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Full Length Research Paper

Effect of bag removal on Fuji apple coloration

QU Shen-chun[1], DU Bei-bei[1], XIA Jing[1], ZHANG Ji-yu[2], LU Dong[1], Gao Zhi-hong[1] and Zhang Zhen[1]*

[1]College of Horticulture, Nanjing Agricultural University, Nanjing, 210095 P. R. China.
[2]Institute of Botany, Jiangsu Province and the Chinese Academy of Sciences, Nanjing, Jiangsu 210014 P. R. China

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Red Fuji apple (*Malus domestica* Borkh, cv. 'Nagafu No. 2') fruit was used to study the variation of pigment content and the activity of phenylalanine ammonia-lyase (PAL) in the peel and sugar content of apple flesh during growth and development after bag removal. The transcription regulatory factor *MYB10d* (GenBank Accession Number: GQ183802) was cloned and its expression in fruit was measured at different stages after bag removal. The results showed that total chlorophyll content significantly declined in double-bagged fruit as compared to control fruit, and this effect was more pronounced for chlorophyll a than chlorophyll b. Carotenoid content declined less than the chlorophyll content. Anthocyanin content was noticeably lower than control fruit at bag removal but rapidly rose following removal. The anthocyanin content exceeded the control value on the 6th day following removal and was approximately double the control group on the 8th day after bag removal. The activity of PAL tended to rise and then declined after bag removal in the treated fruit, while that of the control fruit decreased slowly. On the 2nd day after removal, the activity of PAL in treated fruit exceeded the control fruit and reached a maximum on the 6th day after bag removal. The total soluble sugar content was somewhat reduced by bagging. However, the total soluble sugar content (fructose and glucose) increased after bag removal, although, it was lower than the control fruit and showed a significant positive correlation with anthocyanin content. Sucrose content increased rapidly until the 4th day after bag removal and then declined. *MYB10d*-coded protein, a member of the MYB transcription factor family was related to anthocyanin biosynthesis. Quantitative real time PCR (qRT-PCR) was employed to examine the gene expression levels at different phases after bag removal, and the results indicated that *MYB10d* expression in treated fruit reached a maximum on the 2nd day after bag removal and declined sharply thereafter. Conversely, *MYB10d* expression in control fruit remained stable.

Key words: Apple, *MYB10d*, bag removal.

INTRODUCTION

Red Fuji apples (*Malus domestica* Borkh.*cv.* Red Fuji) are one of the most important cultivated species in China that has the highest cultivation area and yield in the world (http://www.fao.org/corp/statistics/zh/).

This species is popular with producers and consumers because of its large size, high quality and good preservation. Consumers demand high quality fruit; consistent appearance and coloring are major price determinants. Therefore, improving fruit quality to accommodate market demand has become one of the most pressing issues for fruit producers.

Bagging is an important technique for producing high-quality apples. It is thought to promote attractive coloration, improve the smoothness, reduce pesticide residue, expand the overseas market and maintain sustainable development of the fruit industry. Over time, this technology has been widely applied to production, and research into the practice has increased. However, due to the wide variation of climates in China, inappropriate selection of fruit bags and bagging time might

*Corresponding author. E-mail: zhangzh@njau.edu.cn. Tel: +86-025-84395724. Fax: + 86-025-84395266.

Abbreviations: qRT-PCR, Quantitative real time PCR; **TFM,** thiophanate methyl; **M-MLV,** moloney murine leukemia virus; **dNTP,** deoxy-ribonucleoside triphosphate; **AR,** analytical reagent; **IPTG,** isopropyl β-D-1-thiogalactopyranoside; **DTT,** dithiothreitol; **OD,** optical density; **PAL,** phenylalanine ammonia-lyase; **CTAB,** cetyltrimethylammonium bromide; **EST,** expression sequence tag.

result in large product losses.

Jiangsu Province is an important apple production area located in the Southern rim of China's apple region (http://www.jsagri.gov.cn/). Paper bags are required because of the area's abundant rainfall, but systematic research has not been carried out on paper bags for many years. As a result, there is no technical basis for bagging practices.

