSTLGTRWSTIASNLPGRTDNEIKNYWN
PhMYBAN2  KGAWTEEEDLLLRECIDKYGEGKWHLVPVRAGLNRCRKSCRLRWLNYLKPSIKRGKLSSDEVDLLLRLHRLLGNRWSLIAGRLPGRTANDVKNYWN
AtPAP1    KGAWTTEEDSLLRQCINKYGEGKWHQVPVRAGLNRCRKSCRLRWLNYLKPSIKRGKLSSDEVDLLLRLHRLLGNRWSLIAGRLPGRTANDVKNYWN
VlMYBA1-1 KGAWIQEEDVLLRKCIEKYGEGKWHLVPLRAGLNRCRKSCRLRWLNYLKPDIKRGEFALDEVDLMIRLHNLLGNRWSLIAGRLPGRTANDVKNYWH
GMYB10    KGAWTAEEDMLLKNCIERYGEGKWHLVPLKAGLNRCRKSCRLRWLNYLRPNIKRGDFGEDEIDLIIRLHKLLGNRWSLIAGRIPGRTANDVKNWWN
MdMYB1    KGAWTREEDNLLRQCVEIHGEGKWNQVSYKAGLNRCRKSCRQRWLNYLKPNIKRGDFKEDEVDLIIRLHRLLGNRWSLIARRLPGRTANAVKNYWN
MdMYBA    KGAWTREEDNLLRQCVEIHGEGKWNQVSYKAGLNRCRKSCRQRWLNYLKPNIKRGDFKEDEVDLIIRLHRLLGNRWSLIARRLPGRTANAVKNYWN
MdMYB10   KGAWTREEDNLLRQCVEIHGEGKWNQVSYKAGLNRCRKSCRLRWLNYLKPNIKRGDFKEDEVDLIIRLHRLLGNRWSLIARRLPGRTANAVKNYWN
FaMYB10   KGAWTKEEDELLKQFIEIHGEGKWHHVPLKSGLNRCRKSCRLRWLNYLKPNIKRGEFAEDEVDLIIRLHKLLGNRWSLIAGRLPGRTANDVKNYWN
VvMYBCs1  RGPWTPEEDELLANYVKREGEGRWRTLPKRAGLLRCGKSCRLRWMNYLRPSVKRGQIAPDEEDLILKLHRLLGNRWSLIAGRIPGRTDNEIKNYWN
ZmMYBC1   RGAWTSKEDDALAAYVKAHGEGKWREVPQKAGLRRCGKSCRLRWLNYLRPNIRRGNISYDEEDLIIRLHRLLGNRWSLIAGRLPGRTDNEIKNYWN
AtMYB3    KGPWTMEEDLILINYIANHGDGVWNSLAKSAGLKRTGKSCRLRWLNYLRPDVRRGNITPEEQLIIMELHAKWGNRWSKIAKHLPGRTDNEIKNFCR
AtMYB4    KGLWSPEEDEKLLNYITRHGHGCWSSVPKLAGLQRCGKSCRLRWINYLRPDLKRGAFSQDEESLIIELHAALGNRWSQIATRLPGRTDNEIKNFWN

          :* *  :** *    :     *.* *  :.  :** * **** **:***:. ::** : :*  :::.** *.:** **  :***** * :**::.
```

The CLUSTALW2 program was used to align peptide sequences. The sequences were previously reported and retrieved from the GenBank databases [*Malus* × *domestica* MdMYB1 (ABK58136), MdMYBA (AB279598), MdMYB10 (ABB84754); *Arabidopsis thaliana* AtMYB3 (BAA21618), AtMYB4 (BAA21619), AtMYB6 (NP_192684), AtMYB7 (NP_179263), AtMYB32 (NP_195225, AtMYB11 (NM_116126), AtPAP1 (AF325123); *Fragaria ananasa* FaMYB1 (AF401220), FaMYB10 (EU155162); *Vitis labruscana* VlMYBA1-1 (AB073010); *Gerbera hybrida* GMYB10 (AJ554700); *Petunia hybrida* PhMYBAN2 (AF146702); *Zea mays* ZmMYBC1 (AF320614)); ZmMYB31 (NP_001105949); *Vitis vinifera* VvMYBCs1 (AY555190)]. Conserved sequences with 100%, 80%, and 60% homology are marked with asterisks, two dots, and one dot, respectively.

Figure 3.5 Conserved motifs C1 and C2 at C-termini of deduced amino acid sequences of MdMYB11 with other R2R3 MYB transcription factors

```
                  ┌─────────────────┐
                  │LlsrGIDPxᵀ/ₛHRx¹/ₗ│── C1-motif ──────────────────────┐
                  └─────────────────┘
                      ⇩                                                 ⇩
MdMYB11    THIKRKLISRG----------------------------------------LDPQTHRPLNQTTTAAAAA
AtMYB6     THIKRKLLSHG----------------------------------------IDPQTHRQINESKTVSSQV
AtMYB32    THVKRKLLRKG----------------------------------------IDPATHRPINETKTSQDSS
AtMYB7     THIKRKLLSKG----------------------------------------IDPATHRGINEAKIS----
ZmMYB31    THIRRKLLSRG----------------------------------------IDPVTHRPVTEHHASNITI
FaMYB1     SHLKKKILKTG----------------------------------------TTLRPNKPHENNHAP----
AtMYB11    SHLSRKLHGYFRKPTVANTVENAPPPPKRRPGRTSRSAMKPKFILNPKNHKTPNSFKANKSDI
PhMYBAN2   THLRKKLIAP-------------------------------HDQKQESK----NKAVKITENNI
AtPAP1     THLSKKHEPC-------------------------------CKIKMKKRDITPIPTTPALKNNV
VlMYBA1-1  GHHLKKKV-----------------------------QFQEEGR-DKPQTHSKTKA
GMYB10     THLRSRHQ-----------------------------QQQKVHQEDELSQDTTVAI
MdMYB1     TRLRIDSR----------------------------MKTVKNKSQEMRKTN------V
MdMYBA     TRLRIDSR----------------------------MKTVKNKSQEMRKTN------V
MdMYB10    TRLRIDSR----------------------------MKTVKNKSQEMRETN------V
FaMYB10    TYQRKKDQ---------------------------KTASYAKKLKVKPRENTIAYTI
VVMYBCs1   THLSKKLISQGI-----------------DPRTHKPLNPKPNPSPDVNAPVSKSI
ZmMYBC1    STLGRRAGAG-------------------AGAGGSWVVVAPDTGSHATPAATSGA
AtMYB3     TRIQKYIKQS-------------------------------------------
AtMYB4     SCLKKKLRRKG-------------------------------IDPTTHKPLITNELQSLNV


MdMYB11    TPASRLDLRNRSSPSSAVFDHKTIKNNKFELLKHPKMEHEYYNYNIESEANCSTTTGSGTTTD
AtMYB6     VVPIQNDAVEYSFSNLAVKPKTENSSD---------------------NGASTSGTTTD
AtMYB32    -----------DSSKTEDPLVKILSFGP-----------QLEKIANFGDER---------IQK
AtMYB7     -----------DLKKTKDQIVKDVSFVT----------KFEETDKSGDQKQNKYIRNGLVCK
ZmMYB31    SFETEVAAAARDDKKGAVFRLEDEEEEE-----------RNKATMVVGRDRQSQSHSHSHPAG
FaMYB1     -----------NNKLVKLFNKMDDEVVD--------------------------EV
AtMYB11    VLPTTTIENGEGDKEDALMVLSSSSLSG-----AEEPGLGPCGYGDDGDCNPSINGDDGALCL
PhMYBAN2   IKPRPRTFSR----PAMNNFPCWNGKSC--------------NKNTIDKNEGDTEIIKFSDE
AtPAP1     YKPRPRSFTVNNDCNHLNAPPKVDVNPP--------------CLGLNINNVCDNSIIYNKDK
VlMYBA1-1  IKPHPHKFSKALPKFELKTTAVDTFDTQ--------------VSTSSKPSSTS-PQPNDDII
GMYB10     IKPQPRTFSKTLNWFGNRQSVKDHVDIN--------------IIKSSSASDTNNISAPPELI
MdMYB1     IRPQPQKFNR---SSYYLSSKEPILDHI---------------QSAEDLSTPPQTSSSTKNGN
MdMYBA     IRPQPQKFNR---SSYYLSSKEPILDHI---------------QSAEDLSTPPQTSSSTKNGN
MdMYB10    IRPQPQKFNR---SSYYLSSKEPILDHI---------------QSAEDLSTPPQTSSSTKNGN
FaMYB10    VRPRPRTFIKRFNFTERYANIEHNHSEV---------------SYTSSLPTEPPQTLQLENVT
VVMYBCs1   PNANPNPSSSRVGEIGSNHEVKEIESNE--------------NHKEPPNLDQYHSPLAADSN
ZmMYBC1    CETGQNSAAHRADPDSAGTTTTSAAAVW--------------APKAVRCTGGLFFFHRDTTP
AtMYB3     -DVTTTSSVGSHHSSEIN--------------------------DQAASTSSHNVFCTQDQA
AtMYB4     IDQKLTSSEVVKSTGSINNLHDQSMVVSSQQGPWWFPANTTTTNQNSAFCFSSSNTTTVSDQI

                    ┌──────────────┐
                    │pdLNLᴰ/ₑLxiᴳ/ₛ│── C2-motif
                    └──────────────┘
MdMYB11    EDQKQQNKYKC------SDLNLDLSIGL----------------------------EPFQSEP
AtMYB6     EDLRQNGECYYSDNSGHIKLNLDLTLGF----------------------------GSWSGRI
AtMYB32    RVEYSVVEERC------LDLNLELRISP----------------------------PWQDKLH
AtMYB7     EERVVVEEKIG------PDLNLELRISP----------------------------PWQNQRE
ZmMYB31    EWGQGKRPLKC------PDLNLDLCISP----------------------------PCQEEEE
FaMYB1     SSADSAAGCLV------PELNLDLTLSI----------------------------KTSTGMA
AtMYB11    NDDIFDSCFLL------DDSHAVHVSSC----------------------------ESNNVKN
PhMYBAN2   KQKPEES--ID------DGLQWWANLLA----------------------------NNIEIEE
AtPAP1     KKDQLVNNLID------GDNMWLEKFLE----------------------------ESQEVDI
VlMYBA1-1  WWESLLAEHAQ------MDQETDFSASG----------------------------EMLIASL
GMYB10     ASPKILDDAIN------ECRQKLFDGDE----------------------------KEVDIDG
MdMYB1     DWWETLLE-------GEDTFERAAYPSI----------------------------ELEEELF
MdMYBA     DWWETLLE-------GEDTFERAAYPSI----------------------------ELEEELF
MdMYB10    DWWETLLE-------GEDTFERAAYPSI----------------------------ELEEELF
FaMYB10    DWWKDFSEDST------ESIDRTMCSGL----------------------------GLEDHDF
VVMYBCs1   ENWQSADGLVT------GLQSTHGTSND----------------------------DEDDIGF
ZmMYBC1    AHAGETATPMA------GGGGGGGGEAG----------------------------SSDDCSS
AtMYB3     METYSPTPTSY------QHTNMEFNYG-----------------------------NYSAAAV
AtMYB4     VSLISSMSTSS------SPTPMTSNFSPAPNNWEQLNYCNTVPSQSNSIFSAFFGNQYTEASQ
```

C1 and C2 motif are boxed, and placed above their location wherein the protein sequences are indicated as gray color.

Figure 3.6 The marker MdMYB11-SSR was anchored onto linkage group 15 of the apple genetic map

GR15

Position	Marker
0.0	CN862321
5.6	CTG1076069
6.5	CH02e12
7.0	CTG1073209
12.7	Z71981-SSR-1
13.2	CTG1060287 CTG1074058
13.7	CN939907
16.5	BACSSR51
20.4	CH03b06
22.9	CH02d10b
28.6	CO414802-156
32.0	CTG1069215
34.7	Hi15e04
36.6	CN908484
39.1	BACSSR108
41.7	CN907414-1
45.1	CTG1075196
49.3	CTG1069128
50.9	CTG1071157
51.7	CH04a06
54.1	CN911135
54.6	CN911379
59.0	CTG1062468
59.7	CTG1063001
63.0	CH01c08
67.4	CTG1071737
71.0	CTG1064315
72.0	CTG1063606
80.7	CTG1068739
85.0	CN948659
89.5	CH02d11
90.6	CH02b11-2-2
93.1	CH03h06
94.2	CN908658
96.1	CTG1070919
98.0	CTG1073847
103.2	CTG1064914
103.9	CN913419
104.8	Hi09f01
106.7	CTG1070125
107.6	CTG1070264
121.7	KB08-H5-490
123.2	CN888692
123.3	CTG1060546
124.1	CH02c09
127.1	MdMYB11-SSR
127.9	CTG1057509
128.6	CN884916-1
129.2	CTG1057511
136.1	KB02-F5
143.1	BACSSR153-3

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Figure 3.7 Expression profiles of *MdMYB11* in various tissues of apple cvs. Red Delicious and Golden Delicious using qRT-PCR
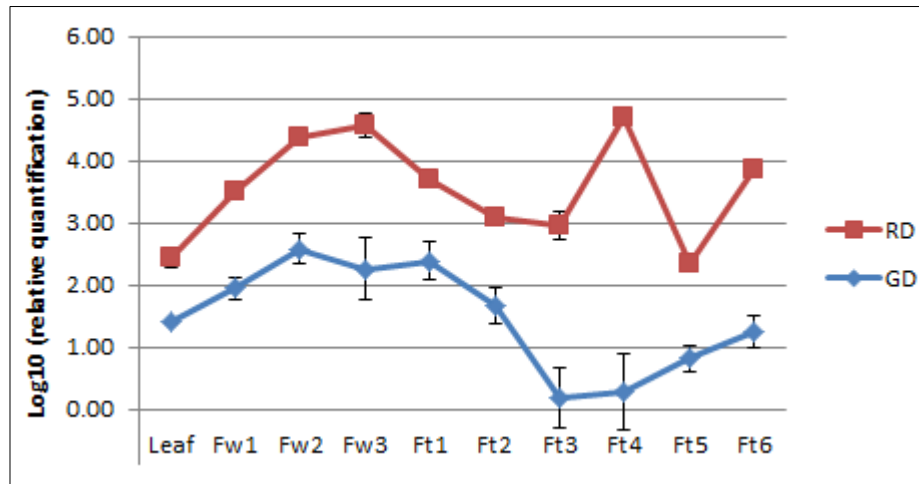
Abbreviations are listed as followings: Fw1: Flower buds at the pink stage; Fw2: flower buds at the balloon stage; Fw3: flowers at full bloom; Ft1: 9 DAP; Ft2: 16 DAP; Ft3: 44 DAP; Ft4: 104 DAP; Ft5: 145 DAP; Ft6: 166 DAP; RD: 'Red delicious'; GD: 'Golden Delicious'. Normalization was made to the expression of actin gene and values are the average of three technical replicates.

Figure 3.8 Copy number of the transgene in transgenic tobaccos from Southern blotting
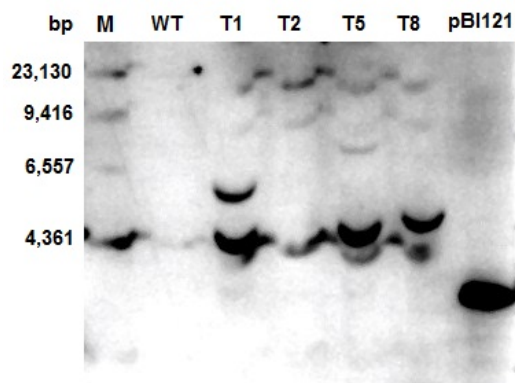
The transgene copy number of transgenic line T1, T2, T5, and T8 was determined by using Southern blotting. Non-transformed tobacco (WT) and the expression vector (pBI121) were served as negative and positive controls, respectively.

Figure 3.9 Flowers of wild-type and transgenic lines carrying *MdMYB11* of tobacco
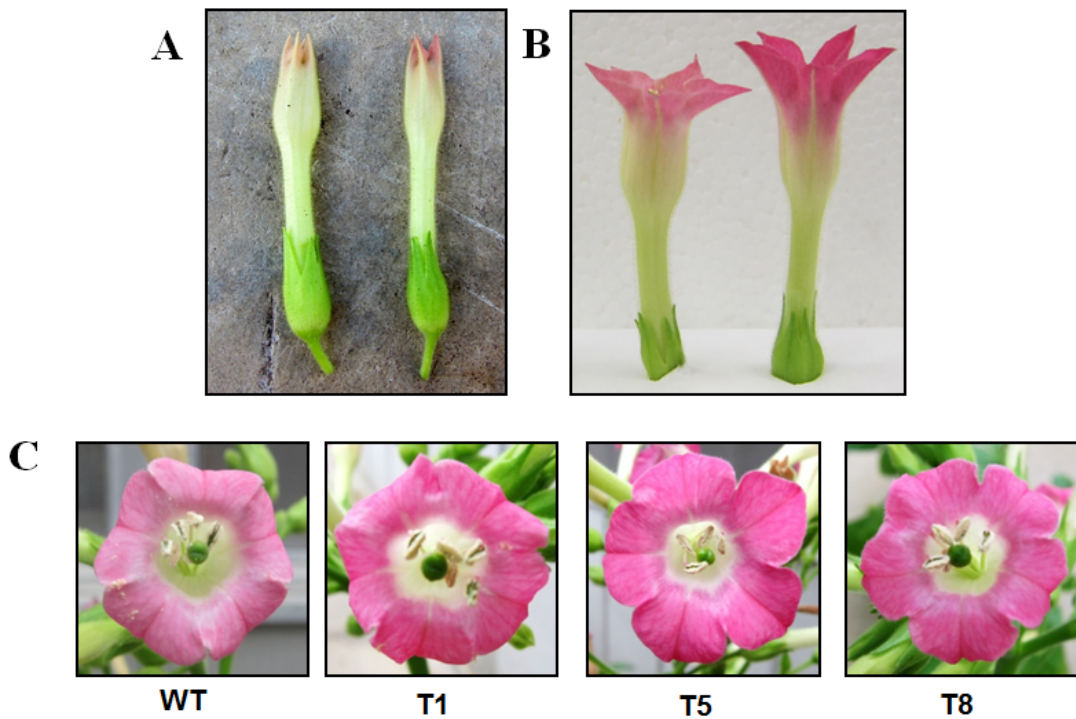
A. Flowers of wild-type (left) and transgenic line T5 (right) at stage I; B. Flowers at stage II of wild type (left) and transgenic line T5 (right); C. Flowers at full bloom stage of WT and transgenic lines T1, T5, and T8.

