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# Transcriptome analysis of the exocarp of apple fruit identifies light-induced genes involved in red color pigmentation

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ABSTRACT

Although the mechanism of light regulation of color pigmentation of apple fruit 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, fruits on trees of apple cv. Red Delicious were covered with paper bags during early stages of fruit development and then removed prior to maturation to analyze the transcriptome in the exocarp of apple fruit. Comparisons of 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 of 40,000 sequences. Expression profiles were investigated over three time points, at one week intervals, during fruit development. Overall, 736 genes with expression values greater than two-fold were found to be modulated by light treatment. Light-induced 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, these 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.

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## 1. 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, are in high demand. 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 structural genes involved in the anthocyanin biosynthesis pathway (Davies and Schwinn, 2003); moreover, these genes are transcriptionally controlled by various transcription factors (TFs), including members of MYB, bHLH, WD40, WRKY, bZIP, and MADS-box protein families (Davies and Schwinn, 2003; Kubo et al., 1999; Ramsay and Glover, 2005).

The anthocyanin biosynthesis pathway has been well characterized genetically and biochemically (Grotewold, 2006; Holton and Cornish, 1995; Martin et al., 2001; 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 occurs early in fruit development 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 influenced 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 following exposure

*Abbreviations:* TF, transcription factor; qRT-PCR, quantitative real time-polymerase chain reaction; DAP, days after pollination; w, week; EST, expressed sequence tag.

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to light (Ban et al., 2007a,b; 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., 2007b; 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 linkage group (LG) 4 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 investigating differential expression of genes regulated by light.

Previously, studies on light-influenced gene expression have been reported in *Arabidopsis*, and these have focused on the impact of high-light on oxidative stress (Kimura et al., 2003; Rossel et al., 2002). Kimura et al. (Rossel et al., 2002) reported that genes involved in the biosynthesis of lignins and flavonoids were activated by high-light, leading to increased accumulation of both lignins and anthocyanins in addition to those scavenging enzymes of reactive oxygen species (ROS). Global expression profiles have been undertaken in apple, but most have been focused on understanding fruit development (Bizjak et al., 2013; Janssen et al., 2008; Lee et al., 2007; Park et al., 2006; Soglio et al., 2009; Soria-Guerra et al., 2011) and aroma production (Schaffer et al., 2007; Seo and Kim, 2009). Previously, suppression subtractive hybridization (SSH) has been used to identify differentially expressed genes in apple exocarp induced by UV-B irradiation (Ban et al., 2007a); however, identification of genes using SSH is limited, and only structural genes related to anthocyanin biosynthesis pathway have been reported. In this study, global gene expression profiles of fruit of red-colored apple cv. Red Delicious have been investigated during dark treatment and following exposure to 14 h of light treatment. By exploiting a 40,000 gene apple microarray, we have been able to identify putative genes whose expression is influenced by light and found to be involved in the flavonoid biosynthesis pathway.

## 2. Materials and methods

### 2.1. 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 (dark treatment) for 97 days after pollination (DAP), 104 DAP, and 111 DAP, designated as week 1 (W1), W2, and W3 groups, respectively. At each time point, bags were removed from six fruits, and these fruits were left on the tree for another period of 24 h and exposed to daylight (essentially 14 h of exposure to light conditions). Following treatment, six fruits were harvested from each of non-bagged fruits (treatment 1), used as control, dark-grown fruits (treatment 2), and 14 h of light-exposed fruits (treatment 3). Each of these fruits was peeled, using an apple peeler, and the peel (exocarp) was immediately frozen in liquid nitrogen, and stored at −80 °C.

Frozen fruit exocarp tissues were ground, and total RNA was isolated from six pooled samples 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 nanodrop (NanoDrop Technologies Inc., Wilmington, DE, USA). cDNA was synthesized three separate times from RNA isolated from these six pooled excocarp samples.

### 2.2. 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 included 13,656 cluster contigs, 6,404 cluster singlets, 16,966 singletons, and 2374 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 Omni Gridarrayer (Newport) in a total of 48 blocks. Moreover, sequences of oligonucleotides were compared with the predicted coding sequences in Genome Database for Rosaceae (GDR) (Velasco et al., 2010).

### 2.3. 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.

### 2.4. Microarray experimental design

The microarray experiments were conducted using a total of three slides for each time point, and including a Cy3-Cy5 dye swap (Table 1). Comparisons of gene expression between treatments 2 (dark grown)

**Table 1**
Microarray experimental design.

| Treatment | 1 | 2 | 3 |
|---|---|---|---|
| | | | |
| Description | Control (standard photoperiod) | Dark-grown (continuous dark) | 14 h light treatment |
| No. technical replicates* | | | 3 |
| Dye-swap | | | 1 |
| Total Cy3/Cy5 hybridizations | | | 3 |

*RNA was isolated from six pooled fruit excocarps. cDNA was synthesized three separate times from this pooled RNA. A microarray comparison was performed separately for each time point (W1, W2, and W3).

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