In this study, Red Fuji apples were bagged in Kobayashi paper bags (Japan); these bags were selected based on standard local practices. We sought to evaluate the effects of paper bags on infections, internal and/or external quality and pigments and sugar content after bag removal. To investigate pigment, we measured the coloration-related *MYB* transcription regulatory factor.

MYB is one of the largest plant transcription factor families involved in cell morphogenesis, environmental responses and cell-cycle progression (Chagné et al., 2007), and it was originally cloned from the Red Fuji apple. R2R3*MYB* is a regulatory protein that participates in metabolic pathways of phenyl propane and anthocyanin biosynthesis (Ludwing et al., 1989). Here, we report preliminary research on gene expression modes at different stages after bag removal, and the molecular mechanism of regulation of coloration functions during bagging is discussed.

MATERIALS AND METHODS

Plant material

The experiments were carried out in 2007 on 'Nagafu No. 2' Fuji apples from Songlou Town, Fengxian County, Xuzhou City, which is the centralized apple production area of Jiangsu Province. The fruit was grown in well-managed, ten-year-old orchards with sandy loam soil with 3 × 5 m row spacing. Strong trees with similar growth and fructification status were selected for the experimental treatments.

Bagging was conducted 41 days after the flowers' wilt (May 18th) on a sunny day. β-cypermethrin (diluted 1: 1000 in water) and thiophanate methyl (TFM, diluted 1: 500 in water) were sprayed and the apples were not bagged until they were air-dried in Kobayashi bags (Japan, manufactured by Kobayashi Co. Ltd., Qingdao) consisting of a greyish-yellow outer bag, a black inner layer and red inner bag chosen as the experimental bag.

Fruit from 4 trees were selected, and unbagged apples served as controls. The fruits were bagged May 18th through September 20th and then the apples were kept at ambient temperature (18-32ºC) for 8 d. Samples were collected once on the removal day, and every other day afterwards for a total of five samples. Each time, 15 fruits were randomly sampled around the crown at the distance of 1.5 m from the ground.

Strain and plasmid

The *Escherichia coli* strain DH5α was preserved by the laboratory and the cloning vector pMD19-T purchased from TaKaRa Corporation.

Reagents

The first strand of cDNA Synthesis Kit, T4DNA ligase and M-MLV were purchased from Promega Co. The restriction endonuclease, rTaq enzyme, four types of dNTP and Gel Extraction Kit were purchased from TaKaRa Co. The quantitative real-time PCR (qRT-PCR) kit was purchased from TOYOBO Co. All primers were synthesized by Yingjun Co. Ltd. (Shanghai). Other analytical grade reagents including isopropyl β-D-1-thiogalactopyranoside (IPTG), imidazole, acrylamide, β-mercaptoethanol and dithiothreitol (DTT) were purchased from Sigma Co.

Pigment determination

Anthocyanin and chlorophyll content in the peel were measured by colorimetric analyses (Ishikura and Hayashi K, 1963; ISO 10260: 1992). The total soluble sugar content was determined with anthrone colorimetric analysis at a wavelength of 620 nm and calculated from the standard curve of sucrose (Yemm and Willis, 1954). By detecting the optical density (OD) at a wavelength of 540 nm through 3, 5-dinitrosalicyclic acid, the content of reducing sugar was calculated from the standard curve of glucose (Somogyi, 1952). Sucrose, glucose and fructose contents were determined with the anthrone colorimetric analysis (Nelson, 1944). The determination of phenylalanine ammonia-lyase (PAL) was conducted by detecting the OD value at 290 nm (Cheng and Breen, 1991; Zucker, 1965).

All of the aforementioned determinations were performed in triplicate on five randomly selected fruits per treatment at each time point.

Extraction of total RNA and cDNA synthesis

Total RNA was isolated using a Cetyltrimethyl ammonium Bromide (CTAB) protocol (Chang et al., 1993). Genomic DNA was eliminated with RNase-free DNase I (TaKaRa Co., Code No: D2215), according to the manufacturer's instructions. Single-strand cDNA synthesis was performed using the ReverTra Ace-α-^{TM} Kit (Toyobo Co., Ltd., Osaka, Japan).