Figure 3.10 Expression profiles of flavonoid-related structural biosynthetic genes in flowers of T2 transgenic tobacco lines carrying the *MdMYB11* transgene using qRT-PCR
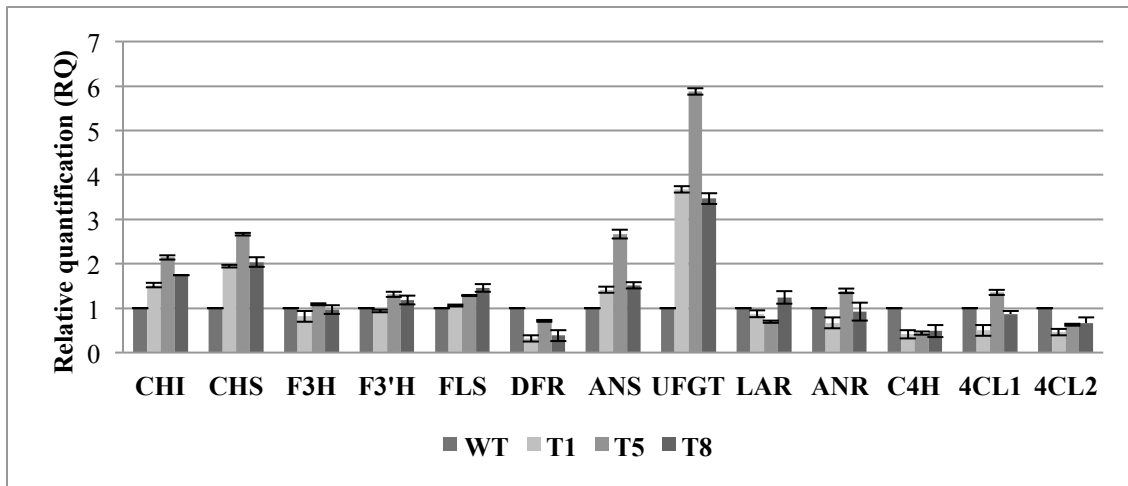
mRNA transcripts in transgenic flowers were quantified relative to those present in wild-type flowers. WT: wild type; CHI: chalcone isomerase; CHS: chalcone synthase; F3H: flavonoid 3-hydroxylase; F3'H: flavonoid 3'-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol reductase; LAR: leucoanthocyanidin reductase; ANS: anthocyanidin synthase; UFGT: UDP-glucose: flavonoid 3-o-glucosyltransferase; ANR: anthocyanidin reductase; C4H: cinnamate-4-hydroxylase; 4CL1/2: 4-coumaroyl-CoA ligase. Normalization was made to the expression of *actin* gene and values are the average of three technical replicates.

Figure 3.11 Flowers of transgenic lines carrying *MdMYB11* and wild-type tobacco
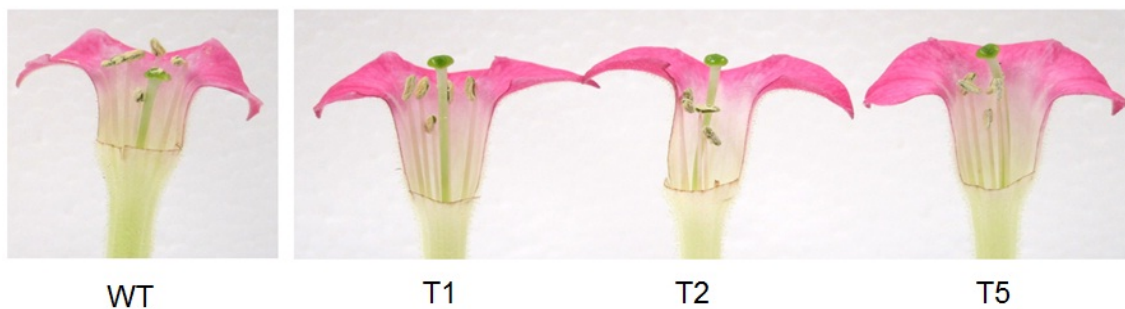
Styles of transgenic lines were longer than those of wild-type resulting in stigmas that are positioned above anthers.

Supplemental Figure S3.1 SSR marker screening of apple parents 'Co-op 16' and 'Co-op 17'
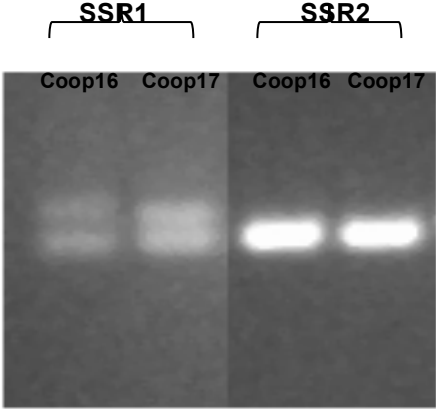
Supplemental Figure S3.2 Segregation of DNA bands of the SSR marker for the
MdMYB11 **gene**
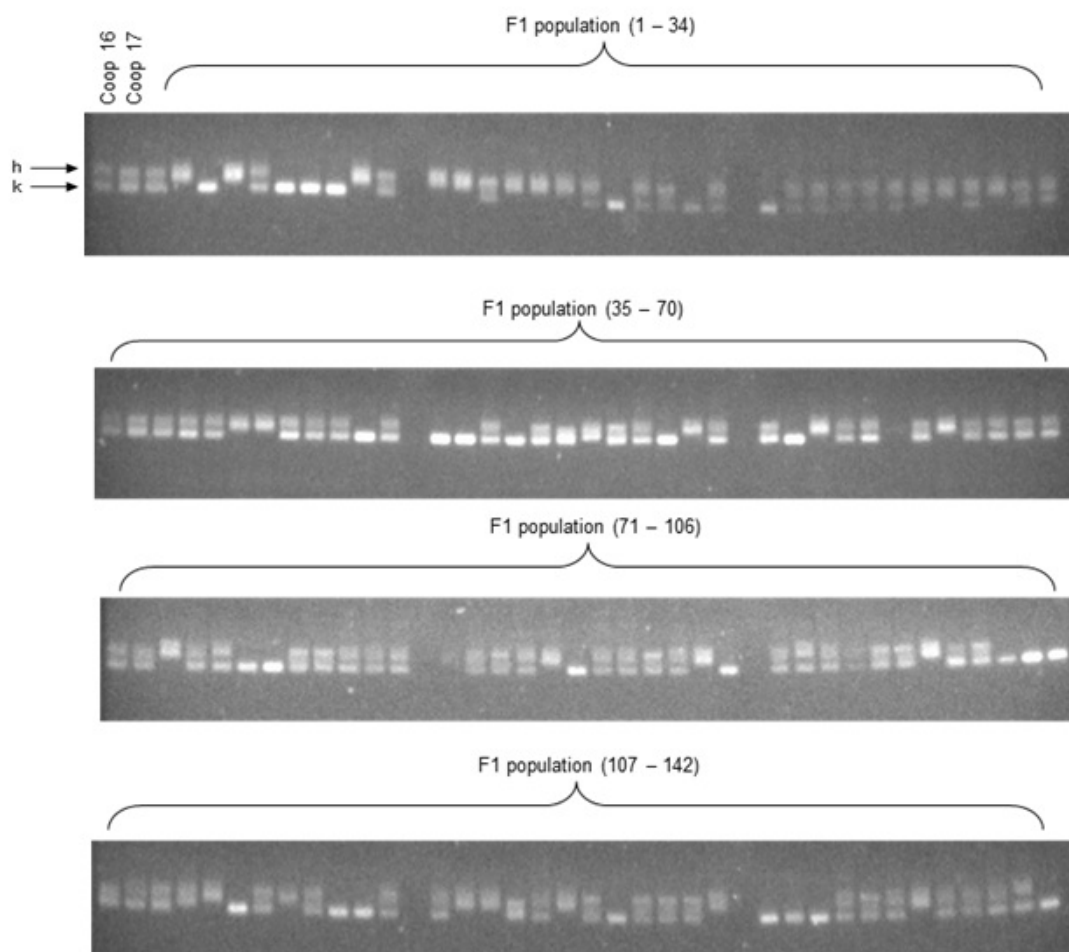
Parents ('Co-op 16' and 'Co-op 17') and 142 individuals of the F1 population of 'Co-op 16' x 'Co-op 17' F1 were analyzed. Arrows indicate segregated DNA bands wherein 'h' represents the upper band and 'k' represents the lower band.

CHAPTER 4

GLOBAL GENE EXPRESSION ANALYSIS OF APPLE FRUIT IDENTIFIES LIGHT-INDUCED GENES REGULATING RED COLOR PIGMENTATION

4.1 Abstract

Light is an important environmental stimulus controlling various fundamental metabolic activities in plants such as those of photosynthesis and photorespiration, among others. It is also involved in the regulation of color pigmentation of fruit skin. Although the mechanism is not fully understood, it has been shown that light can regulate expression of genes in the anthocyanin biosynthesis pathway by inducing transcription factors (TFs). Moreover, expression of genes encoding enzymes involved in this pathway may be coordinately regulated by multiple TFs. In this study, fruit on trees of apple cv. Red Delicious were covered with paper bags during the early stages of fruit development and then removed prior to maturation. Comparisons of global gene expression profiles of fruit covered with paper bags (dark-grown treatment) and those subjected to 14 h light treatment following removal of paper bags were investigated using an apple microarray consisting of 40,000 sequences. Expression profiles were investigated over three time points, with one week intervals, during fruit development using three biological replicates for each time point along with a dye swap. Overall, 815 genes with expression values greater than two-fold were found to be modulated by light treatment. Putative light-induced protein products were classified into 19 categories with highest scores in primary metabolism (17%) and transcription (12%). Based on the *Arabidopsis* gene ontology annotation, 18 genes were identified as TFs. To further confirm expression patterns of flavonoid-related genes, they were subjected to quantitative RT-PCR (qRT-PCR) using fruit of red-skinned apple cv. Red Delicious and yellow-skinned apple cv. Golden Delicious. Of these, two genes showed higher levels of expression in 'Red Delicious' than in 'Golden Delicious', and were likely involved in the regulation of fruit red color pigmentation.

Keywords: apple; light; coloration; anthocyanin; transcription factor; microarray analysis

4.2 Introduction

Apple (*Malus × domestica* Borkh.) is an important fruit crop that is widely grown all over the world. With increasing apple consumption, apple cultivars with highly desirable fruit quality characters such as fruit color, taste, flavor, and texture, among others. Among these characters, fruit coloration is a major important determinant of fruit quality as consumers have been reported to perceive red skin coloration to be correlated with better taste and flavor (King and Cliff, 2002).

Red color of apple fruit is derived from accumulation of anthocyanin pigments. It has been well known that coloration is genetically regulated by structurally genes involved in the anthocyanin biosynthesis pathway (Davies and Schwinn, 2003); moreover, these genes are transcriptionally controlled by various transcription factors (TFs) including MYB, bHLH, WD40, WRKY, bZIP, and MADS-box (Davies and Schwinn, 2003; Kubo et al., 1999; Martin et al., 2001; Ramsay and Glover, 2005).

The anthocyanin biosynthesis pathway has been well characterized genetically and biochemically (Grotewold, 2006; Holton and Cornish, 1995; Lancaster, 1992). The regulation of anthocyanin biosynthesis is dependent on critical stages of apple fruit development, known to occur at two peaks. The first peak is at the early developmental stage in both red and non-red cultivars; however, this stage is not economically important (Lancaster, 1992). The second peak occurs at the mature fruit stage, and it is markedly influence by environmental factors, including temperature and light. It is well documented that environmental conditions such as light impact color development of apple fruit by inducing regulatory genes to act upstream of the anthocyanin biosynthesis cascade (Davies and Schwinn, 2003). Several studies have demonstrated that genes in the anthocyanin biosynthetic pathway are upregulated upon light exposure (Ban et al., 2007; Dong et al, 1998; Kim et al., 2003; Ubi et al., 2006). Recently, MYB10/MYB1/MYBA have been reported to be critical TFs regulating several anthocyanin biosynthetic genes in apple (Ban et al., 2007; Espley et al., 2007; Takos et al., 2006). Moreover, expression of the *MdMYB1* gene is light inducible (Takos et al., 2006). In both apples and pears, the MYB10 gene has been mapped on linkage group 9 (LG9) of the apple genetic map; whereas, QTL analysis has indicated that a critical gene

regulating red coloration in pear cultivar Max Red Bartlett is mapped onto LG4 of the pear linkage map (Pierantoni et al., 2010). These findings have suggested that additional studies should be undertaken to better understand the mechanism and regulation of coloration as well as studying differential expression of genes regulated by light.

Previously, studies on light-influenced gene expression have been reported in *Arabiodopsis*, and these focused on the impact of high light on oxidative stress (Kimura et al., 2003; Rossel et al., 2002). Kimura et al. (2003) reported that genes involved in the biosynthesis of lignins and flavonoids were activated by high-light and resulted in increased accumulation of both lignins and anthocyanins in addition to scavenging enzymes of reactive oxygen species (ROS). Recently, global expression profiles have been undertaken in apple, but most have been focused on understanding fruit development (Lee et al., 2007; Janssen et al., 2008; Park et al., 2006; Soglio et al., 2009; Soria-Guerra et al., 2011) and aroma production (Schaffer et al., 2007; Seo and Kim, 2009). However, there are no studies on the influence of light on regulation of genes involved in the anthocyanin biosynthesis pathway. In this study, global gene expression profiles of fruit of red-skinned apple cv. Red Delicious was investigated during dark treatment and following exposure to 14 h of light treatment.

4.3 Materials and methods

4.3.1 Experimental design, fruit collection, and RNA isolation

Fruits (> 0.5 cm in size) of apple tree cv. Red Delicious, growing at the Pomology Research Center of the University of Illinois at Urbana-Champaign, were covered with brown paper bags for 97 days after pollination (DAP), 104 DAP, and 111 DAP. At each time point, bags were removed, and fruits were left on the tree for another period of 24 h, and exposed to 14 h of daylight. Then, at least six fruits were harvested from each of light-treated and untreated apples. Non-bagged fruits were used as a control group (Group 1). Dark-grown and 14 h-light-exposed fruits were designated as Groups 2 and 3, respectively. For each fruit, skin was peeled, using an apple peeler, immediately frozen in liquid nitrogen, and stored at -80 °C.

Frozen fruit skin tissues were ground, and total RNA was isolated according to a previously described protocol (Gasic et al., 2004). The quality of RNA was assessed by running samples on a formamide-agarose gel. RNA concentration was measured using an ND-1000 nandrop.

4.3.2 Microarray design

A 40,000 long-oligonucleotide apple microarray (Invitrogen) was developed as described by Soria-Guerra et al. (2011). Briefly, this microarray was developed using 40,000 sequences, along with positive and negative controls, obtained from 34 cDNA apple libraries constructed from both vegetative and reproductive tissues at different stages of development, varying genotypes, and under different biotic and abiotic stresses. The total number of probes include 74 apple seed cluster contigs, 35 seed cluster singlets, 13,582 cluster contigs, 6,369 cluster singlets, 16,966 singletons, and 2,374 novel clusters for a total of 39,400 probes, along with 50 control-positive, 50 control-negative, 150 control-distance, and 350 control-mismatch probes. These oligo probes were spotted onto ultragap coated slides (Corning) using a GeneMachine OmniGrid arrayer (Newport) in a total of 48 blocks.

4.3.3 Preparation of cDNA labeling with aminoallyl

Aminoallyl labeling of RNA was conducted following an established protocol (W.M. Keck Center, University of Illinois at Urbana-Champaign). Total DNaseI-treated RNA (15 μg) was reverse transcribed and aminoallyl labeled. The reaction was carried out overnight at 46 °C to a final volume of 30 μL and in the presence of 100 μM aminoallyl-dUTP, 2 μg oligo(dT)18-mer, 2 μg random hexamer (Invitrogen), 250 μM of each of dATP, dCTP, and dGTP, 150 μM dTTP, 20 units RNase inhibitor RNaseOUT (Invitrogen), and 10 μM dithiothreitol in the provided buffer. Samples were treated with 0.25 N NaOH, and 0.12 N EDTA for 15 min at 65 °C, and neutralized by 0.25 N HCl. Unincorporated aa-dUTP and free amines were removed using the Qiagen PCR Purification Kit (Qiagen). Samples were vacuum-dried using a speed vac system (AES2010, Savant) and coupled by adding 9 μl of either 3' Cy3 N-hydroxysuccinimide

ester (Cy3) or 3' Cy5 N-hydroxysuccinimide ester (Cy5) and incubated for 1 h in the dark at room temperature. The reaction was then purified to remove uncoupled dye with the Qiagen PCR Purification Kit (Qiagen). The labeled cDNA probe was quantified using Nanodrop. For each microarray slide, two probes were combined, and vacuum-dried for 1 h.

4.3.4 Microarray experimental design

The microarray experiments were conducted using a total of three slides for each time point, and each consisting of three biological replicates including a dye swap (Table 4.1).

4.3.5 Microarray hybridization and analysis

Slides were incubated in a prehybridization buffer [20% formamide, 6× SSC, 0.1% SDS and 5× Denhardt's solution, with 25 μg mL^{-1} tRNA (Sigma)] for 45 min at 42°C, washed five times in water and once in isopropanol, and spin-dried for 1 min. cDNA probes were dissolved in 42 μL of 1X hybridization solution (Ambion, Austin, TX), denatured at 95 °C for 2 min, and cooled down to 42°C. Hybridizations were carried out at 42°C for 16 h in the Maui chamber system (BioMicro systems, Salt Lake City, UT). Slides were placed in Coplin jars, washed along with gentle agitation in 1× SSC and 0.2% SDS at 42°C for 5 min, 0.1× SSC, 0.2% SDS at 25°C for 5 min, and twice in 0.1× SSC for 5 min, and immediately dried by centrifugation at 400g for 1 min.

Microarray slides were scanned using a Genepix 4000 B fluorescence reader (Axon Instruments Inc., Foster City, CA). Image files were analyzed using Genepix 3.0 image acquisition software adjusted for Cy3 and for Cy5. Non-homogeneous and aberrant spots were visually inspected and flagged. All data were standardized and normalized by the Lowess method using SAS software. Those genes with means of normalized log2 intensity ratio of ≥1 were identified as differentially expressed genes. Q-plot analysis was used to adjust p-values, and number of genes that were significantly different at p-value < 0.05 and ≥3 fold change were selected.