Cloning of *MYB10d* gene

In accordance with the expression sequence tag (EST) of apple registered in GenBank, gene-specific primers were designed:

F1: 5'-GGTGCCTGGACTCGAGAGGAAGACAATC-3' and

F2: 5'-CAGGCAGTGCGTTGAGATTCATGGAGAG-3'.

Reverse transcription was performed using F1 and F2 primers to amplify the *MYB10d* gene (PCR amplification conditions: 94℃ 4 min; 94℃ 30 s, 58℃ 45 s, 72ºC 1 min, 35 cycles; 7 2ºC 10 min). The purified PCR products were inserted into the pMD19-T vector (TaKaRa) after recycling. Competent *E. coli* DH5α cells were transformed through thermal stimulation. The bacterial solution (100 µL) was spread in ampicillin-containing selection medium with X-gal and IPTG and cultured at 37ºC for 16 h. Then, single white colonies were randomly selected to carry out the inoculation in LB liquid medium containing ampicillin (50 mg/L). After an overnight cultivation at 37ºC, PCR identification was conducted with the bacterial solution as the template. cDNA sequence analysis was performed with software including DNAman and DNAclub as well as, the NCBI website (http://www.ncbi.nlm.nih.gov) and confirmed by Yingjun Biotech Co., Ltd.