4.3.6 Functional classification

Functional classification of all differentially expressed genes was performed using database of the web site at the Munich Information Center from Protein Sequence (http://mips.gsf.de/proj/plant/jsf/athal/index.jsp).

4.3.7 Quantitative real-time PCR (qRT-PCR)

Total RNA (2 μg) from each sample was treated with DNase I (Invitrogen), and used for cDNA synthesis. The first-strand cDNA synthesis was performed with Oligo (dT) primer using a SuperScript III RT kit (Invitrogen) according to the manufacturer's instructions. Specific primers were designed to perform qRT-PCR and these are listed in Table 4.2.

The SYBR Green real-time PCR assay was carried out in a total volume of 15 μl, containing 7.5 μl of 2× SYBR Green I Master Mix (Applied Biosystems), 0.2 μM (each) specific primers, and 100 ng of template cDNA. The amplification program consisted of 1 cycle of 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. The fluorescent product was detected at the last step of each cycle. Following amplification, melting temperatures of PCR products were analyzed to determine specificity of these PCR products. Melting curves were obtained by slow heating at 0.5 °C /sec, from 60 °C to 90 °C, while continuously monitoring the fluorescence signal. A negative control without a cDNA template was run along with each analysis to normalize data and evaluate the overall specificity.

All reactions were carried out in 96-well plates in either a 7300 Real Time PCR System (Applied Biosystems) or in 384-well plates in a 7900 Real Time PCR System (Applied Biosystems). All experimental samples were run in duplicate. The *actin* gene was used as an internal control, and expression of the *actin* gene was used to normalize all data. Relative transcript levels of target genes were determined following normalization.

4.4 Results

4.4.1 Global gene expression profiles of apple fruit skin are modulated by light

A microarray analysis was conducted to evaluate expression profiles of genes in skin of fruit of the apple cultivar Red Delicious, collected in Weeks 1, 2, and 3 as modulated by light. Comparison of "treatment 3 (bagged fruit subjected to 14 h light) vs. treatment 2 (bagged fruit and dark grown)" was performed. Genes exhibiting changes in expression values that were higher than two-fold change were deemed as significant, selected at P value <0.05, and were subjected to Q plot analysis. Of 40,000 sequences, 815 sequences that demonstrated significant changes across all three time points (with three replications per time point) were selected. All 815 sequences that were modulated in response to light were grouped into seven clusters based on timing of gene expression (Figure 4.1). Cluster 1 consisted of 131 genes with changes observed at all time points. Cluster 2 consisted of 232 genes expressed in both Weeks 1 and 2. Cluster 3 consisted of 167 genes expressed in both Weeks 2 and 3. Cluster 4 consisted of 128 genes expressed in both Weeks 1 and 3. While, clusters 5, 6, and 7 consisted of genes that were differentially expressed only in Week 1 (41 genes), Week 2 (80 genes), or Week 3 (36 genes), respectively. These genes were annotated based on the *Arabidopsis* protein database (following the Munich Information Center for Protein Sequences [MIPS; http://mips.gsf.de/proj/plant/jsf/athal/index.jsp]), arranged by cluster, and summarized in Table 4.3.

All significant 815 modulated genes detected in the apple microarray were classified into 19 functional categories (Figure 4.2). Regardless of the timing of expression, most genes were involved in primary metabolism (17%), transcription (12%), and protein with binding function (11%), along with those of unknown function (20%) with no hits to any *Arabidopsis* proteins. When considering function of genes within each cluster, not all functional categories fell into a single cluster (Table 4.4). There were few genes involved in transposase and regulation of metabolism. Furthermore, it was interesting to note that only a single gene expressed at all three time points was involved in regulation of metabolism. Moreover, a single transposase gene was expressed in Weeks 2 and 3 (Cluster 3); while, another transposase gene was expressed only in Week 1 (Cluster 5). This indicated that these genes functioned differently, depending on the

developmental stage of the fruit; moreover, not all genes were functional at the same developmental stage of fruit development. Finally, those genes classified into the same functional category might be temporally regulated depending on the developmental stage of the fruit.

When bagged fruit was exposed to 14 h light treatment, numerous genes were differentially expressed in bagged fruit that were subjected to 14 h light exposure. Separated by timing of expression, 111 genes from Week 1, 408 from Week 2, and 307 from Week 3, exhibiting three-fold change in levels of expression were selected (Table 4.5). Overall, most of these genes (70%) were upregulated in all three time points. However, some genes (30%) were downregulated following exposure of fruit to light. Over all three time points, genes expressed in Week 2 were highly modulated by light.

4.4.2 Light-induced expression of flavonoid-related genes at different time points

RNA pools of peel of fruit of treatments 1 (never-bagged fruit and exposed to normal light conditions), 2 (bagged fruit and dark-grown throughout), and 3 (bags were removed for 14 h of light treatment) were prepared over three time points, each at one-week intervals (see Materials and Methods), and designated as Week 1, Week 2, and Week 3. cDNA was synthesized from each of these pools, and used for qRT-PCR analysis. To compare levels of gene expression under normal light-grown and 14 h light-grown conditions, relative gene expression levels of 'treatment 1 vs. treatment 2' and 'treatment 3 vs. treatment 2' were compared. Expression of flavonoid-related genes including *CHS*, *CHI*, *F3H*, *DFR*, *FLS*, *ANS*, *LAR*, and *UFGT* encoding chalcone synthase, chalcone isomerase, flavonol-3-hydroxylase, dihydroflavonol reductase, flavonol synthase, anthocyanidin synthase, leaucoanthocyanidin reductase, and UDP-glucose:flavonoid 3-O-glucosyltransferase, respectively, were monitored over the three time points (Figure 4.3). For fruit grown under normal light conditions (treatment 1), all genes were highly expressed in Week 1, but these dropped slightly in Week 2. Only *CHI*, *CHS*, *FLS*, *ANS*, and *UFGT* genes were upregulated in Week 3. This indicated that flavonoid-related genes were temporally expressed during fruit development, and their expression was highest at Week 1. For fruit grown in bags and then subjected to 14 h

light treatment (treatment 3), all flavonoid-related genes, except for *LAR* and *ANR*, were upregulated at Week 1, and these were even at higher levels than those of fruits grown under normal light conditions (treatment 1). At Week 2, only transcripts of *ANS*, *CHI*, and *FLS* remained high; whereas, at Week 3, transcripts of only *CHI*, *FLS*, and *UFGT* genes were upregulated.

Altogether, these findings suggested that light exposure significantly induced expression of flavonoid-related genes, even following short duration (14 h) of exposure.

4.4.3 Expression of genes linked to red color development are influenced by light

Structural genes, particularly those involved in flavonoid biosynthesis, were preferentially expressed following exposure of bagged fruit to 14 h light treatment. Although some genes were not highly expressed after 14 h of light exposure over all three weeks, they were significantly modulated at least during two time points. Based on microarray results, *CHS*, *FLS*, and *ANS/LDOX* genes were highly expressed, greater than three-fold change when bagged fruits were exposed to light. Moreover, these results were similar to those of qRT-PCR for the first two time points. In addition, expression of 18 genes encoding several TF families including MYB, bHLH, bZIP, and WD-40 proteins were also influenced by light. Selected apple ESTs along with their accession numbers, based on the putative TF family they belong to, *Arabidopsis* accession number with their description, global gene expression profiles, and validation of expressionby qRT-PCR are listed in Table 4.6. Expression profiles observed following microarray analysis were confirmed by qRT-PCR (Figure 4.4). Candidate genes encoding those TF families are likely responsible for fruit color pigmentation.

4.4.4 Expression profiles of putative regulatory genes linked to red coloration in apple

To further identify candidate genes linked to coloration of apple fruit, expression profiles of these candidate genes were evaluated in fruit of a red-skinned cultivar Red Delicious and a yellow-skinned cultivar Golden Delicious at five stages of development, from early fruitlet (9 DAP) to full ripening (145 DAP) (Figure 4.5). Most candidate

genes were expressed at steady state levels at all developmental stages and differentially expressed in both cultivars. However, two putative genes including EST45 and EST55 were expressed at significantly higher levels in fruit of 'Red Delicious' than in 'Golden Delicious'; moreover, their expression steadily increased during fruit development. Expression of these two candidate genes was upregulated by light, thus suggesting that these were highly likely linked to red color pigmentation of skin of apple fruit.

4.4.5 Validation of gene expression by qRT-PCR

The reliability of patterns of gene expression patterns identified following microarray analysis was verified by quantitative reverse transcriptase-PCR (qRT-PCR). All significant genes (815 genes) selected from the microarray were sorted by their p-value, from the highest to lowest. Eighteen genes were randomly selected at regular intervals, approximately every 50th gene, and subjected to qRT-PCR. In addition, 18 genes likely to be involved in fruit color pigmentation were also selected and their expression patterns were investigated by qRT-PCR. Expression profiles of all 36 genes from both qRT-PCR and microarray were compared (Figures 4.4 and 4.5), and found to be highly similar. Overall, 72% (26 out of 36) of genes demonstrated the same expression patterns in both microarray and qRT-PCR analyses.

4.5 Discussion

An apple oligonucleotide-based microarray consisting of 39,400 apple genes selected from 34 cDNA libraries along with 600 control genes (Soria-Guerra et al., 2011) was used to study global gene expression in skin of apple fruit. Comparison of gene expression profiles of Red Delicious bagged fruit vs. fruit that was bagged and then exposed to 14 h light treatment at three time points of one week intervals identified a total of 815 genes that were differentially modulated over all time points. Light regulates expression of genes mostly engaged in basic functions (Schäfer and Nagy, 2006). In this study, most differentially expressed genes were mainly involved in primary metabolism, transcription, protein with binding function, and cell rescue/defense. This is not uncommon since light is probably the most important environmental stimulus for plants,

controlling primary functions such as photosynthesis, photorespiration, and central developmental processes such as germination, de-etiolation, and the transition from the vegetative to the reproductive phase (Schäfer and Nagy, 2006).

Regulation of gene expression by light is in conjunction with the fruit developmental stage. In this study, about 800 genes significantly changed in their expression following 14 h light exposure were clustered into 7 groups, depending on the time of expression. Regardless of the developmental stage of the fruit, only 16% of all genes were expressed together at all time points. The majority of genes were expressed together either in Week 1 and Week 2, Week 2 and Week 3, or Week 1 and Week 3. Even though these genes were temporally expressed at different times during fruit development, they primarily belonged to the same set of functional categories including primary metabolism, transcription, and protein with binding function. This indicated that light has a greater impact on gene expression than fruit developmental stage.

In an earlier study on young developing apple fruits, it was reported that the second highest group of differentially expressed genes (~10%) was involved in the cell cycle (Soria-Guerra et al., 2011). However, in this study, only 3% of genes belonged to the cell cycle functional category in fruit following light treatment. In this study, genes belonging to the transcription functional category ranked second highest (12%). This suggested that light has a strong effect on gene expression, particularly at the transcriptional levels. These findings are not unexpected as genes involved in apple fruit development at mature stages such as those of starch synthesis, cell division, and cell expansion are no longer in high demand, and are not highly expressed. This could also explain lack of highly differentially expressed genes observed in fruit analyzed across all three weeks.

Previously, it has been reported that light influenced oxidative stress in Arabidopsis (Rossel et al., 2002; Kimura et al., 2003). In this study, similar genes have also been modulated by light; for example genes encoding heat shock proteins, such as HSP70-3, were upregulated by light treatment. Moreover, flavonols such as kaempferol and quercetin play an important role against the deleterious effects of UV radiation in plants (Smith and Markham, 1998; Ryan et al., 2001; 2002). In addition, flavonols

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accumulated in red-skinned apple are significantly important in protection against UV-B radiation (Solovchenko and Schmitz-Eiberger, 2003). *FLS* gene encoding flavonol synthase is light inducible and essential in flavonol biosynthesis (Ferreyra et al., 2010). An Arabidopsis *FLS* mutant was unable to produce flavonol in response to UV radiation (Wisman et al., 1998). In our study, *FLS* gene was upregulated 5- to 20-fold after light exposure, indicating that light induced *FLS* expression in apple skin. It has been reported that expression of *FLS* was regulated by transcription factors (TFs) such as Arabidopsis AtMYB12, AtMYB11, AtMYB111 (Mehrtens et al., 2005; Stracke et al., 2007), and grape VvMYBF1 (Czemmel et al., 2010). These Arabidopsis TFs upregulated several flavonol biosynthetic genes including *CHS*, *CHI*, *FLS*, and *UFGT* (Mehrtens et al., 2005; Stracke et al., 2007). Moreover, light induced expression of *VvMYBF1* in grape (Czemmel et al., 2010). In this study, several light-induced putative MYB TF genes were identified. It is worth noted that some of them may encode a TF regulating flavonol synthesis by inducing expression of flavonoid biosynthesis genes, particularly *FLS*. Further studies should be conducted to identify and verify their functions.

In addition to flavonol biosynthesis, anthocyanin biosynthesis has been influenced by light. Previously, the influence of light on anthocyanin biosynthesis genes has revealed that *CHS*, *F3H*, *DFR*, *ANS*, and *UFGT* genes are coordinately expressed in response to light (Kim et al., 2003; Ubi et al., 2006). In this study, color of bagged apple fruit was green throughout development, but turned red in color following 14 h light treatment. Expression of genes involved in anthocyanin biosynthesis as well as those genes involved in flavonoid and proanthocyanin biosynthesis, as determined by qRT-PCR, was downregulated when fruit was bagged (dark-grown), but these were highly expressed in fruit that were either grown under normal light conditions or those subjected to only 14 h light treatment. It is important to note that *CHI*, *CHS*, *ANS*, and *UFGT* genes are constitutively expressed during fruit ripening; however, *CHI* and *FLS* quickly respond to light treatment (within 14 h).

The observed upregulation of flavonoid-related genes including *CHI*, *FLS*, *ANS* and *UFGT* by light in this study is similar to those reported previously. For example, the apple *MYBA* gene, which regulates fruit coloration, is reported to be highly induced after

a one-day light exposure (Takos et al., 2006); moreover, anthocyanin production is reported to gradually increase, reaching a peak at 14 h of light treatment in tomato fruit (Guo and Wang, 2010). These findings suggest that coloration of apple fruit is highly dependent on light, which is involved in signal transduction in anthocyanin synthesis. Induction of genes involved in the anthocyanin biosynthesis pathway is elicited within a very short period of light exposure, although accumulation of anthocyanin pigments increases with continued exposure time to light. Steyn et al. (2004) have previously reported that anthocyanin could be degraded in the absence of light when shading of previously light-exposed pear fruit have been found to contribute to rapid loss of anthocyanin and red coloration of fruit.

It has been reported that genes engaged in the anthocyanin biosynthesis pathway are coordinately regulated, suggesting control by common TFs. In fact, MYB, bHLH, WD40, WRKY, bZIP, and MADS-box are among several TF families that have been identified (Kubo et al., 1999; Martin et al., 2001; Davies and Schwinn, 2003; Ramsay and Glover, 2005). In apple, MdMYB1, MdMYBA, and MdMYB10 are members of a MYB TF family that control the anthocyanin biosynthesis pathway by regulating expression of many genes in the pathway. Sunlight is an environmental factor that induces the expression of the *MdMYB1* gene (Takos et al., 2006). It has been reported that red coloration of fruit is detected soon after dark-grown apple fruits are exposed to sunlight. It is also known that light can regulate coloration by inducing regulatory genes to act upon upstream genes in the anthocyanin biosynthesis pathway (Davies and Schwinn, 2003).

Taken together, global gene expression analysis in reddened apple color induced by light has contributed to identification of novel regulatory genes that are linked to red color pigmentation in apple fruit. In this study, 18 candidate regulatory genes encoding WD40 protein, MYB, bHLH, and bZIP TFs have been found to be modulated, either positively or negatively, by light. Among these genes, two genes encoding an MYB and a bHLH TFs are highly regulated by light, and are highly expressed in the red-skinned cultivar Red Delicious, but down-regulated in the non-red-skinned cultivar Golden Delicious. It is known that MYB and bHLH protein families are conserved in the

regulation of anthocyanin and condensed tannin pathways in all plant species (Koes et al., 2005). Therefore, these two candidate genes are proposed to encode novel regulatory genes responsible for red color pigmentation in apple fruit. Further studies must be conducted to further characterize the functional roles of these genes.

TABLES

Table 4.1 Microarray experimental design

Treatment			
Description	Control (continuous light)	Dark-grown (continuous dark)	14 h light treatment
No. biological replicates			3
Dye-swap			1
Total Cy3/Cy5 hybridizations			3

Table 4.2 List of primers for quantitative RT-PCR

Detector	Accession No.	Forward	Reward
EST1	CN488634	gaaattcgatgcagcaacag	acctacaacagcagccatgc
EST2	GO553903	acccgtcacaaactccagac	ggaaaaaccaactcgctgac
EST5	CN492670	agacaccaacgatgggattc	tttgttgttggcgctattga
EST9	CV130009	cattcgatcagcgtccact	ctccgggtatgtttttgacc
EST10	GO527390	ccatatgctcctgtgggtct	tgcagccaactttgtcacat
EST11	CN578548	cgcggtaataacgaaaagga	tgagcagctttcaagcaaaa
EST17	CN879874	caattagttccagccctcca	ggttgaaggcgaatcagaag
EST25	EB123548	tggcgatttcttctctgctc	cctgcatgggtagaagatgc
EST26	GO517077	tgagagagtgagaaaccgaaaa	accttcctccagaagcccta
EST27	CN494975	caactcgtttgcgtagctga	agttcctgcctctcctgtca
EST28	EB141460	gccgtagatgcttttgcact	cggcattacgaaaaactcac
EST29	CV523993	tagcaaagatgcgggagatt	taaaccaaccggctttcttg
EST30	GO539182	tggtgtcgctcaactctctc	cccgggattgggtttatact
EST31	GO541355	tcgtctcgatgagatcgttg	gacaactccgtcaccacctt
EST32	GO542224	ctggcaccagaatgtctgaa	agaaaaagcggtgaaagcaa
EST33	GO568753	cagcataattccagccatga	gctacatgtggtaaaatgtaagggta
EST34	CV882930	tcccaagaggaaattgagaag	ggcgccaatcttctcgtc
EST35	CN493874	cgataagaaaccgagacacca	tatgtctcctttcggcgact
EST36	CV122001	ttccattaggaatactttctttgg	cactcttcacacaatgaacctca
EST37	GO504629	caacgaaagctagggtgctg	tcgcaggctgtacacttttg
EST38	GO502632	gagggcacagtcacagatga	cggttgcttgacaaaaggtt
EST40	GO548015	ctgtgatgaagccgagtgtg	aggcaaccaactccaaacac
EST41	GO521117	tttcaggacggtgagagctt	ttttggctgctttcttgttg
EST42	GO509579	tttcccgagaaaatgtgtcc	gaattcctcccattcgatca
EST43	DT042835	cttgatgcaccagagttgga	ccaacctctccacaaattgc
EST44	GO544170	caaccccataaaacccaatg	ttgtcgaagttgtgcctgag
EST45	GO514125	tgcatgtgaggagaaactgg	tggatatgtcctcccaccat
EST47	CV880909	atcttggttagcgcaacagc	aagggtcatccagcaaacac
EST49	GO553206	agctcttcctgcacgtgttt	gttgggcaggcagataagag
EST50	GO513701	caaaaataaactcggaattgttga	cgtctatcagccttccaacc
EST52	GO556069	ttttgattcataaatgggaagc	attcggattgagcaacaacc
EST53	GO521117	tttcaggacggtgagagctt	ttttggctgctttcttgttg
EST55	GO556160	gtttaggtccgggtgcctat	tccagttgatgacgatgctc
EST56	CO416737	acccatggcacagaaccata	caaatctatctctcatcctcatgg
EST57	CO067294	ggttccaatcttgggagtga	tcgcaatacaaatgctctgc
EST58	GO563507	ctgctcaagtggcctttct	gggcagaaagcagaagtgac

Table 4.3 Distribution of selected array features clustered based on time of expression

Numbers of EST hits to the *Arabidopsis* protein as well as those of unknown function.

Cluster	Time of expression[a]	ESTs	ESTs hit to *Arabidopsis* protein[b]	ESTs with unknown function[c]
Selected				
815		815	632	183
1	W1, W2, W3	131	100	31
2	W1, W2	232	186	46
3	W2, W3	167	127	40
4	W1, W3	128	97	31
5	W1	41	29	12
6	W2	80	68	12
7	W3	36	25	11

a W1 = week 1, W2 = week 2, W3 = week 3.
b Apple ESTs were compared with *Arabidopsis* protein to identify similar *Arabidopsis* genes.
c Apple ESTs were matched to unknown protein or no hit to any *Arabidopsis* protein.

Table 4.4 Distribution of ESTs expressed in each functional category within each cluster

ESTs presented as percentof total number of ESTs from all categories distributed in each cluster.

Functional category	Whole microarray[a]	Cluster[b] 1	2	3	4	5	6	7
Primary metabolism	17.3	17.6	30.5	22.9	15.3	6.9	9.2	5.3
Transcription	11.5	10.7	17.6	16.8	12.2	5.3	6.9	2.3
No hit	11.2	16.0	20.6	10.7	10.7	2.3	4.6	4.6
Protein with binding function	11.0	6.9	19.8	16.8	7.6	2.3	11.5	3.8
Unknown	9.3	7.6	20.6	11.5	13.0	2.3	3.1	0.0
Hypothetical protein	5.8	6.9	9.2	7.6	4.6	2.3	3.8	1.5
Cell rescue/ defense	5.4	5.3	3.1	8.4	6.9	4.6	3.8	1.5
Cellular comunication /signal transduction mechanism	5.3	5.3	11.5	4.6	6.1	0.8	2.3	2.3
Development	4.2	3.8	5.3	9.2	3.8	0.8	1.5	1.5
Cellular transport/ transport facilities	3.4	2.3	7.6	3.8	4.6	0.0	1.5	1.5
Subcellular localization	3.2	3.1	6.9	3.1	2.3	0.8	3.1	0.8
Cell cycle and DNA processing	3.1	1.5	7.6	3.1	3.1	0.8	2.3	0.8
Cell proliferation and differentiation	2.9	3.1	4.6	3.1	3.1	0.0	3.8	0.8
Secondary metabolism	2.7	5.3	6.9	2.3	0.8	0.8	0.0	0.8
Energy	1.5	2.3	1.5	0.8	0.8	0.0	3.8	0.0
Protein Fate (folding, modification, destination)	1.1	1.5	1.5	2.3	1.5	0.0	0.0	0.0
Protein synthesis	0.7	0.0	2.3	0.0	1.5	0.8	0.0	0.0
transposase	0.2	0.0	0.0	0.8	0.0	0.8	0.0	0.0
Regulation of metabolism	0.1	0.8	0.0	0.0	0.0	0.0	0.0	0.0

[a] Selected features that are differentially expressed with reliable signals.
[b] Cluster: 1 = all three weeks; 2 = weeks 1 and 2; 3 = weeks 2 and 3; 4 = weeks 1 and 3; 5 = week1; 6 = week 2; and 7 = week 3.

Table 4.5 Number of ESTs differentially expressed with log_2-ratio ≥ 1.6 or ≤ 1.6

Expression ratio[a] (log_2-ratio)	Treatment 3/ Treatment 2[b]		
	W1	W2	W3
≥ 1.6	93(11.4%)[c]	295(36.2%)	190(23.3%)
≤ -1.6	18(2.2%)	113(13.8%)	117(14.3%)

[a] Log-ratio of 1.6 denotes three fold differential expression signals in transcript level.
[b] Treatment 2: bagged fruit; treatment 3: bagged fruit exposed to 12 h light.
[c] Percentage based on 815 features showing a reliable signal.

Table 4.6 List of candidate apple ESTs linked to coloration

Family	Name	Apple EST[a]	Accession No.[b]	Description[b]	Microarray[c]	qRT-PCR[d]
MYB	EST45	GO514125	AT1G20950	AtY53, AtMYB67 | AtMYB67/AtY53 (myb domain protein 67)	Up	Yes
	EST47	CV880909	AT2G21280	AtMYB51, BW51A, BW51B, MYB51 | MYB51 (myb domain protein 51)	Up	Yes
	EST9	CV130009	AT1G74080	AtMYB122, MYB122 | MYB122 (myb domain protein 122)	Up	Yes
	EST49	GO553206	AT1G74080	AtMYB122, MYB122 | MYB122 (myb domain protein 122)	Up	Yes
	EST52	GO556069	AT5G01200	myb family transcription factor	Up	Yes
	EST50	GO513701	AT5G17300	myb family transcription factor	Up	Yes
	EST11	CN578548	AT5G02320	AtMYB3R5, MYB3R-5 | MYB3R-5 (myb domain protein 3R-5)	Up	No
	EST17	CN879874	AT2G20400	myb family transcription factor	Up	No
	EST44	GO544170	AT5G55020	AtMYB120, MYB120 | MYB120 (myb domain protein 120)	Up	No
bHLH	EST55	GO556160	AT1G32090	basic helix-loop-helix (bHLH) family protein	Up	Yes
	EST58	GO563507	AT2G31210	basic helix-loop-helix (bHLH) family protein	Up	Yes
	EST56	CO416737	AT1G05805	basic helix-loop-helix (bHLH) family protein	Up	No
	EST53	GO521117	AT1G10120	basic helix-loop-helix (bHLH) family protein	Down	Yes
	EST57	CO067294	AT2G31280	basic helix-loop-helix (bHLH) protein-related	Down	Yes
WD40	EST10	GO527390	AT5G18525	WD-40 repeat family protein	Up	Yes
	EST1	CN488634	AT1G04140	transducin family protein / WD-40 repeat family protein	Down	Yes
	EST2	GO553903	AT2G46560	transducin family protein / WD-40 repeat family protein	Down	Yes
bZIP	EST5	CN492670	AT4G37730	AtbZIP7 | bZIP transcription factor family protein	Up	No

[a] Accession numbers of apple ESTs that were used to design its primers.

[b] Annotation based on similarity to *Arabidopsis* protein database: accession numbers and their functions.

[c] Trend of gene expression level regulated by light.

[d] 'Yes' means that the microarray result is confirmed by qRT-PCR, 'no' means that the result is not confirmed (it shows not significant variations or an opposite trend of expression)

Figure 4.1 The number of genes expressed in each time point and overlapping expression among three time points
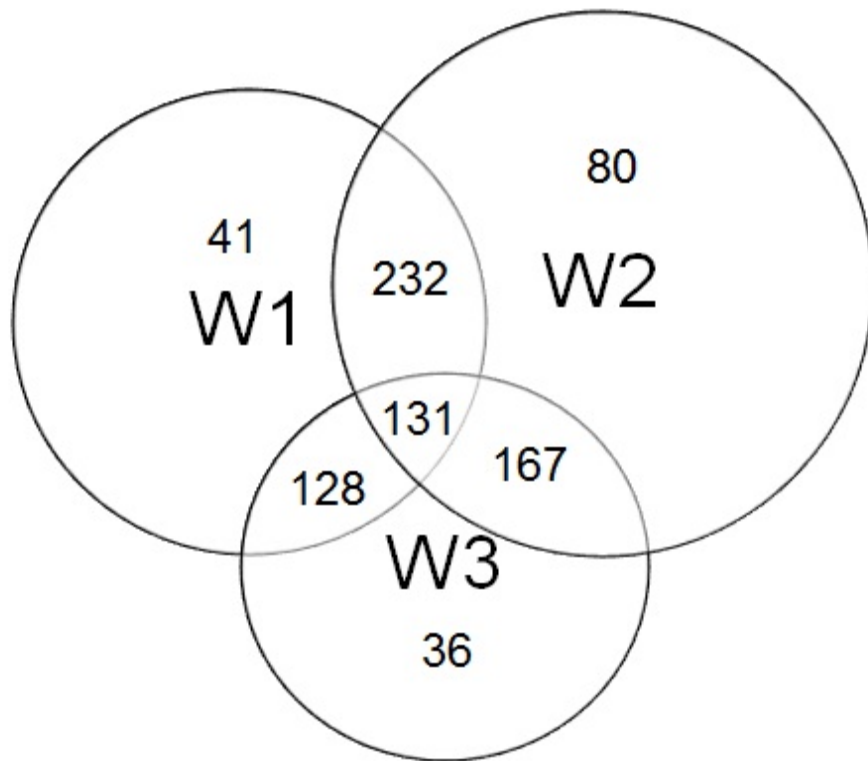
Abbreviations are listed as follows. W1: Week 1; W2: Week 2; W3: Week 3.

Figure 4.2 Functional categories of light-influenced genes
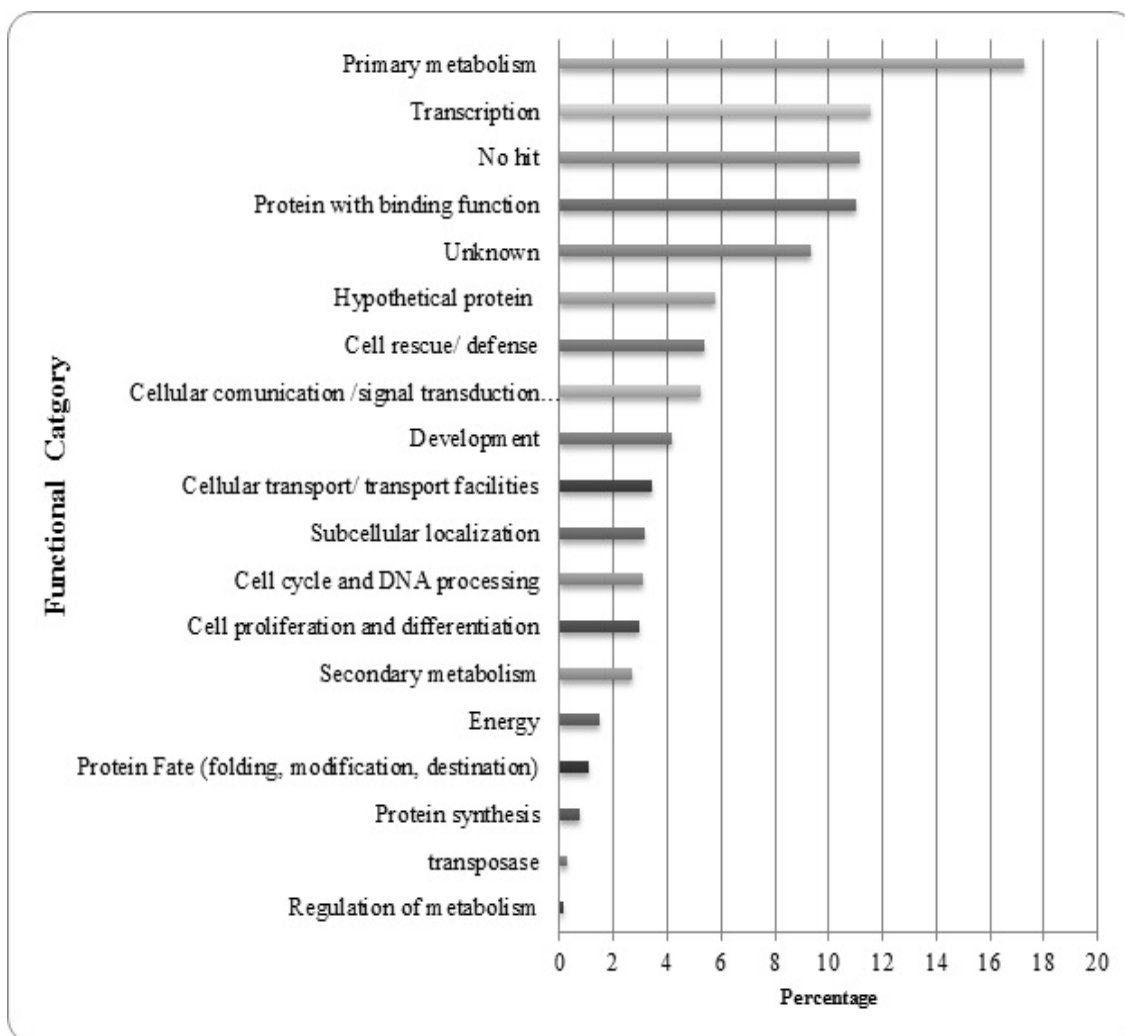
Figure 4.3 Expression profiles of apple flavonoid-related genes in response to continuous vs. 14 h light treatment
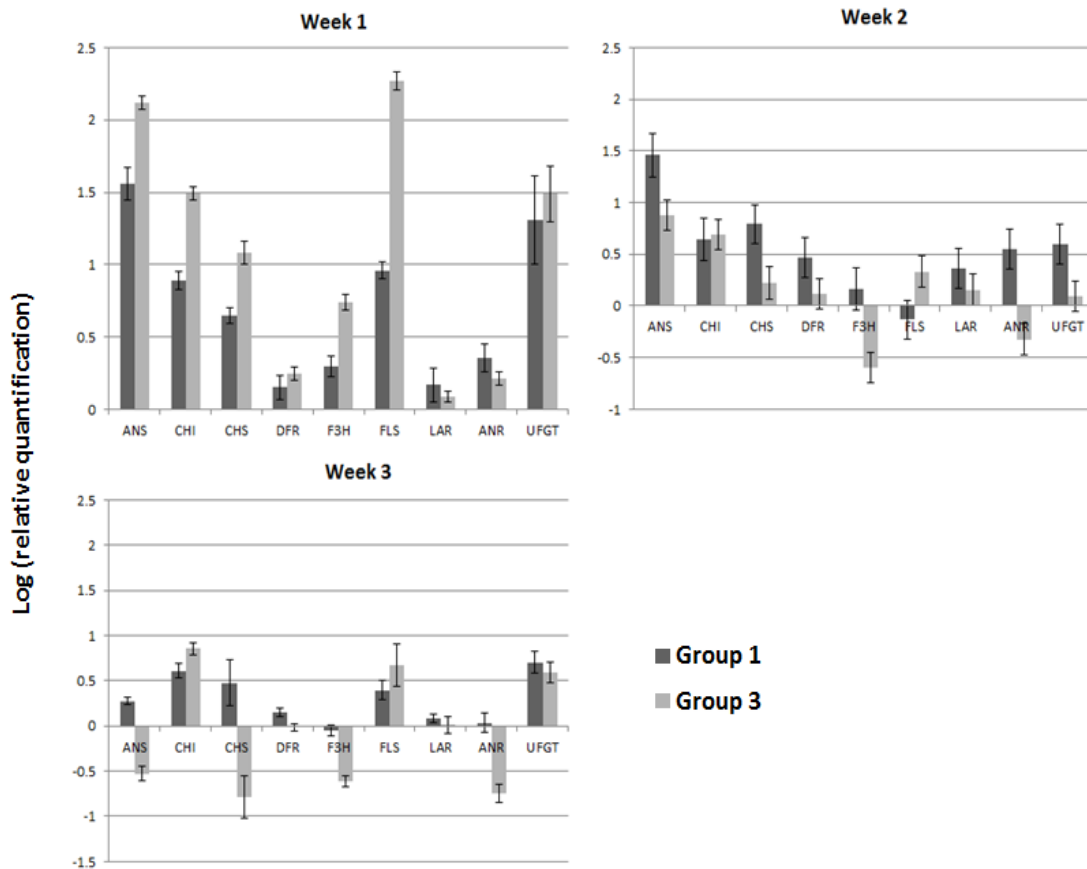
Fruit samples were collected at three time points at one-week intervals, identified as Week 1, Week 2, and Week 3. Differential gene expression levels were calculated by comparing each of groups 1 (never-bagged, continuous light conditions) and 3 (14 h light exposure) to group 2 (dark-grown, continuous dark). CHI: chalcone isomerase; CHS: chalcone synthase; F3H: flavonoid 3-hydroxylase; FLS: flavonol synthase; DFR: dihydroflavonol reductase; LAR: leucoanthocyanidin reductase; ANS: anthocyanidin synthase; UFGT: UDP-glucose: flavonoid 3-o-glucosyltransferase; and ANR: anthocyanidin reductase. All expression values were adjusted to those of *actin* gene. Values correspond to means of three technical replications.