Expression of MYB10d after bag removal

The total RNA was isolated from apple peels on the 2nd, 4th, 6th and

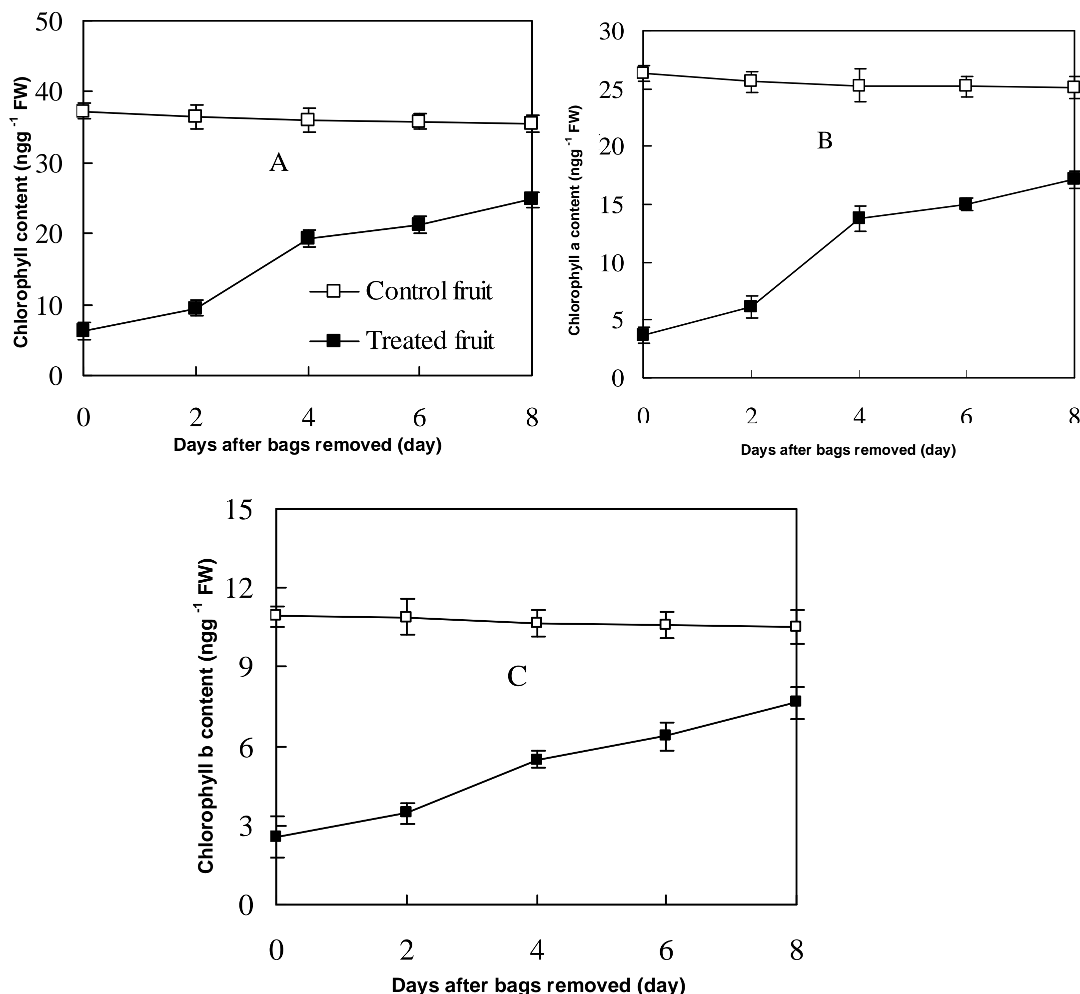

Figure 1. Samples were measured every 2 d over 8 d after bag removal using 15 apples per sampling time per group. The experiment was replicated twice, and was performed in triplicate each time. Vertical bars represent the mean ± standard deviation (SD). The same below. Dynamic changes of total chlorophyll content (Panel A), chlorophyll a content (Panel B) and chlorophyll b content (Panel C) in red Fuji apples.

8th days after bag removal. Relative quantification was performed with SYBRgreen II *MYB10d* gene expression (primers detailed previously) was determined based on the relative quantification with the actin standard internal primers of Red Fuji apple:

ACT-F1: 5'- AGGTCCATCCATTGTCCACAG -3' and

ACT-R1: 5'-TGCCAACCAAACTGACTTCAC -3'.

Real-time quantitative fluorescent PCR was amplified with the specific primers:

qPCRMYB-F: 5'-AAGACCTCAGCCCCAAAAAT-3' and

qPCRMYB-R: 5'-TGTCCTTCAGGAAAATTGGC -3'

(PCR conditions: 94ºC 2 min; 94ºC 10 s, 60ºC 15 s; 72ºC 15 s, 45 cycles; 72ºC, 10 min). After the target gene fragments were amplified, the expression profiles under hormone treatments and different interventions were produced.

RESULTS

Dynamic changes in pigment content, PAL and chlorophyll content in red fuji apple peels

Chlorophyll a (bluish-green) and chlorophyll b (yellowish-green) are mainly produced in higher plants. After bag removal, the content of chlorophyll in the treated apples began to increase on the 2nd day, and a marked rise occurred from the 2nd day to the 6th day before slowing. However, the total chlorophyll content of control fruit remained stable and was always higher than the bagged fruit (Figure 1A). The content change trends of chlorophyll a and chlorophyll b were similar to that of the total chlorophyll content (Figure 2B). After bag removal, chlorophyll a content was always significantly higher than that of chlorophyll b (Figure 1B to C), suggesting that