Figure 4.4 Expression profiles of the candidate regulatory genes in apple fruit
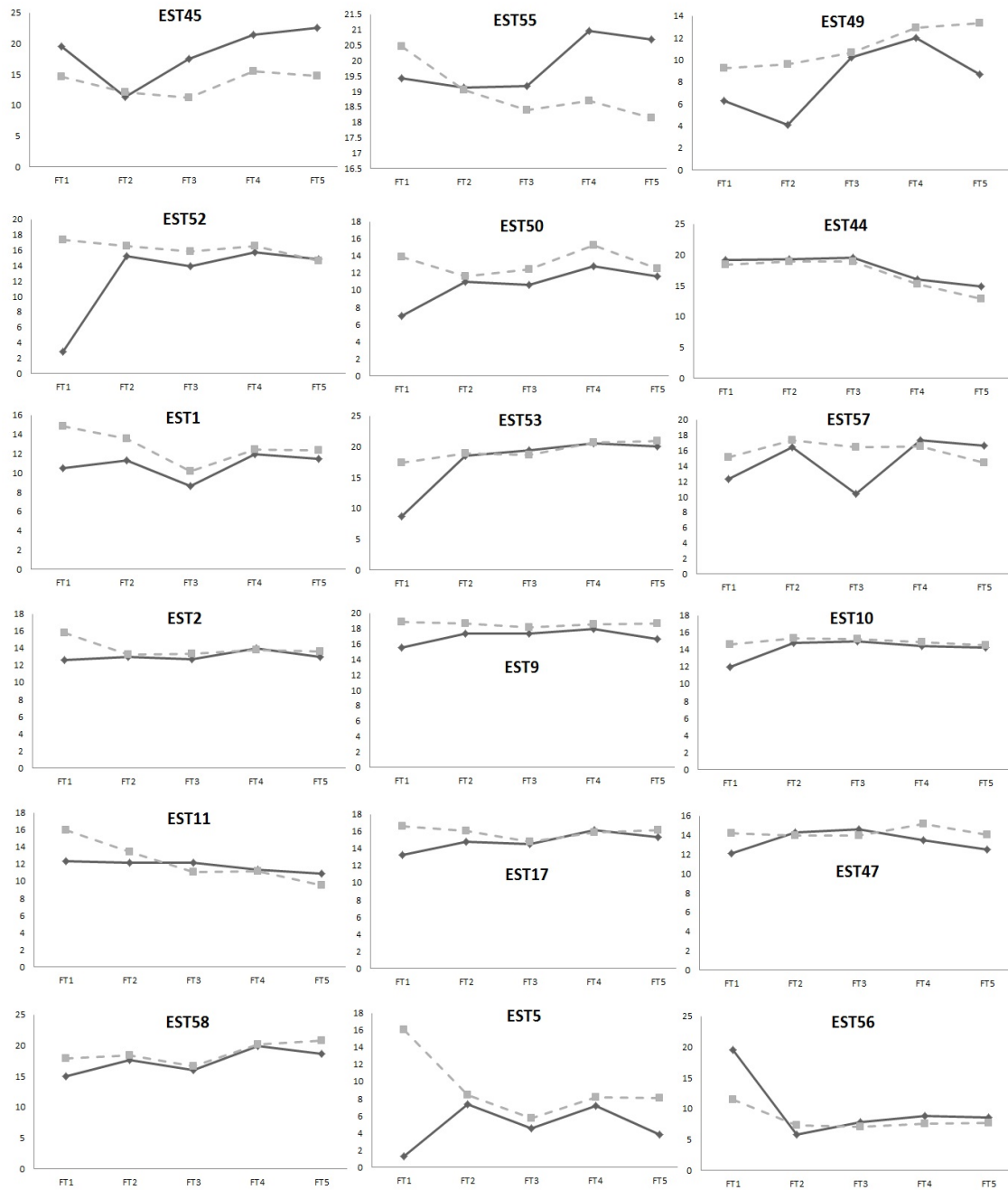
Expression levels of candidate genes were determined in fruit collected at five developmental stages of apple cvs. Red Delicious (solid lines) and Golden Delicious (dashed lines). Fw1: Flower buds at the pink stage; Fw2: flower buds at the balloon stage; Fw3: flowers at full bloom; Ft1: 9 DAP; Ft2: 16 DAP; Ft3: 44 DAP; Ft4: 104 DAP; and Ft5: 145 DAP.

Figure 4.5 Validation of microarray expression pattern of the candidate regulatory genes linked to coloration
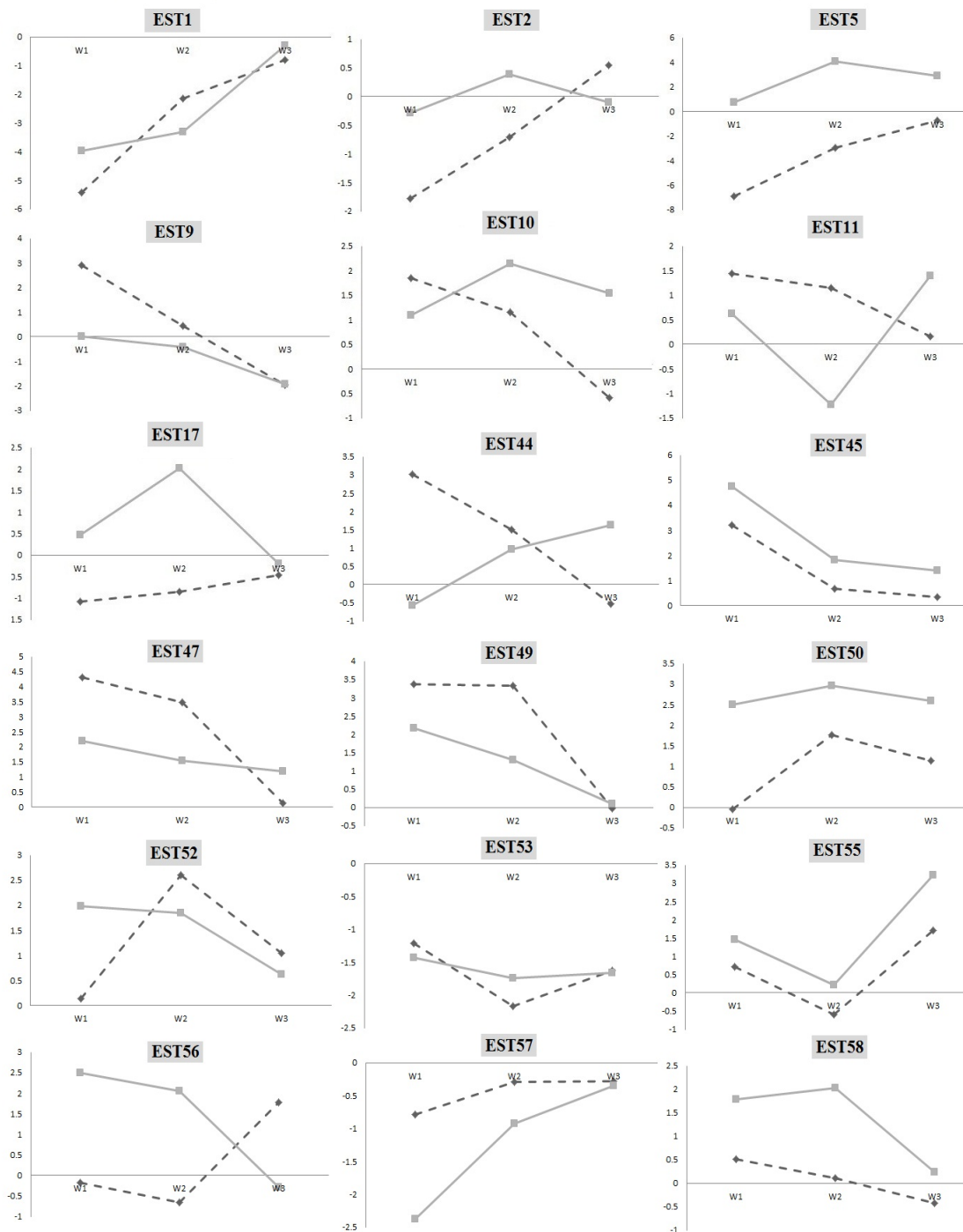
Graph includes gene expression data from the array (solid lines) and qRT-PCR (dashed lines). X axes show the time points including Week 1 (W1), Week 2 (W2), and Week 3 (W3). Y axes show log$_2$-relative quantification.

Figure 4.6 Validation of microarray expression patterns
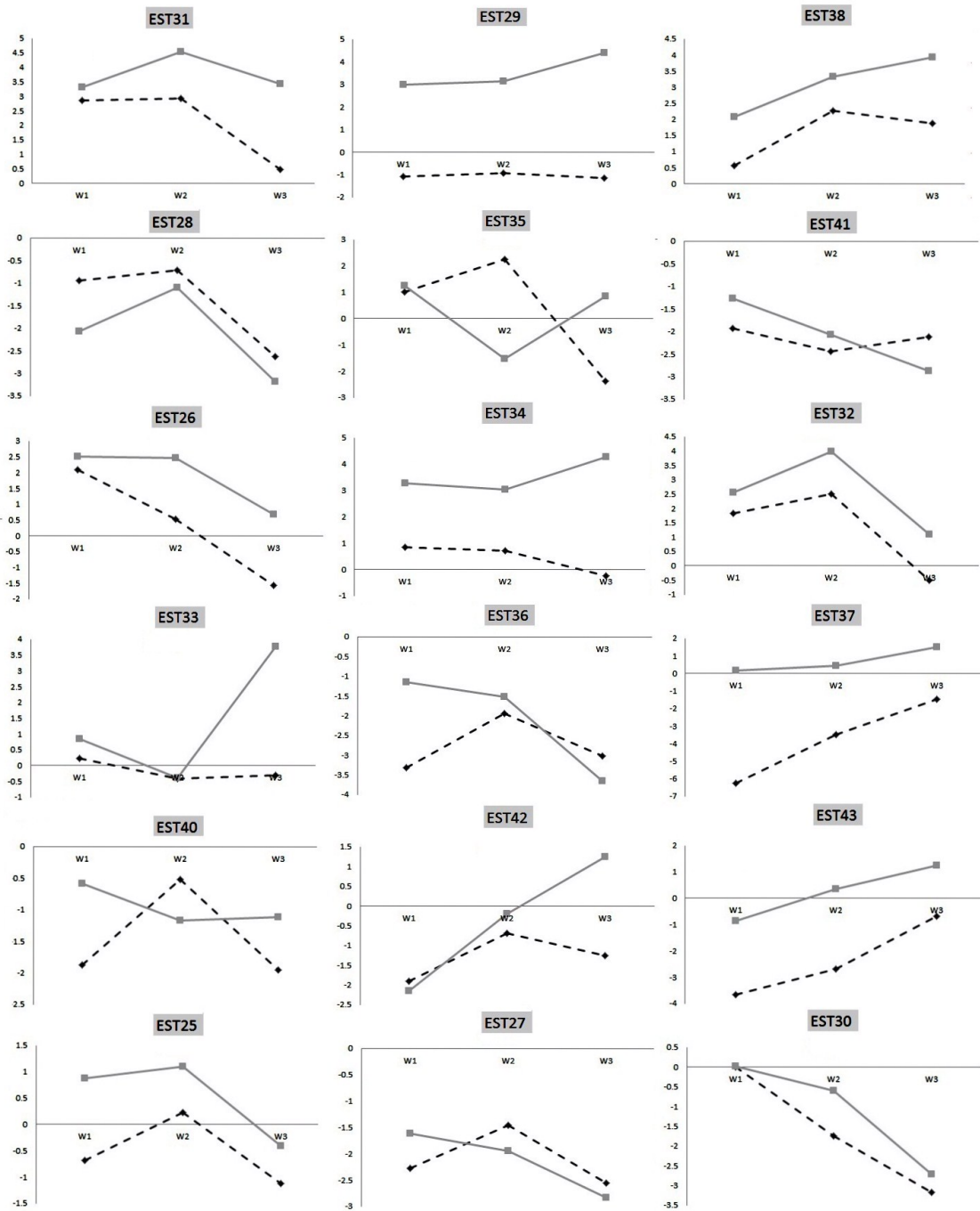
Sorted by p-value, eighteen ESTs from reliable 815 ESTs were selected by every 50th position and confirmed their expression by qRT-PCR using primers designed close to the array oligo. Transcript levels from the array (solid lines) and qRT-PCR (dashed lines) were compared in all three time points. X axes show the time points including Week 1 (W1), Week 2 (W2), and Week 3 (W3). Y axes show log2-relative quantification.

CHAPTER 5

CONCLUSIONS

Findings obtained in this study have provided critical and important information on genes and transcription factors involved in the anthocyanin biosynthesis pathway in apple. Moreover, these findings have provided knowledge on regulation of color pigmentation and patterns in plants. These findings will contribute to future scientific research towards manipulating genes involved in the anthocyanin biosynthetic pathway, and towards enhancing color pigmentation in apple and in other plants. The following is a listing of major findings obtained:

- The apple *anthocyanidin reductase* (*ANR*) gene family consists of three genes, including *MdANR1*, *MdANR2a*, and *MdANR2b* genes. Moreover, *MdANR2a* and *MdANR2b* are allelic. Two indel genetic markers have been developed from genomic sequences, and used to map the *MdANR1* onto linkage group (LG) 10 and the *MdANR2a/b* onto LG5 of the apple genetic map.

- The functionality of *MdANR* genes was evaluated in tobacco, *Nicotiana tabacum* cv. Petite Havana SR1. Overexpression of these genes in tobacco resulted in the recovery of transgenic lines with flowers of modified color, from pink to pale-pink, to white. It was determined that *MdANR* genes were capable of disrupting pigment development by promoting proanthocyanin biosynthesis, but inhibiting anthocyanin production via down-regulation of expression of endogenous *chalcone isomerase* (*CHI*) and *dihydroflavonol reductase* (*DFR*) genes.

- Incomplete inhibition of anthocyanin production was observed in transgenic tobacco lines expressing *ANR1* and resulting in new floral pigmentation patterns.

- Catechin was highly accumulated in transgenic tobacco lines whose *leucoanthocyanidin reductase* (*LAR*) gene was repressed, and suggesting that the ANR might have a redundant role to that of LAR in the synthesis of catechin.

- a novel MYB transcription factor (TF) gene regulating fruit coloration in apple was identified, and designated as *MdMYB11*. The *MdMYB11* was located on LG15 of the apple genetic map.

- The MdMYB11 TF modulates regulation of gene expression in the flavonoid biosynthesis pathway. Expression of *MdMYB11* in transgenic tobacco enhanced anthocyanin biosynthesis by inducing expression of *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), and *UDP-glucose: flavonoid 3-o-glucosyltransferase* (*UFGT*) genes in tobacco. However, this TF downregulated *cinnamate-4-hydroxylase* (*C4H*) and *4-coumaroyl:CoA-ligase* (*CL4*) genes encoding enzymes in the phenylpropanoid pathway. The observed long floral styles in transgenic tobacco lines expressing *MdMYB11*, when compared to those of wild-type plants, suggested that *MdMYB11* was involved in floral morphogenesis, and in particular in pistil development.

- Following global gene analysis of the apple transcriptome, a total of 815 genes were identified that were modulated in response to light treatment in peel of fruit of apple cv. Red Delicious. Moreover, two putative regulatory genes were identified that were expressed at higher levels in red-skinned apple cv. Red Delicious than that of the non-red skinned apple cv. Golden Delicious. Thus, these genes maybe linked to red coloration in apple fruit.

The above findings and studies have also presented new scientific questions that deserve further investigation. These include the following:

- What is the mechanism of the ANR enzyme in the biosynthesis of catechin, and does it directly convert anthocyanin to catechin?

- What is the mechanism of co-suppression of *LAR* and *DFR* genes by the *ANR* gene? Moreover, how do these interact at the molecular and biochemical levels? How does the *ANR* gene influence the regulatory role of the *anthocyanin2* (*AN2*) gene?

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- How does the *MdMYB11* gene interact with flavonoid-related genes; i.e., protein-DNA interactions in the promoter region or protein-protein interactions?

- How is expression of *MdMYB11* regulated? Do other TFs activate its function?

- How does the *MdMYB11* gene affect floral morphogenesis? Is it due to its negative regulatory role on flavonol biosynthesis and/or its relation to lignin biosynthesis?

- What are the functions of the two identified putative transcription factor genes, EST45 and EST53, in plants? Are they really involved in anthocyanin production or the flavonoid biosynthesis pathway? How are the functions of other TFs influenced by light?

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APPENDIX

Table A1 Full list of significant genes differentially expressed in three time points

Expression ratios and accession numbers were arranged by functional category.

ID	Week1	Week2	Week3	Accession Number
Cell cycle and DNA processing				
MdUI18255	4.14	4.63	1.19	AT5G08230.1
MdUI03540	3.18	3.82	1.52	AT5G64420.1
MdUI08669	2.68	6.16	-1.79	AT3G46230.1
MdUI13482	2.56	4.15	2.90	AT4G31870.1
MdUI15570	1.97	2.66	0.88	AT5G64040.1
MdUI27413	1.84	2.28	2.87	AT3G55180.1
MdUI35680	1.73	1.58	-0.41	AT2G31690.1
MdUI35558	1.48	-1.63	-1.94	AT1G54130.1
MdUI18685	1.36	2.81	4.25	AT5G23940.1
MdUI34679	0.84	-0.41	1.99	AT3G54890.2
MdUI26208	0.69	-0.10	0.82	AT1G63160.1
MdUI38498	0.44	0.33	1.45	AT2G25050.1
MdUI26169	0.25	2.84	0.71	AT1G44910.1
MdUI04654	0.24	-0.87	-0.08	AT2G29770.1
MdUI39263	0.22	1.80	0.12	AT2G38180.1
MdUI39541	-0.16	0.55	0.80	AT2G27010.1
MdUI33935	-0.19	1.04	-0.49	AT3G18300.1
MdUI21260	-0.24	-0.12	1.24	AT3G07360.1
MdUI26318	-0.25	-1.72	-1.15	AT3G26430.1
MdUI29690	-0.29	-0.56	1.10	AT3G05040.1
MdUI25736	-0.58	1.62	-0.20	AT1G12640.1
MdUI03642	-0.64	-4.42	-1.45	AT5G57160.1
MdUI36923	-0.80	-2.89	-1.54	AT2G24450.1
MdUI31610	-0.89	0.51	-0.55	AT1G52700.1
MdUI14044	-2.41	0.03	-3.23	AT5G08120.1
Cell proliferation and differentiation				
MdUI02138	2.67	3.02	0.62	AT3G14340.1
MdUI03379	2.53	1.63	3.11	AT4G37460.1
MdUI20902	1.78	2.31	1.50	AT3G29200.1
MdUI39850	1.19	-0.62	1.49	AT1G01510.1
MdUI27452	0.82	0.86	-0.18	AT5G10300.1
MdUI35460	0.81	0.57	1.09	AT1G12600.1
MdUI01053	0.65	-2.52	-3.41	AT1G33030.1
MdUI32589	0.57	1.85	3.44	AT5G27110.1
MdUI25721	0.42	3.39	1.99	AT1G68050.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Cell proliferation and differentiation				
MdUI38921	0.06	-0.99	-0.51	AT1G21760.1
MdUI32379	0.05	2.28	0.18	AT2G04860.1
MdUI29612	-0.17	1.37	0.08	AT2G44580.1
MdUI38571	-0.23	0.98	-0.58	AT1G61870.1
MdUI23431	-0.24	-0.02	-2.37	AT4G01570.1
MdUI26885	-0.25	1.82	-0.18	AT1G15520.1
MdUI26278	-0.27	0.18	1.87	AT3G63400.1
MdUI18586	-0.31	-2.16	-0.87	AT1G35080.1
MdUI22357	-0.55	0.99	-0.21	AT3G06920.1
MdUI39421	-0.59	-1.19	1.12	AT1G14770.1
MdUI04186	-0.59	-0.77	-1.91	AT1G63150.1
MdUI26230	-0.60	1.80	0.48	AT5G64813.1
MdUI32241	-0.71	-0.49	1.63	AT4G25270.1
MdUI12789	-1.31	-3.13	-3.29	AT4G26340.1
MdUI28471	-1.91	-3.57	-1.19	AT2G17210.1
Cell rescue/ defense				
MdUI11352	5.95	5.55	2.68	AT1G07400.1
MdUI16042	5.23	5.20	4.97	AT1G16030.1
MdUI01410	4.40	-0.46	2.59	AT4G22880.1
MdUI32174	4.19	0.14	2.88	AT4G22870.1
MdUI03405	3.94	3.57	1.31	AT3G03930.1
MdUI32329	3.92	0.65	2.85	AT4G22880.1
MdUI32173	3.77	-0.30	2.28	AT4G22880.1
MdUI22535	3.67	-0.46	2.63	AT4G22880.1
MdUI32175	3.51	0.68	1.55	AT4G22880.1
MdUI29298	3.49	3.59	4.90	AT3G09440.1
MdUI10258	3.32	4.53	3.44	AT1G74310.1
MdUI10257	3.10	4.70	3.76	AT1G74310.1
MdUI19017	3.00	1.33	1.58	AT3G09640.1
MdUI14498	2.72	3.97	3.64	AT1G74310.1
MdUI32700	2.71	0.08	2.98	AT4G22880.1
MdUI28908	2.69	4.07	1.57	AT5G17680.1
MdUI19682	2.21	1.36	2.31	AT1G30410.1
MdUI32330	2.10	-0.06	2.08	AT4G22880.1
MdUI29141	1.51	1.39	1.34	AT4G33390.1
MdUI30514	1.45	-0.10	1.80	AT1G58440.1
MdUI23297	1.37	4.78	0.67	AT1G52560.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Cell rescue/ defense				
MdUI21299	1.14	3.65	2.27	AT4G17850.1
MdUI38355	1.13	0.82	0.85	AT2G18980.1
MdUI30851	0.97	1.87	2.51	AT1G03400.1
MdUI13573	0.77	4.10	2.93	AT4G37730.1
MdUI33837	0.70	2.09	1.89	AT4G34360.1
MdUI04401	0.68	-1.41	-3.84	AT1G48910.1
MdUI00568	0.39	-1.53	-2.20	AT2G26550.1
MdUI27797	0.28	1.21	1.27	AT2G31800.1
MdUI35204	0.26	1.64	1.42	AT5G39580.1
MdUI30705	0.24	2.98	3.57	AT1G28135.1
MdUI39447	0.24	-2.94	0.23	AT3G07040.1
MdUI39756	0.11	-0.66	0.61	AT1G75640.1
MdUI38007	-0.14	2.56	1.22	AT4G19500.1
MdUI25456	-0.14	-0.32	-1.26	AT2G29970.1
MdUI39312	-0.15	2.32	0.80	AT4G08450.1
MdUI21601	-0.23	-0.10	-1.05	AT1G27170.1
MdUI39364	-0.47	-1.67	-0.15	AT3G21295.1
MdUI13936	-0.69	-1.47	2.37	AT5G26990.1
MdUI35511	-1.27	-2.06	-2.87	AT3G15700.1
MdUI24014	-1.85	-4.42	-6.89	AT2G07240.1
MdUI11838	-2.14	-2.67	-3.80	AT1G49570.1
Cellular communication /signal transduction mechanism				
MdUI18889	3.94	4.65	0.95	AT1G67090.1
MdUI03543	3.46	3.11	0.89	AT4G40050.1
MdUI32268	3.11	3.43	0.15	AT1G06430.1
MdUI05028	3.03	4.01	1.59	AT2G18710.1
MdUI12325	2.73	2.41	1.08	AT5G13630.1
MdUI18547	2.57	2.55	1.68	AT5G13630.1
MdUI21351	2.54	-0.59	0.55	AT1G45180.1
MdUI22840	2.45	3.74	1.38	AT1G17870.1
MdUI34488	2.20	0.14	2.56	AT5G65420.1
MdUI39605	1.91	0.12	1.76	AT4G02010.1
MdUI23751	1.83	2.41	1.47	AT4G13000.1
MdUI32720	1.62	2.44	3.30	AT4G23882.1
MdUI39514	1.52	2.40	1.56	AT2G27580.1
MdUI38732	1.39	-0.09	2.07	AT2G20470.1
MdUI29286	1.37	0.34	1.50	AT1G53730.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Cellular communication /signal transduction mechanism				
MdUI28088	1.13	-0.36	0.76	AT1G32360.1
MdUI10496	1.05	-1.20	-2.00	AT2G17040.1
MdUI17151	1.03	1.82	4.37	AT1G61430.1
MdUI38689	0.81	1.19	0.97	AT2G17090.1
MdUI29247	0.71	-0.12	-0.17	AT2G17820.1
MdUI39409	0.62	1.97	-0.30	AT1G49050.1
MdUI38402	0.49	0.97	-0.06	AT1G09440.1
MdUI39679	0.48	1.84	1.40	AT5G47500.1
MdUI21619	0.45	2.87	0.59	AT2G26140.1
MdUI35437	0.39	-1.19	2.68	AT5G35560.1
MdUI36788	0.38	1.80	1.53	AT3G26700.1
MdUI39237	0.33	1.00	1.13	AT1G08750.1
MdUI37159	0.33	-1.38	0.88	AT3G09770.1
MdUI38438	0.25	0.91	0.17	AT5G28680.1
MdUI37252	0.08	-1.20	-0.65	AT4G34440.1
MdUI20724	0.07	3.68	2.26	AT3G53920.1
MdUI31859	-0.17	0.92	1.84	AT1G55545.1
MdUI25294	-0.24	0.24	0.70	AT3G58590.1
MdUI32023	-0.25	0.40	1.23	AT1G12370.2
MdUI36692	-0.30	2.21	1.65	AT1G33770.1
MdUI33856	-0.43	-2.01	1.42	AT3G58920.1
MdUI33859	-0.44	0.00	-1.99	AT2G34140.1
MdUI38484	-0.63	-0.13	0.72	AT2G19110.1
MdUI25752	-0.63	-1.91	1.42	AT5G48250.1
MdUI02948	-0.84	-0.55	-4.96	AT1G64255.1
MdUI37043	-1.61	-2.03	-0.72	AT1G53050.1
MdUI06227	-2.04	-3.01	-3.52	AT4G01340.1
MdUI00515	-3.97	-3.30	-0.29	AT1G17620.1
Cellular transport/ transport facilities				
MdUI22818	4.97	2.21	5.18	AT3G12750.1
MdUI21093	3.18	3.23	0.78	AT5G65230.1
MdUI19547	3.08	4.59	3.36	AT1G21270.1
MdUI05029	2.64	3.53	0.50	AT3G19553.1
MdUI03608	2.60	6.28	3.54	AT5G17020.1
MdUI38899	1.76	1.55	2.69	AT3G49650.1
MdUI03775	1.76	4.77	2.56	AT2G39510.1
MdUI28904	1.56	0.43	2.51	AT1G48240.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Cellular transport/ transport facilities				
MdUI23956	1.54	0.97	2.34	AT4G28630.1
MdUI39457	0.70	-1.05	2.25	AT5G62050.1
MdUI37587	0.57	-0.72	0.70	AT5G13800.2
MdUI34038	0.52	-1.48	1.27	AT3G10290.1
MdUI33002	0.51	0.06	-2.09	AT5G06510.1
MdUI02301	0.27	1.92	0.05	AT1G71810.1
MdUI24037	0.26	1.36	-1.79	AT2G39810.1
MdUI25748	0.25	1.99	0.36	AT1G54580.1
MdUI20811	0.16	-1.45	-0.35	AT4G11810.1
MdUI26787	0.01	1.77	0.87	AT5G61730.1
MdUI29249	-0.23	-1.56	0.68	AT5G09550.1
MdUI25174	-0.23	1.44	-0.91	AT2G37980.1
MdUI33189	-0.26	-1.53	-0.67	AT1G16380.1
MdUI26099	-0.37	-0.76	1.23	AT5G19640.1
MdUI16107	-0.45	-2.48	-0.64	AT1G66020.1
MdUI12599	-0.66	-2.91	-3.73	AT3G47420.1
MdUI39088	-0.76	-0.70	0.95	AT1G77140.1
MdUI02558	-0.77	1.00	1.58	AT4G02050.1
MdUI19464	-0.79	-3.28	-4.54	AT3G11910.1
MdUI18680	-2.00	-3.03	-4.34	AT3G06450.1
Development				
MdUI02132	3.34	3.76	0.77	AT5G38410.1
MdUI01565	3.29	5.74	3.40	AT4G23990.1
MdUI19946	2.80	6.93	4.38	AT1G23850.1
MdUI27691	2.64	3.74	2.69	AT5G59000.1
MdUI01755	2.30	4.05	2.91	AT3G20920.1
MdUI34836	2.21	4.85	1.59	AT1G24340.1
MdUI20710	1.77	3.82	2.03	AT5G05780.1
MdUI26148	1.53	2.52	1.02	AT2G04650.1
MdUI25918	1.43	2.81	3.43	AT1G34430.1
MdUI32117	1.42	3.69	3.64	AT1G16130.1
MdUI32278	1.36	0.72	1.91	AT2G39770.1
MdUI21106	1.32	1.82	0.23	AT3G13870.2
MdUI29597	1.16	0.38	1.61	AT1G25350.1
MdUI29222	1.14	1.78	1.53	AT1G03380.1
MdUI26961	1.13	0.33	1.36	AT5G65080.2
MdUI38796	1.05	1.44	1.54	AT5G64050.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Development				
MdUI37777	0.89	1.00	-0.99	AT4G33090.1
MdUI29237	0.33	-2.08	1.48	AT3G12080.1
MdUI21156	0.30	1.53	0.84	AT5G66055.1
MdUI16171	0.22	-0.09	-2.13	AT1G01550.2
MdUI20069	0.14	0.92	-1.31	AT4G17620.1
MdUI21552	0.05	1.83	3.04	AT1G02205.1
MdUI32542	0.03	-2.12	-1.35	AT3G61070.1
MdUI38222	-0.05	-1.53	1.80	AT5G40480.1
MdUI33620	-0.23	-0.19	-1.66	AT2G34880.1
MdUI38851	-0.30	-1.86	2.87	AT3G44735.1
MdUI35390	-0.36	-1.54	-0.70	AT4G31100.1
MdUI21967	-0.48	0.19	-2.48	AT2G20870.1
MdUI22060	-0.84	0.12	-3.36	AT1G34210.1
MdUI26291	-0.86	-1.15	4.14	AT4G16120.1
MdUI09870	-0.94	-2.74	-3.53	AT1G16590.1
MdUI09056	-1.39	-0.26	-4.26	AT1G64140.1
MdUI21663	-2.00	-3.36	-4.45	AT2G30800.1
MdUI22893	-2.32	0.26	-3.18	AT5G25140.1
Energy				
MdUI14849	2.71	4.27	2.92	AT2G05100.1
MdUI19678	2.51	4.33	2.62	AT2G05100.1
MdUI19541	2.43	3.42	2.46	AT2G05100.1
MdUI19042	2.34	3.28	2.74	AT2G34430.1
MdUI18475	2.33	4.40	1.75	AT2G05100.1
MdUI32733	2.22	3.54	1.37	AT2G39730.1
MdUI04177	1.82	4.31	2.55	AT5G54270.1
MdUI39035	1.45	3.29	0.01	ATCG00340.1
MdUI31943	1.22	-0.45	2.94	AT1G17260.1
MdUI38953	1.19	2.49	0.73	ATCG00490.1
MdUI23050	1.12	-2.56	1.45	AT3G21740.1
MdUI33963	0.48	0.78	0.86	AT3G27950.1
Hypothetical protein				
MdUI02141	4.36	4.58	0.54	AT1G60270.1
MdUI02133	4.16	4.93	0.76	AT1G75980.1
MdUI21992	4.09	4.53	2.26	AT5G17890.1
MdUI12034	4.08	3.32	3.88	AT3G46810.1
MdUI03880	4.05	3.10	0.19	AT5G11680.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Hypothetical protein				
MdUI31996	2.78	4.59	0.98	AT2G28370.1
MdUI39158	2.66	2.60	2.67	AT1G11970.1
MdUI13478	2.34	3.92	3.08	AT1G56710.1
MdUI12516	2.25	3.90	3.19	AT5G44770.1
MdUI15859	2.06	4.01	1.17	AT4G06601.1
MdUI29304	1.98	2.57	0.43	AT4G37090.1
MdUI01756	1.52	3.78	2.28	AT3G46430.1
MdUI28204	1.38	2.05	1.68	AT5G32590.1
MdUI27796	1.31	1.42	2.26	AT5G22830.1
MdUI06661	1.28	4.36	3.32	AT2G15040.1
MdUI20016	1.21	1.32	1.31	AT1G71380.1
MdUI32237	1.01	0.96	4.41	AT4G15530.2
MdUI29621	0.94	-1.58	0.87	AT1G22335.1
MdUI21322	0.75	-1.54	1.21	AT5G20280.1
MdUI34677	0.58	-1.04	2.21	AT3G30300.1
MdUI35033	0.52	0.31	-2.18	AT5G23610.1
MdUI38544	0.51	1.51	0.94	AT5G53170.1
MdUI38552	0.44	2.22	2.34	AT5G63270.1
MdUI39253	0.36	-0.38	0.53	AT4G13030.2
MdUI39359	0.34	2.22	1.80	AT2G25720.1
MdUI28815	0.32	1.59	0.58	AT3G20720.1
MdUI38227	0.12	-2.43	0.18	ATMG00660.1
MdUI24779	0.05	1.31	0.73	AT2G39950.1
MdUI26276	0.02	-0.87	-0.75	AT4G31040.1
MdUI33733	-0.02	-1.11	-2.93	AT2G34580.1
MdUI23455	-0.06	-2.12	-3.83	AT1G60680.1
MdUI32984	-0.11	-2.54	-1.04	AT5G65400.1
MdUI28828	-0.15	-1.08	-0.03	AT5G34686.1
MdUI10082	-0.28	-0.36	0.72	AT1G02870.1
MdUI37729	-0.38	0.42	1.25	AT1G70390.1
MdUI27202	-0.49	0.37	-0.61	AT3G26744.1
MdUI26127	-0.50	1.68	1.59	AT1G05430.1
MdUI29673	-0.75	0.74	-1.57	AT1G29355.1
MdUI32934	-1.03	2.07	0.33	AT1G29170.1
MdUI00683	-1.45	-1.25	-0.18	AT3G24480.1
MdUI03228	-1.56	-0.15	-0.31	AT5G09860.1
MdUI01075	-1.59	-1.53	-3.46	AT5G45480.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Hypothetical protein				
MdUI29646	-1.66	2.52	-0.36	AT5G26240.1
MdUI32314	-1.78	-1.69	-3.55	AT2G36485.1
MdUI21881	-2.20	-4.83	-6.28	AT2G30460.1
MdUI10722	-2.40	-1.36	-2.97	AT3G10680.1
MdUI00467	-3.13	1.88	-3.12	AT5G48360.1
Primary metabolism				
MdUI04863	5.73	3.48	5.35	AT1G06930.1
MdUI19989	5.58	5.76	6.83	AT5G08640.1
MdUI21390	4.58	5.65	4.70	AT5G48570.1
MdUI19314	4.46	3.96	0.72	AT1G67090.1
MdUI19219	4.40	3.91	0.75	AT1G67090.2
MdUI14098	4.38	3.51	0.90	AT5G38410.1
MdUI19209	4.21	4.39	0.93	AT5G38430.1
MdUI18717	4.14	3.95	0.57	AT1G67090.1
MdUI01067	4.14	3.04	1.45	AT3G11402.1