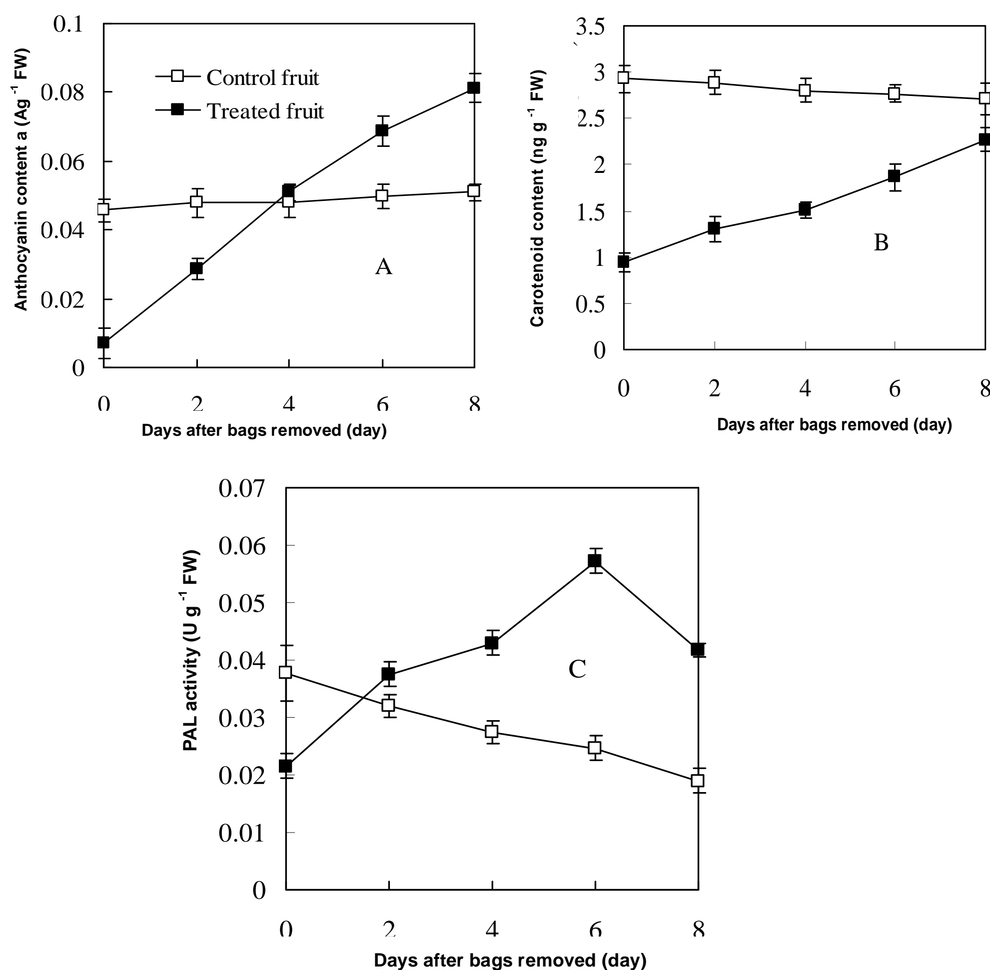

Figure 2. Dynamic changes of anthocyanin content (Panel A), carotenoid content (Panel B) and PAL activity (Panel C) in red Fuji apples.

total chlorophyll elevation was mainly due to chlorophyll a.

Dynamic change of carotenoid content

Compared with chlorophyll, carotenoid content was very low. The carotenoid content of treated apples increased following bag removal, whereas controls had a slow and slight decrease (Figure 2B). However, anthocyanin, the most crucial pigment, is obviously increased after bag removal. The anthocyanin content of treated fruit exceeded the level of control fruit after 4 days and eventually was double the value measured in control fruit (Figure 2A).

Change of PAL activity after bag removal

PAL is a key anthocyanin synthesis enzyme and therefore, its activity is an indirect measure of anthocyanin synthesis. Enzyme activity increased for the first six days after bag removal and was higher than controls from the 2nd day onwards. Activity began to decline on the 6th day after removal, but it was still higher on the 8th day than it was on the first day (Figure 2C) .

Dynamic change of sugar content

After bag removal, total sugar, fructose, sucrose and glucose levels tended to increase with the exception of a decreasing trend for sucrose from the 4th day on. Although, the treated fruit had lower sugar levels throughout, the disparities diminished over time (Figure 3). Fructose was the main soluble sugar (50 to 70 mg/g.FW), followed by sucrose (20 to 40 mg/g.FW) and glucose (10 to 30 mg/g.FW). From the 2nd to the 6th day, glucose content in treated fruit increased rapidly but remained significantly lower than that of the control group