MdUI03878	3.97	2.29	0.09	AT2G23093.1
MdUI31651	3.78	2.36	4.94	AT1G76140.1
MdUI02140	3.77	4.39	0.78	AT3G16250.1
MdUI14451	3.42	4.04	1.60	AT3G17720.1
MdUI02395	3.18	3.21	1.80	AT1G64680.1
MdUI18635	3.09	2.67	5.07	AT1G76140.1
MdUI37339	2.93	4.55	2.81	AT4G15130.1
MdUI39321	2.84	3.93	1.36	AT2G44480.1
MdUI32189	2.80	3.61	3.14	AT1G07720.1
MdUI13477	2.76	4.26	3.11	AT1G16460.1
MdUI02270	2.60	3.27	3.08	AT1G07720.1
MdUI19225	2.59	5.41	2.36	AT1G59610.1
MdUI30507	2.55	-2.84	1.23	AT1G19300.1
MdUI07994	2.50	2.54	0.39	AT3G54050.1
MdUI19234	2.49	3.76	2.52	AT5G15450.1
MdUI07147	2.43	3.48	1.77	AT3G01680.1
MdUI16015	2.38	2.23	4.14	AT1G76140.1
MdUI32188	2.35	2.64	3.50	AT5G04530.1
MdUI32011	2.34	1.67	1.47	AT2G23540.1
MdUI18784	2.30	4.41	2.87	AT1G14100.1
MdUI34056	2.26	0.44	0.20	AT3G10740.1
MdUI38587	2.23	1.21	1.44	AT3G07160.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Primary metabolism				
MdUI19138	2.21	3.61	3.24	AT2G45810.1
MdUI02258	2.08	1.09	1.80	AT5G39660.1
MdUI31764	2.04	1.23	1.46	AT1G27440.1
MdUI31560	2.03	0.59	2.05	AT1G02410.1
MdUI38224	2.02	0.60	0.60	AT3G50230.1
MdUI10487	1.97	3.42	1.88	AT3G32400.1
MdUI30698	1.97	-1.05	1.20	AT2G39930.1
MdUI33712	1.96	0.37	1.45	AT2G16500.1
MdUI16180	1.82	0.37	-0.72	AT4G15396.1
MdUI19632	1.61	2.79	2.05	AT2G37790.1
MdUI32608	1.58	-0.71	2.32	AT2G17010.1
MdUI38063	1.50	2.64	-0.19	AT2G16370.1
MdUI39294	1.50	0.95	1.56	AT5G61480.1
MdUI30576	1.46	1.48	1.72	AT3G09150.3
MdUI39779	1.42	-0.06	1.90	AT4G11820.1
MdUI33771	1.38	1.29	3.06	AT3G17760.1
MdUI35578	1.38	-2.87	0.83	AT5G37830.1
MdUI34573	1.37	2.59	0.26	AT1G01780.1
MdUI38350	1.36	2.38	2.13	AT1G22190.1
MdUI33736	1.34	-0.01	1.04	AT1G44110.1
MdUI21406	1.24	2.72	2.62	AT5G64760.1
MdUI21669	1.24	3.65	1.61	AT4G39790.1
MdUI26017	1.14	1.47	1.34	AT1G61670.1
MdUI35576	1.11	0.95	0.52	AT2G36050.1
MdUI26195	1.11	-1.41	1.52	AT4G39400.1
MdUI08415	1.08	1.94	3.71	AT5G49340.1
MdUI26218	1.06	0.96	1.78	AT2G35770.1
MdUI31818	1.04	1.34	1.23	AT5G19010.1
MdUI21556	1.03	-0.90	-2.31	AT1G77670.1
MdUI20723	0.95	2.26	0.30	AT1G35620.1
MdUI39726	0.94	2.44	2.04	AT1G29400.1
MdUI06662	0.94	4.13	3.00	AT5G11040.1
MdUI18972	0.93	0.48	3.87	AT4G39350.1
MdUI38485	0.89	1.92	-0.11	AT3G11850.1
MdUI20112	0.83	2.06	1.81	AT3G57630.1
MdUI39308	0.80	2.07	1.22	AT4G19240.1
MdUI32350	0.79	-2.40	-5.71	AT4G08900.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Primary metabolism				
MdUI19778	0.78	0.40	1.20	AT3G23750.1
MdUI38950	0.68	1.70	-0.29	AT2G36390.1
MdUI31985	0.66	1.77	0.13	AT3G16110.1
MdUI25528	0.63	-1.22	1.39	AT5G02320.1
MdUI39964	0.56	1.10	1.18	AT2G16870.1
MdUI39098	0.41	-0.54	0.31	AT5G18410.1
MdUI32724	0.37	2.32	2.30	AT1G62640.1
MdUI38526	0.37	1.50	-1.22	AT1G55420.1
MdUI31463	0.35	-0.13	-0.98	AT1G77670.1
MdUI24240	0.34	1.73	-0.26	AT1G65760.1
MdUI39116	0.31	2.39	0.19	AT2G01210.1
MdUI27701	0.30	3.32	2.19	AT5G08670.1
MdUI38430	0.28	-0.59	1.07	AT3G54640.1
MdUI25357	0.27	0.26	1.82	AT3G45010.1
MdUI30408	0.26	-1.35	1.72	AT1G11800.1
MdUI29199	0.23	-0.31	1.30	AT1G50030.1
MdUI29307	0.21	0.12	1.27	AT5G11130.1
MdUI31515	0.21	0.92	-1.06	AT4G28300.1
MdUI18423	0.12	0.01	-1.48	AT3G27060.1
MdUI35592	0.07	2.38	-0.29	AT1G62440.1
MdUI24292	0.07	0.80	-0.32	AT4G38600.2
MdUI32014	0.03	-3.56	-1.81	AT5G13930.1
MdUI38747	-0.01	1.72	0.33	AT2G42270.1
MdUI24695	-0.03	1.24	0.62	AT4G22730.1
MdUI38830	-0.04	1.51	1.86	AT2G23470.1
MdUI34548	-0.04	1.44	-0.84	AT1G68400.1
MdUI06425	-0.07	-4.03	-0.16	AT2G26600.1
MdUI23899	-0.08	1.01	0.16	AT3G01090.1
MdUI39927	-0.10	-1.73	0.68	AT5G22940.1
MdUI33884	-0.14	0.69	2.03	AT1G51270.1
MdUI17916	-0.14	-0.37	-3.98	AT2G06020.1
MdUI26901	-0.17	-1.76	-2.97	AT5G04500.1
MdUI21164	-0.17	0.24	0.72	AT1G11720.1
MdUI37089	-0.22	-2.11	-1.60	AT5G43810.1
MdUI39276	-0.22	2.24	1.72	AT3G02350.1
MdUI30177	-0.29	0.35	-1.54	AT5G27380.1
MdUI01622	-0.38	0.11	-0.47	AT1G62030.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Primary metabolism				
MdUI39110	-0.39	1.03	0.09	AT5G53840.1
MdUI27667	-0.44	-0.28	-2.54	AT5G17200.1
MdUI30801	-0.49	-0.16	1.82	AT5G05730.1
MdUI20436	-0.57	-0.56	-1.94	AT5G04540.1
MdUI31506	-0.70	2.27	0.09	AT5G36890.1
MdUI36717	-0.73	-0.74	-2.35	AT1G73850.1
MdUI38874	-0.74	1.50	-0.22	AT1G69040.1
MdUI32838	-0.82	-1.29	-0.74	AT4G18570.1
MdUI38473	-0.84	0.08	1.54	AT2G36810.1
MdUI29293	-0.92	3.33	-2.08	AT3G52840.1
MdUI24220	-1.00	-2.17	-2.08	AT3G51000.1
MdUI20033	-1.05	2.88	-1.81	AT2G32810.1
MdUI03270	-1.06	-0.28	-3.81	AT4G30950.1
MdUI23867	-1.10	0.85	-0.97	AT5G27620.1
MdUI35966	-1.13	-1.66	-1.81	AT1G54926.1
MdUI14546	-1.15	-0.55	-1.62	AT5G45810.1
MdUI28221	-1.15	-1.52	-3.65	AT1G49490.1
MdUI30556	-1.19	-0.86	-1.88	AT5G44640.1
MdUI35583	-1.21	-2.39	-2.47	AT3G20170.1
MdUI20007	-1.28	4.16	-1.89	AT3G52840.1
MdUI04341	-1.30	2.41	-1.44	AT3G09570.1
MdUI00059	-1.40	-1.93	-1.84	AT1G67510.1
MdUI33923	-1.51	-1.72	-2.29	AT1G07350.1
MdUI36700	-1.53	-0.88	-1.91	AT3G43190.1
MdUI36453	-1.54	-1.99	-3.31	AT3G25805.1
MdUI27749	-1.54	-1.68	-3.75	AT2G33170.1
MdUI22271	-1.87	-1.32	-1.97	AT4G16580.1
MdUI35156	-1.90	-1.60	0.11	AT1G68010.1
MdUI06463	-2.07	-1.10	-3.19	AT5G23670.2
MdUI16702	-2.17	-3.98	-4.77	AT4G01400.1
MdUI08706	-2.23	-3.22	-4.19	AT3G07980.1
MdUI19220	-2.54	-4.05	-4.68	AT4G07960.1
MdUI00491	-2.68	-0.97	-3.48	AT1G22015.1
MdUI01164	-3.19	1.10	-4.02	AT1G18520.1
MdUI27763	-3.70	-5.65	-5.91	AT1G76930.1
MdUI33149	-4.06	0.09	-0.21	AT5G49180.1
Protein Fate (folding, modification, destination)				

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Protein Fate (folding, modification, destination)				
MdUI21557	4.52	6.13	5.11	AT4G16900.1
MdUI19308	4.18	3.85	1.20	AT1G67090.1
MdUI16033	2.32	5.01	3.82	AT5G23110.1
MdUI30890	2.01	-1.15	2.02	AT1G04820.1
MdUI32745	1.53	3.04	1.15	AT1G09780.1
MdUI26706	1.19	1.07	0.61	AT4G25640.1
MdUI18631	-0.58	0.29	-0.17	AT1G31810.1
MdUI28563	-0.92	2.27	1.17	AT3G16570.1
MdUI19411	-0.99	-1.26	-1.21	AT4G21910.3
Protein synthesis				
MdUI25431	0.97	2.25	0.81	AT4G22300.1
MdUI02544	0.81	-0.09	2.07	AT1G08540.1
MdUI33261	0.45	-1.57	-1.28	AT2G02320.1
MdUI14606	-0.55	-1.15	-0.88	AT5G42690.1
MdUI29220	-0.63	1.13	0.47	AT3G11820.1
MdUI39600	-1.26	0.57	1.82	AT1G14610.1
Protein with binding function				
MdUI25045	5.11	5.57	6.23	AT1G53750.1
MdUI31981	5.04	6.76	5.90	AT5G52640.1
MdUI20044	4.35	4.43	5.17	AT3G20720.1
MdUI03541	4.26	5.13	1.28	AT1G79190.1
MdUI13209	4.26	4.10	2.24	AT1G10657.1
MdUI14378	4.05	5.68	5.94	AT5G52640.1
MdUI19281	3.71	4.80	0.95	AT4G02540.1
MdUI02139	3.70	4.25	0.70	AT3G23780.1
MdUI19093	3.70	4.61	0.80	AT5G38430.1
MdUI18100	3.57	2.73	4.13	AT4G25800.1
MdUI20027	3.44	5.16	4.47	AT3G12580.1
MdUI18480	3.25	4.82	2.06	AT2G44140.1
MdUI20048	3.07	5.09	2.24	AT1G70020.1
MdUI08535	3.00	3.15	4.41	AT1G56410.1
MdUI19980	2.78	2.36	3.74	AT5G02500.1
MdUI19106	2.75	3.85	2.77	AT1G29930.1
MdUI04826	2.74	3.82	1.66	AT4G08180.3
MdUI13480	2.74	3.56	2.14	AT3G60190.1
MdUI31491	2.72	3.61	4.35	AT3G47910.1
MdUI03669	2.65	6.22	1.12	AT5G57110.2

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Protein with binding function				
MdUI13504	2.62	3.28	2.17	AT3G12580.1
MdUI02800	2.52	3.61	2.05	AT5G01450.1
MdUI29219	2.47	0.96	0.61	AT5G47970.1
MdUI19051	2.45	3.09	2.48	AT1G29910.1
MdUI23813	2.42	5.11	1.74	AT3G54890.1
MdUI08980	2.41	3.73	0.82	AT1G55480.1
MdUI19981	2.09	3.21	4.20	AT3G12580.1
MdUI21919	2.08	-1.53	-2.79	AT5G04940.1
MdUI23688	2.07	3.03	4.29	AT3G12580.1
MdUI18316	1.99	3.23	2.36	AT1G29910.1
MdUI35463	1.97	2.55	0.94	AT4G12750.1
MdUI22216	1.94	4.43	3.08	AT1G29910.1
MdUI28059	1.92	2.62	0.38	AT4G02560.1
MdUI28587	1.89	-1.03	2.07	ATCG00350.1
MdUI03773	1.75	4.85	2.56	AT3G07000.1
MdUI24201	1.70	2.79	1.01	AT5G47840.1
MdUI01754	1.69	3.74	2.94	AT1G29910.1
MdUI38395	1.58	-1.01	0.61	AT4G17330.1
MdUI18538	1.55	-1.48	1.10	AT3G12000.1
MdUI38413	1.37	2.57	1.86	AT2G37550.1
MdUI31172	1.29	1.93	1.44	AT5G13690.1
MdUI37966	1.09	0.68	1.17	AT1G12800.1
MdUI35567	1.02	1.67	0.67	AT5G21080.1
MdUI14412	0.90	-0.15	0.32	AT1G44446.1
MdUI34477	0.84	0.28	1.45	AT2G28360.1
MdUI39309	0.80	1.00	1.35	AT1G13110.1
MdUI28712	0.69	-0.75	-2.74	AT5G20490.1
MdUI26913	0.65	1.61	2.17	AT4G02260.3
MdUI39475	0.64	1.32	-0.22	AT4G23660.1
MdUI35483	0.38	2.00	-0.58	AT5G53150.1
MdUI38530	0.36	1.08	-0.18	AT4G15396.1
MdUI27317	0.36	1.01	-0.05	AT1G33560.1
MdUI37745	0.32	-0.85	1.82	AT1G32240.1
MdUI21312	0.29	-0.39	1.49	AT5G50780.1
MdUI37657	0.28	2.62	1.40	AT3G07300.1
MdUI19258	0.25	0.33	-0.78	AT5G55070.1
MdUI35079	0.24	-2.41	1.29	AT1G02335.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Protein with binding function				
MdUI18962	0.17	0.11	0.03	AT3G22370.1
MdUI39346	0.14	2.08	0.61	AT1G08860.1
MdUI39677	0.03	1.08	3.52	AT2G31290.1
MdUI34349	0.02	-1.24	-1.73	AT5G13800.2
MdUI26301	0.01	2.17	3.62	AT5G43990.2
MdUI15847	0.01	0.12	-3.67	AT1G01240.1
MdUI36458	-0.04	1.58	1.31	AT3G02830.1
MdUI38981	-0.09	-3.77	2.16	AT3G18215.1
MdUI20030	-0.12	1.54	1.63	AT2G28380.1
MdUI37785	-0.19	-2.04	-0.06	AT1G07920.1
MdUI38876	-0.27	2.13	0.50	AT5G12430.1
MdUI35063	-0.33	1.31	-1.62	AT2G07560.1
MdUI21697	-0.35	0.13	1.34	AT4G36950.1
MdUI19998	-0.36	-0.39	-1.17	AT5G42080.1
MdUI15984	-0.38	-1.27	-3.10	AT1G45976.1
MdUI34456	-0.47	-1.01	-1.64	AT1G78160.1
MdUI37450	-0.49	-2.25	-1.22	AT5G28540.1
MdUI27873	-0.55	-2.17	-1.90	AT1G09320.1
MdUI27643	-0.58	1.03	0.21	AT4G32200.1
MdUI38503	-0.71	0.13	1.86	AT5G61960.1
MdUI24246	-0.90	-1.84	-1.37	AT1G64750.1
MdUI09858	-1.11	-2.92	-3.37	AT3G13070.1
MdUI39904	-1.21	-0.97	-2.59	AT2G48160.1
MdUI30484	-1.27	-0.85	1.53	AT4G26630.1
MdUI16818	-1.33	0.46	-1.02	AT2G01275.2
MdUI39301	-1.46	0.25	1.38	AT2G23460.1
MdUI18663	-1.49	-3.15	-4.48	AT5G37470.1
MdUI33955	-1.49	-1.90	-2.20	AT3G27600.1
MdUI06136	-1.58	-2.11	-2.43	AT5G67110.1
MdUI10127	-1.66	-0.22	-1.99	AT5G04550.1
MdUI19978	-1.80	-1.74	-3.11	AT4G20090.1
MdUI06535	-2.11	-4.06	-5.25	AT1G78900.2
MdUI18640	-2.50	-3.33	-5.27	AT3G17660.1
Regulation of metabolism				
MdUI23814	-0.58	0.58	-2.03	AT3G49160.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Secondary metabolism				
MdUI02985	5.46	4.19	3.34	AT1G11750.1
MdUI02135	4.59	4.88	1.26	AT5G05890.1
MdUI27341	4.28	4.56	0.98	AT4G33620.1
MdUI02142	4.21	4.22	0.50	AT5G17730.1
MdUI18789	3.72	3.07	0.99	AT5G38410.1
MdUI23762	1.53	0.96	0.60	AT4G16360.1
MdUI35459	1.36	0.74	2.28	AT5G65740.2
MdUI39345	1.06	0.35	1.26	AT5G36790.1
MdUI32771	0.87	0.93	-0.14	AT5G14130.1
MdUI38335	0.86	-3.35	2.02	AT1G22660.1
MdUI29359	0.73	-0.84	-1.36	AT4G39550.1
MdUI26193	0.57	-2.19	1.84	AT3G19020.1
MdUI37656	0.50	2.97	0.53	AT3G54690.1
MdUI34673	0.43	-1.72	-1.96	AT3G28490.1
MdUI32604	0.43	2.04	-0.49	AT4G19925.1
MdUI01960	0.23	-0.64	-0.64	AT1G17680.1
MdUI36500	0.07	2.54	-0.88	AT5G11120.1
MdUI25410	-0.01	1.45	1.53	AT2G18950.1
MdUI36323	-0.10	-1.40	-2.31	AT5G20550.1
MdUI38926	-0.60	1.72	0.98	AT3G46100.1
MdUI12465	-0.71	-0.37	-3.45	AT1G03350.1
MdUI38801	-1.43	-1.74	-0.71	AT1G44880.1
Subcellular localization				
MdUI04634	5.54	-1.07	0.61	AT2G18680.1
MdUI02983	4.61	3.33	2.45	AT1G16030.1
MdUI18429	4.04	3.62	0.80	AT5G38430.1
MdUI32013	3.09	3.49	1.98	AT5G01270.1
MdUI03575	2.55	4.51	2.65	AT3G62400.2
MdUI27347	2.43	-1.83	3.35	AT5G54860.1
MdUI18974	2.13	4.03	3.54	AT5G25960.1
MdUI20015	1.55	1.65	1.52	AT5G63180.1