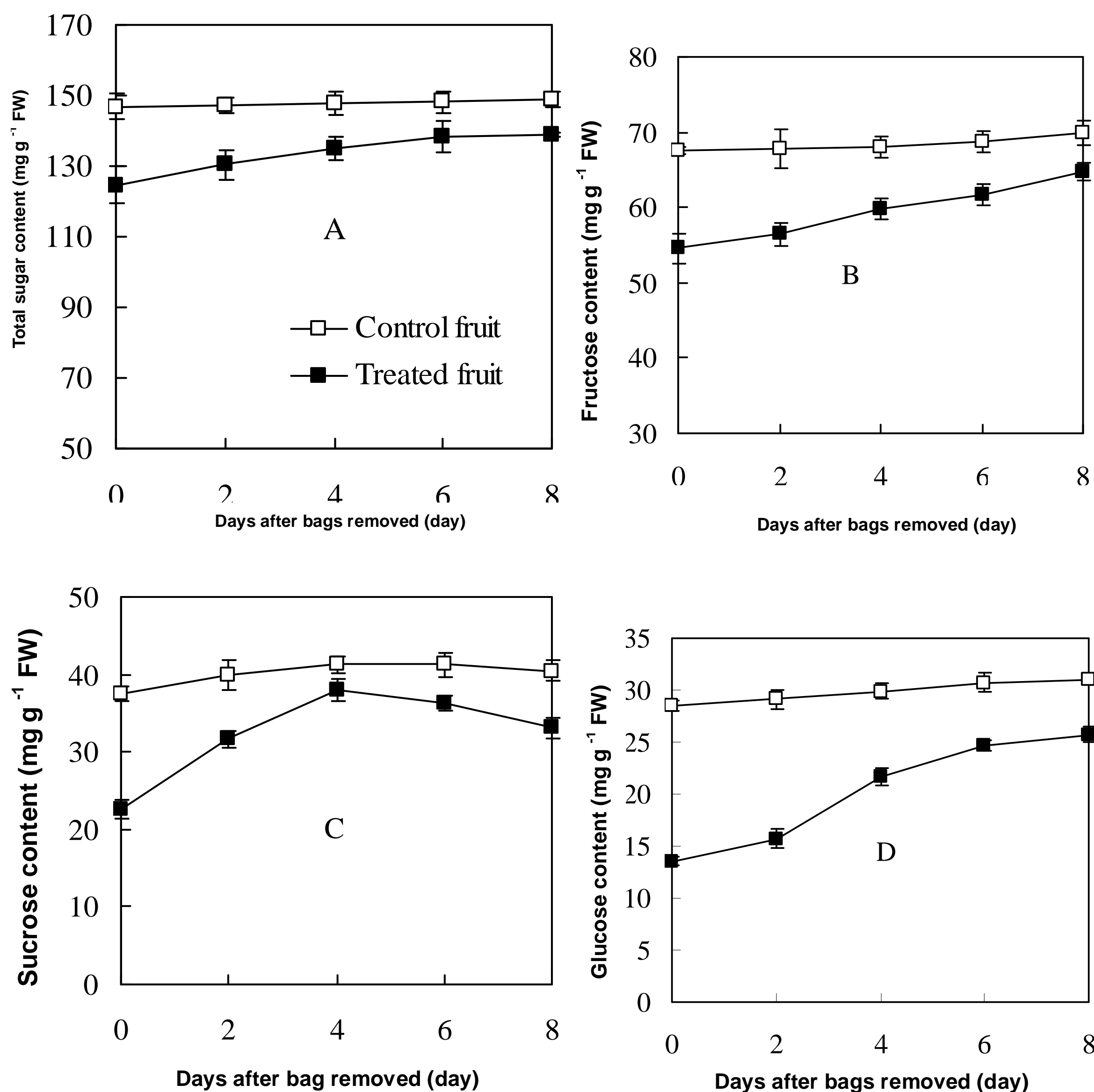

Figure 3. Dynamic change of total soluble sugar content (Panel A), fructose content (Panel B), sucrose content (Panel C) and glucose content (Panel D) after bag removal in red Fuji apples.

($P < 0.01$) (Figure 3 D).

MYB10d cloning and real-time quantitative fluorescent PCR analysis

Cloning and sequential analysis of cDNA sequence at the 3'-terminal of MYB10d

A cDNA fragment of the *MYB10d* gene was cloned from apple peels two days after bag removal using RT-PCR. The cloned fragment of cDNA was 508 bp in length and resulted from a sequence encoding a 170 amino acid-long protein (GenBank Accession Number: GQ183802). DNAman analysis showed that the homology between *MYB10d* in red Fuji apple peels and other *MYB* genes relevant to plant anthocyanidin synthesis was extremely high, up to 99% according to the reported *MYB* gene of apple (GenBank Accession Number: ABK58138).

Analysis of the expression mode of MYB10d gene

Further investigations were carried out on *MYB10d* gene expression at different stages after bag removal, and the cDNA fragment of *MYB10d* was used as a probe. In accordance with the qRT-PCR results, there were significant differences in *MYB10d* gene expression for 8

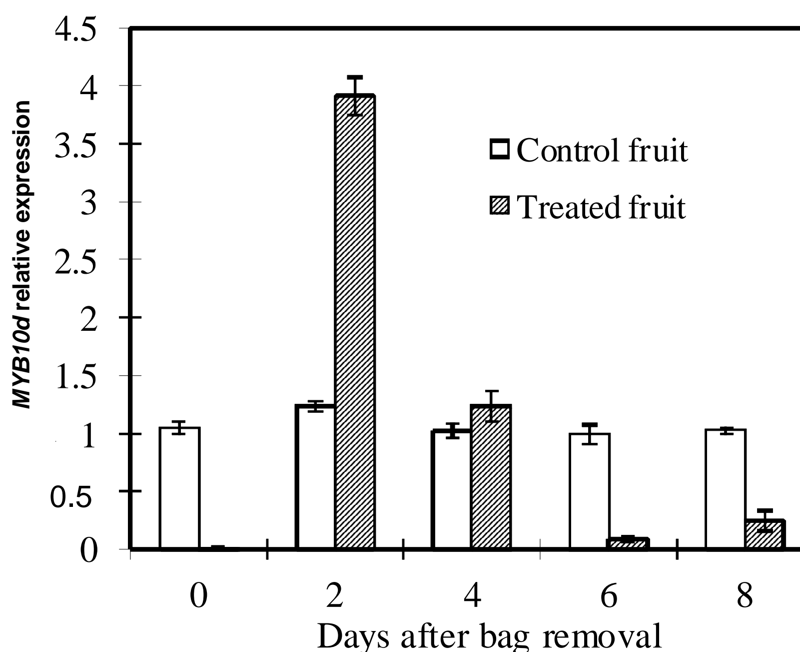

Figure 4. Relative *MYB10d* expression levels at different times after bag removal.

days after bag removal. As indicated in Figure 4, the expression of *MYB10d* in treated fruit reached a maximum of two days after bag removal, followed by a sharp decline and nearly undetectable expression six days after removal, this result was consistent with the degree of fruit coloration. However, the expression amount of *MYB10d* in control fruit was about actin and remained stable throughout the experiment. The results suggested that bagging affects anthocyanin-synthesis-relevant transcription factor activity, and this has an effect on pigmentation.

DISCUSSION

The ultimate manifestation of pericarp color in red fruits was determined by the integrated effects of chlorophyll, carotenoid and anthocyanin; of these pigments, anthocyanin has a decisive role in coloration, and chlorophyll masks the red hue (Lancaster et al., 1994).

In apples, elevated anthocyanin content promotes cardinal red, deep (purple) red, and some other colors. When the anthocyanin content was relatively stable, the fruit was dark red with high chlorophyll content, and the color was cardinal red with low chlorophyll content (Wang et al., 2002a). The experimental outcome showed that the contents of chlorophyll and carotenoid had declined in the peels of bagged fruits. Although, these values rose after bag removal, the chlorophyll content of bagged fruits remained significantly lower than that of the control fruit which contained approximately 1.83 times more chlorophyll. Thus, the interference effect of chlorophyll on the chromogenic background which had been beneficial to the fruit coloration was decreased.