MdUI34716	1.34	2.26	0.08	AT2G27300.1
MdUI24087	1.25	-1.52	0.86	AT1G25280.1
MdUI39179	1.12	1.82	1.05	AT3G27473.1
MdUI39555	1.04	-0.19	1.04	AT5G57210.1
MdUI38784	0.56	0.25	1.72	AT1G11480.1
MdUI36631	0.47	2.02	-0.20	AT2G20400.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Subcellular localization				
MdUI26563	0.08	1.67	0.27	AT2G29940.1
MdUI39118	0.01	2.34	1.26	AT2G47760.1
MdUI31053	-0.18	1.47	0.27	AT1G64450.1
MdUI32910	-0.20	1.23	-1.49	AT3G50650.1
MdUI31952	-0.23	-0.41	-0.54	AT1G73860.1
MdUI00707	-0.38	-0.81	1.51	AT5G64050.1
MdUI28829	-0.48	0.64	1.93	AT1G04730.1
MdUI38727	-0.93	0.75	1.19	AT3G48380.1
MdUI37585	-1.04	-1.05	-1.57	AT1G14130.1
MdUI00611	-1.22	-2.00	-1.49	AT2G13660.1
MdUI05765	-1.36	-2.62	-0.90	AT4G39220.1
MdUI16047	-2.00	-4.21	-3.10	AT4G23630.1
Transcription				
MdUI04336	5.47	1.58	1.38	AT1G20950.1
MdUI36965	4.49	3.16	-0.03	AT1G09420.1
MdUI05272	4.25	5.40	3.94	AT1G12720.1
MdUI06494	3.73	4.15	3.21	AT4G06604.1
MdUI04825	3.41	3.98	1.81	AT3G46990.1
MdUI29973	3.37	2.73	2.44	AT4G32551.1
MdUI27138	3.32	3.53	0.39	AT5G60990.1
MdUI28090	3.11	1.44	3.54	AT3G33097.1
MdUI21189	2.97	1.08	1.56	AT4G33790.1
MdUI21691	2.92	3.86	1.64	AT5G58910.1
MdUI03609	2.69	6.14	3.16	AT4G02540.1
MdUI39172	2.52	-2.28	2.62	AT5G28056.1
MdUI04178	2.41	4.86	3.28	AT3G51390.1
MdUI29819	2.28	3.22	1.96	AT2G18360.1
MdUI11013	2.21	3.99	1.10	AT3G54910.1
MdUI39208	2.18	1.57	1.49	AT5G61140.1
MdUI28975	2.01	-2.49	0.67	AT2G34750.1
MdUI28778	1.95	1.71	0.54	AT3G10980.1
MdUI09509	1.66	4.58	1.71	AT1G02952.1
MdUI04816	1.66	3.78	2.19	AT3G46780.1
MdUI21294	1.60	2.36	-0.62	AT5G23550.1
MdUI38766	1.48	-0.53	0.70	AT4G14360.2
MdUI30310	1.40	-1.32	1.62	AT1G63360.1
MdUI26014	1.34	-0.83	1.82	AT1G03740.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Transcription				
MdUI19012	1.30	-1.98	0.82	AT4G08131.1
MdUI34576	1.19	-3.24	0.91	AT3G29290.1
MdUI29181	1.18	1.96	2.80	AT1G53290.1
MdUI38441	1.15	-1.48	-4.03	AT3G03620.1
MdUI33716	1.11	-1.17	-1.11	AT4G34860.1
MdUI37876	1.11	2.71	0.87	AT1G35405.1
MdUI38385	1.10	-0.19	1.25	AT4G00300.1
MdUI38556	1.04	3.11	0.94	AT2G23380.1
MdUI22938	1.02	-0.17	1.22	AT3G50510.1
MdUI38762	0.99	3.23	1.95	AT1G11100.1
MdUI33962	0.96	0.74	1.87	AT1G19430.1
MdUI38320	0.96	1.26	2.69	AT3G04680.1
MdUI27158	0.95	-1.71	-0.62	AT5G11010.1
MdUI23709	0.91	-2.41	1.52	AT1G52150.1
MdUI30456	0.89	4.10	0.62	AT1G76920.1
MdUI19971	0.85	-0.40	3.78	AT5G16680.1
MdUI35409	0.80	0.23	1.67	AT3G01790.1
MdUI39196	0.78	-0.43	1.79	AT1G35140.1
MdUI38460	0.76	2.61	2.64	AT4G01460.1
MdUI37832	0.74	-1.91	1.56	AT4G33090.1
MdUI35458	0.72	1.60	0.65	AT4G21865.1
MdUI37202	0.71	-0.37	-1.48	AT1G04430.1
MdUI34775	0.67	0.60	2.09	AT5G08520.1
MdUI33094	0.64	2.12	-1.11	AT1G07728.1
MdUI37682	0.63	-1.77	2.06	AT3G02000.1
MdUI26456	0.51	2.36	0.11	AT2G33120.1
MdUI39994	0.49	3.25	0.77	AT5G26660.1
MdUI21265	0.40	-0.58	-1.28	AT1G66510.1
MdUI29642	0.33	2.25	1.08	AT2G27980.1
MdUI31768	0.31	0.46	1.52	AT1G10200.1
MdUI34718	0.31	-0.22	0.53	AT1G35380.1
MdUI34717	0.27	2.94	1.47	AT3G56700.1
MdUI26111	0.26	2.85	2.77	AT1G36403.1
MdUI33783	0.24	1.34	-0.02	AT4G33600.1
MdUI38911	0.23	2.59	0.44	AT4G07935.1
MdUI02607	0.21	0.65	1.16	AT4G14980.1
MdUI19038	0.21	0.24	-0.25	AT3G15500.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Transcription				
MdUI33559	0.11	-1.49	-1.06	AT4G06594.1
MdUI10713	0.11	-0.41	0.45	AT1G69560.1
MdUI34538	0.08	1.92	0.36	AT1G74088.1
MdUI37474	0.07	1.25	-0.06	AT1G08800.1
MdUI23872	0.06	0.21	0.34	AT2G40340.1
MdUI23528	0.01	-0.42	-1.92	AT1G74080.1
MdUI29740	-0.03	-0.41	-1.57	AT3G20770.1
MdUI33866	-0.05	-1.14	0.51	AT2G46270.1
MdUI39540	-0.09	1.39	0.18	AT5G23570.1
MdUI39580	-0.13	0.77	1.43	AT2G46530.1
MdUI37707	-0.25	-0.76	1.69	AT1G31640.1
MdUI39804	-0.33	-0.87	1.18	AT5G45670.1
MdUI38557	-0.33	-0.39	0.29	AT1G58590.2
MdUI36301	-0.44	-1.81	0.52	AT1G71070.1
MdUI12743	-0.50	-0.98	-1.73	AT3G28153.1
MdUI26460	-0.65	-0.17	-3.17	AT2G44150.1
MdUI37265	-0.76	1.00	-0.03	AT5G37210.1
MdUI22343	-0.78	-0.05	-0.31	AT1G36795.1
MdUI30915	-1.04	-0.75	-0.67	AT5G29574.1
MdUI14692	-1.06	-3.74	-1.11	AT2G43730.1
MdUI37091	-1.23	0.46	-2.22	AT1G30500.2
MdUI23072	-1.28	-2.13	0.21	AT4G06593.1
MdUI14124	-1.33	-3.81	-4.94	AT3G58250.1
MdUI00011	-1.34	-2.70	-1.35	AT2G36190.1
MdUI06536	-1.51	-4.19	-4.85	AT4G02190.1
MdUI35559	-1.51	1.92	0.22	AT4G12100.1
MdUI37673	-1.60	-0.91	-1.14	AT2G35110.1
MdUI12758	-1.74	4.31	-1.50	AT4G13259.1
MdUI11839	-1.77	-3.01	-4.17	AT2G18980.1
MdUI13631	-2.17	-0.38	-3.43	AT5G45780.1
MdUI20988	-2.44	-4.66	-6.36	AT1G65540.1
MdUI00767	-3.36	-5.07	-6.18	AT5G26642.1
MdUI33289	-3.57	-0.04	0.33	AT5G05410.1
Transposase				
MdUI35543	0.24	1.30	0.74	AT3G18550.1
MdUI33916	-1.35	-1.92	-0.35	AT4G02610.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Unknown				
MdUI19237	4.66	5.26	0.04	AT1G67120.1
MdUI02129	4.55	4.04	0.82	AT1G67090.1
MdUI18445	4.52	4.56	0.83	AT5G38430.1
MdUI18644	4.47	3.87	0.81	AT5G05480.1
MdUI18655	4.45	4.12	1.08	AT1G67090.1
MdUI02136	4.31	4.98	1.00	AT3G07070.1
MdUI18602	4.24	5.31	1.02	AT5G38420.1
MdUI13158	4.11	3.36	2.45	N/A
MdUI18603	4.08	4.90	1.07	AT5G38430.1
MdUI18891	3.89	6.41	2.32	AT5G38430.1
MdUI19982	3.87	4.04	4.43	AT2G46980.1
MdUI19052	3.83	-0.67	2.43	AT4G22870.1
MdUI37115	3.81	3.24	0.12	N/A
MdUI28884	3.48	6.26	3.44	N/A
MdUI19983	3.18	4.41	5.27	AT2G47370.1
MdUI31940	3.18	4.89	2.68	AT1G62421.1
MdUI27852	3.15	3.62	2.22	AT4G18080.1
MdUI26198	3.13	1.71	2.55	N/A
MdUI19370	2.98	3.18	1.84	AT2G30950.1
MdUI12002	2.91	4.08	4.32	AT5G49830.1
MdUI03579	2.85	3.92	1.98	AT4G19220.1
MdUI28330	2.66	2.16	0.72	AT1G67090.1
MdUI25211	2.64	2.93	3.59	AT1G17790.1
MdUI18650	2.57	3.40	1.53	AT5G42450.1
MdUI21609	2.41	2.72	2.86	N/A
MdUI04179	2.37	5.44	3.66	AT4G01590.1
MdUI33910	2.30	4.58	1.95	N/A
MdUI03774	2.16	5.00	2.73	AT4G10640.1
MdUI39268	2.12	-0.14	1.63	AT3G04450.1
MdUI38499	2.10	0.07	2.37	AT3G54230.1
MdUI11015	2.10	3.75	0.94	N/A
MdUI31796	2.08	3.34	3.94	AT5G18760.1
MdUI19445	1.89	3.14	4.82	N/A
MdUI29246	1.84	3.66	1.91	AT5G03900.1
MdUI30209	1.81	-2.48	-3.00	AT1G18950.1
MdUI34621	1.77	0.86	-0.03	N/A
MdUI26040	1.68	1.82	2.22	N/A

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Unknown				
MdUI38502	1.63	-2.46	2.21	AT4G12750.1
MdUI18506	1.61	3.79	3.06	AT1G78270.1
MdUI33747	1.51	-1.39	0.74	AT1G71340.1
MdUI29341	1.50	2.00	-1.39	N/A
MdUI05817	1.48	6.33	0.90	AT5G64470.2
MdUI39388	1.45	-1.64	0.55	AT2G28840.1
MdUI33743	1.44	2.08	1.62	AT5G22390.1
MdUI28886	1.44	-0.72	0.10	AT5G46880.1
MdUI19300	1.42	3.57	3.10	AT3G62480.1
MdUI34058	1.32	0.65	1.09	AT1G64690.1
MdUI19037	1.30	0.65	-0.02	AT4G28260.1
MdUI36242	1.29	-0.69	-1.56	N/A
MdUI32783	1.24	2.00	0.48	AT4G36500.1
MdUI21130	1.22	1.52	0.83	AT1G34220.1
MdUI37601	1.17	1.59	1.19	N/A
MdUI38365	1.13	1.45	1.03	N/A
MdUI20722	1.12	0.76	-0.94	N/A
MdUI32518	1.10	0.76	2.18	AT1G03090.2
MdUI23968	1.09	2.14	1.54	AT5G18525.1
MdUI33774	1.05	0.62	0.16	AT5G35980.1
MdUI35556	1.04	-1.84	0.44	AT2G28880.1
MdUI32423	1.03	-0.65	-0.04	AT3G25185.1
MdUI03627	1.03	0.99	3.58	N/A
MdUI38403	1.00	-0.12	2.12	AT3G06510.1
MdUI29736	0.98	1.07	1.07	AT3G13010.1
MdUI29545	0.96	1.66	1.68	N/A
MdUI28110	0.89	0.16	0.67	AT1G35470.1
MdUI33821	0.87	1.46	1.66	AT5G04550.1
MdUI38065	0.86	1.83	0.76	AT5G06440.1
MdUI26659	0.86	0.96	-0.55	N/A
MdUI35363	0.76	1.34	1.93	N/A
MdUI28254	0.75	-0.31	0.28	AT3G03640.1
MdUI37649	0.74	-1.07	-2.57	N/A
MdUI32658	0.73	-0.14	0.78	AT2G01180.1
MdUI35447	0.64	1.72	0.60	AT4G01290.1
MdUI29544	0.63	0.72	2.11	AT4G35730.1
MdUI30362	0.59	-0.83	-1.34	N/A

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Unknown				
MdUI20019	0.57	3.73	1.80	N/A
MdUI33769	0.56	0.65	0.65	AT5G24570.1
MdUI28209	0.54	-1.39	1.80	AT5G47800.1
MdUI35384	0.51	1.12	0.99	N/A
MdUI23212	0.50	0.27	1.69	AT3G44670.1
MdUI38005	0.49	1.32	3.94	N/A
MdUI23754	0.49	1.04	1.43	N/A
MdUI33772	0.47	0.59	1.81	AT1G80480.1
MdUI16500	0.45	-1.92	-1.83	AT2G46280.1
MdUI34763	0.44	-1.59	1.88	AT1G49890.1
MdUI23849	0.39	0.59	1.78	AT3G14850.2
MdUI38213	0.38	-0.04	1.26	AT4G34880.1
MdUI19728	0.37	2.38	1.33	N/A
MdUI26513	0.30	0.13	-2.51	AT2G22010.1
MdUI30459	0.28	0.45	0.99	N/A
MdUI38309	0.27	-3.01	-0.18	AT4G39610.1
MdUI03679	0.26	1.70	4.99	AT2G14530.1
MdUI28826	0.26	3.10	1.04	N/A
MdUI35388	0.25	-1.28	0.39	N/A
MdUI39141	0.23	1.28	-0.45	AT5G18740.1
MdUI26224	0.23	1.28	0.89	AT2G22720.2
MdUI39373	0.23	1.67	0.59	AT1G71980.1
MdUI35750	0.22	-1.44	-1.01	N/A
MdUI35268	0.19	1.20	-0.35	N/A
MdUI10909	0.17	-1.39	-2.69	AT5G33200.1
MdUI30363	0.15	-1.47	2.12	AT1G73260.1
MdUI39271	0.13	2.31	1.52	N/A
MdUI21643	0.12	1.52	0.87	AT3G49410.1
MdUI38610	0.10	-2.62	-0.29	N/A
MdUI36953	0.09	0.41	1.52	AT5G49220.1
MdUI35308	0.08	-1.58	-2.43	N/A
MdUI29183	0.00	0.02	-2.47	AT3G27280.2
MdUI02008	-0.03	-0.78	-0.97	N/A
MdUI38582	-0.06	2.19	1.69	AT1G69510.3
MdUI28363	-0.07	-1.61	-1.55	AT5G25820.1
MdUI24615	-0.07	0.59	1.60	N/A
MdUI00539	-0.08	-2.45	-1.16	AT5G04110.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Unknown				
MdUI34707	-0.08	-0.45	-1.16	N/A
MdUI19015	-0.09	-0.73	1.45	AT2G27630.1
MdUI15522	-0.11	-0.47	-3.78	AT5G39400.1
MdUI34822	-0.14	2.12	0.30	AT3G49720.1
MdUI27304	-0.16	1.31	2.04	AT1G75420.1
MdUI37923	-0.17	-1.76	-0.84	AT5G67030.1
MdUI21896	-0.19	-1.34	-4.41	AT5G53905.1
MdUI36511	-0.19	0.62	-0.67	AT1G67340.1
MdUI25127	-0.23	1.57	1.20	N/A
MdUI34019	-0.23	1.42	-0.88	AT5G12150.1
MdUI36584	-0.24	0.50	1.13	AT1G04800.1
MdUI31543	-0.24	1.24	1.98	AT3G42120.1
MdUI33814	-0.26	-1.47	-0.85	N/A
MdUI23091	-0.28	1.24	-1.50	AT3G62770.2
MdUI00563	-0.29	0.38	-0.10	AT2G46560.1
MdUI22309	-0.31	2.11	0.04	AT5G66950.1
MdUI32410	-0.35	-2.13	-1.23	AT4G27870.1
MdUI39651	-0.37	-1.37	-0.37	N/A
MdUI38685	-0.39	1.38	0.16	N/A
MdUI38248	-0.39	1.40	0.63	AT3G03305.1
MdUI37337	-0.43	-1.60	0.60	N/A
MdUI37736	-0.46	-2.07	1.00	AT1G74350.1
MdUI33367	-0.46	-1.76	-0.94	AT4G32260.1
MdUI33317	-0.46	0.49	-1.94	AT3G06550.1
MdUI35960	-0.47	-1.94	-1.94	AT3G05670.1
MdUI23413	-0.54	-0.30	-3.20	AT3G14920.1
MdUI33644	-0.56	0.07	2.33	AT2G35150.1
MdUI39418	-0.58	1.84	1.51	AT3G27020.1
MdUI38044	-0.60	0.34	2.08	AT2G14830.1
MdUI28514	-0.66	0.25	-2.08	AT5G45840.1
MdUI32230	-0.69	-0.52	2.20	AT2G31560.1
MdUI08804	-0.71	-2.13	-3.34	AT5G04940.1
MdUI39834	-0.75	-1.99	-0.42	AT1G29200.1
MdUI32925	-0.78	0.24	-1.80	N/A
MdUI35833	-0.90	-2.06	-2.29	N/A
MdUI34306	-1.01	-0.65	-3.09	N/A
MdUI20662	-1.07	-1.15	-2.62	AT2G24920.1

Table A1 (cont.) Full list of significant genes differentially expressed in three time points

ID	Week1	Week2	Week3	Accession Number
Unknown				
MdUI37593	-1.13	-2.40	-1.90	N/A
MdUI01973	-1.14	-4.53	-5.06	N/A
MdUI00442	-1.15	-1.50	-0.04	AT2G42950.1
MdUI38691	-1.20	1.26	-1.51	AT1G04090.1
MdUI16466	-1.21	-1.74	-1.38	AT1G58120.1
MdUI32351	-1.34	-4.97	-5.28	N/A
MdUI00587	-1.40	-0.75	-5.03	N/A
MdUI35234	-1.41	-1.13	-2.44	N/A
MdUI39072	-1.55	1.99	1.02	AT1G50710.1
MdUI32703	-1.64	-3.18	-5.13	N/A
MdUI05894	-1.81	-1.34	-3.04	AT2G38840.1
MdUI02994	-1.93	-1.05	-3.70	N/A
MdUI00443	-2.04	-2.45	-4.12	AT2G27790.1
MdUI00635	-2.09	-4.89	-0.72	AT5G17530.1
MdUI08705	-2.13	-3.09	-4.45	AT5G26270.1
MdUI06717	-2.91	-3.77	-5.67	AT1G43205.1
MdUI28027	-3.32	-5.02	-5.07	AT1G71680.1
MdUI31856	-3.70	1.40	-3.00	AT5G64570.1
MdUI11708	-4.40	1.16	-4.09	AT1G02640.1