In accordance with the total chlorophyll content of peels from bagged apples, the content and variation range of chlorophyll a was significantly higher than that of chlorophyll b, which suggested that total chlorophyll content elevation might be mainly due to an increased amount of chlorophyll a. Compared with the controls, bagged fruit had lower carotenoid content from bag removal to harvest. The anthocyanin content rapidly increased after removal and this was similar to control fruit on the 4th day, and was two-fold greater than unbagged fruits on the 8th day.

PAL is the first enzyme in the processes of phenylpropanoid metabolism and anthocyanin synthesis; its activity is regulated by light and accomplished with phytochrome (Lancaster et al., 1994). After bag removal, fruit peels were white or yellowish-white with a high level of phytochrome. Exposure to light activated and induced PAL could be the reason for the rapid synthesis of anthocyanin after bag removal (Proctor and Lougheed, 1976). However, chlorophyll synthesis was also inhibited in the bag. Thus, the content of chlorophyll had been artificially low, and the masking effect on anthocyanin was greatly weakened, and this promoted the brilliant color of the bagged fruits.

Consistent with the trend of anthocyanin content in the fruit peel, PAL activity was significantly elevated for the first six days after bag removal, which might suggest a relationship between PAL activity and anthocyanin synthesis. As in previous studies (Wang et al., 2002b), the activity of PAL could be reduced through bagging with

improvement of activity after removal.

The function of sugar in anthocyanin synthesis is complex; it participates in the formation of anthocyanin with anthocyanidin within the vacuole, but could also be a crucial transduction signal for anthocyanin synthesis (Uddin et al., 2001; Hiratsuka et al., 2001). Anthocyanin is the major pigment for the red peel coloration. There was a significant positive correlation between anthocyanin content and coloration in red Fuji apples as well as, the contents of reducing and soluble sugars in fruit flesh.

The total soluble sugar in bagged Fuji apples was similar to that of the controls, although, it was slightly lower (Xia et al., 2009). The accumulation laws of reducing sugar and total soluble sugar contents were similar; however, the content of reducing sugar was lower than that of total soluble sugar. After bag removal, total sugar, fructose, sucrose, and glucose levels increased; sucrose decreased slightly after the 4th day postharvest, but fructose and glucose continued to increase. It is tempting to speculate that sucrose in the peel instigated the increase in anthocyanin content.

The MYB family of transcription regulatory factors has extensive functions, including regulating secondary metabolism, cell morphogenesis, environmental response and the cell cycle (Espley and Roger, 2007). To investigate MYB transcription regulatory factors related to the coloration of red Fuji apple, a segment of color-related EST sequence was identified from the EST Database of *Malus Mill. MYB10d*, which had been cloned from red Fuji apple by RACE-PCR homology cloning, was considered as a *MYB* transcription factor. Similar to other *MYB* genes, the *MYB10d* sequence contained a conserved DNA-binding domain consisting of 24 amino acids that is important in binding DNA and activating related genes. *MYB10d* expression reached its peak value two days after bag removal, 4 days earlier than the peak of PAL activity (one key enzyme of anthocyanin synthesis). It could be argued that after bag removal, the *MYB10d* expression was induced by light, which stimulated the activities of anthocyanin-related genes (for example, PAL) that are beneficial for the active synthesis of anthocyanin in fruit.

The expression of *MYB10d* sharply declined and was nearly undetectable from the 6th day on, suggesting that the function of the gene might be to increase the activities of genes important for anthocyanin synthesis. The level of expression decreased once these genes had been transcribed because the transcription factors were no longer required. The same could be inferred of PAL activity, which began to decrease on the 6th day after bag removal, possibly because the synthesis substrate of anthocyanin had accumulated in the prophase. However, the accumulation of anthocyanin increased rapidly, which suggests that the function of *MYB10d* might be the transcription regulation of the anthocyanin structural gene.

Further research should be carried out to determine the specific details of anthocyanin-related transcription regulation of *MYB10d*.

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