The Methylation of the *PcMYB10* Promoter Is Associated with Green-Skinned Sport in Max Red Bartlett Pear

Zhigang Wang, Dong Meng, Aide Wang, Tianlai Li, Shuling Jiang, Peihua Cong, and Tianzhong Li*

Laboratory of Fruit Cell and Molecular Breeding, College of Agronomy and Bio-tech, China Agricultural University, Beijing 100193, China (Z.W., D.M., T.Z.L.); Research Institute of Pomology, Chinese Academy of Agricultural Sciences, Xingcheng, Liaoning 125100, China (Z.W., S.J., P.C.); Shenyang Agricultural University, Shenyang 110866, China (A.W., T.L.L.); and Key Laboratory of Biology and Genetic Improvement of Horticultural Crops (Germplasm Resources Utilization), Ministry of Agriculture, Xingcheng, Liaoning 125100, China (Z.W., S.J., P.C.)

Varieties of the European pear (*Pyrus communis*) can produce trees with both red- and green-skinned fruits, such as the Max Red Bartlett (MRB) variety, although little is known about the mechanism behind this differential pigmentation. In this study, we investigated the pigmentation of MRB and its green-skinned sport (MRB-G). The results suggest that a reduction in anthocyanin concentration causes the MRB-G sport. Transcript levels of *PcUFGT* (for UDP-glucose:flavonoid 3-O-glucosyltransferase), the key structural gene in anthocyanin biosynthesis, paralleled the change of anthocyanin concentration in both MRB and MRB-G fruit. We cloned the *PcMYB10* gene, a transcription factor associated with the promoter of *PcUFGT*. An investigation of the 2-kb region upstream of the ATG translation start site of *PcMYB10* showed the regions −604 to −911 bp and −1,218 to −1,649 bp to be highly methylated. A comparison of the *PcMYB10* promoter methylation level between the MRB and MRB-G forms indicated a correlation between hypermethylation and the green-skin phenotype. An *Agrobacterium tumefaciens* infiltration assay was conducted on young MRB fruits by using a plasmid constructed to silence endogenous *PcMYB10* via DNA methylation. The infiltrated fruits showed blocked anthocyanin biosynthesis, higher methylation of the *PcMYB10* promoter, and lower expression of *PcMYB10* and *PcUFGT*. We suggest that the methylation level of *PcMYB10* is associated with the formation of the green-skinned sport in the MRB pear. The potential mechanism behind the regulation of anthocyanin biosynthesis is discussed.

Anthocyanin is a water-soluble flavonoid compound. It confers various different colors to plants and is essential in plant resistance, pollen spread, and UV protection (Feild et al., 2001; Regan et al., 2001; Schaefer et al., 2004). In addition, the coloration resulting from anthocyanin is an important agronomic trait, with roles in landscaping, the food industry, and health care (Lila, 2004; He and Giusti, 2010). The biosynthesis of anthocyanin is a complex process involving the regulation of a number of structural and regulatory genes (Koes et al., 2005). Mutations of these genes have led to aberrant anthocyanin biosynthesis and color changes of plant organs. For example, mutations in the structural genes *CHS* (for chalcone synthase), *DFR* (for dihydroflavonol 4-reductase), and *ANS* (for anthocyanidin synthase) involved in anthocyanin biosynthesis cause flower color changes (Stracke et al., 2009; Tanaka et al., 2009; Vanholme et al., 2010). Genetic mutations causing color changes of plant organs also occur in genes regulating anthocyanin biosynthesis, such as transcription factors (Boss et al., 1996; Morita et al., 2006; Park et al., 2007; Allan et al., 2008; Chiu et al., 2010). The transcription factors involved in anthocyanin biosynthesis include R2R3-MYB, bHLH (for basic helix-loop-helix), and WDR (for WD-repeat protein), which regulate the downstream structural genes by forming the MYB-bHLH-WD40 complex, which is involved in the anthocyanin biosynthesis pathway (Holton and Cornish, 1995; Winkel-Shirley, 2001; Schwind et al., 2006; Gonzalez et al., 2008). Mutations including nucleotide substitutions, deletions, and insertions in the MYB gene result in color changes in plant organs (Boss et al., 1996; Morita et al., 2006). Transposon insertions in the *bHLH* gene cause white flowers in common morning glory (*Ipomoea purpurea*; Park et al., 2007), whereas simultaneous mutations of a nucleotide deletion in MYB and a nucleotide insertion in WDR change morning glory flower color from blue to white (Chiu et al., 2010).

Several cis-acting elements in the promoter of key genes regulating anthocyanin biosynthesis are activated by a series of signal transduction processes under the light and temperature receptors (Vitrac et al., 2000; Dela et al., 2003; Zhang et al., 2003). Transposon insertions and deletions in the promoter region of grape

Wang et al.

(Vitis vinifera) VoMYBA1 lead to a peel color change from green to red (Poudel et al., 2008), a single-nucleotide polymorphism in an apple (Malus domestica) MdMYB1 promoter causes anthocyanin biosynthesis abnormalities (Ban et al., 2007), and a microsatellite insertion in the MdMYB10 promoter results in red flesh of apple (Espley et al., 2009).

The natural occurrence of DNA methylation is another cause of plant mutation. In toad (Linaria vulgaris), radially symmetric floral mutants arise from epialleles of the CYCLOIDEA gene, which encodes a transcription activator of the floral asymmetry development process (Cubas et al., 1999). Hypermethylation of the phosphoribosylanthranilate isomerase1-4 (pai1-4) locus in Arabidopsis (Arabidopsis thaliana) is associated with transcriptional repression of PAI (Bender and Fink, 1995). Furthermore, hypermethylation in the promoter of the white pericarp/red cob (P1-wr) gene causes maize (Zea mays) color change, and hypermethylation in the 3’ untranslated region of the allele gene of PI (P1-Bh) leads to the formation of red-blotting maize (Cocciolone and Cone, 1993; Sekhon and Chopra, 2009). Hypermethylation in the promoter of MdMYB10 causes a striped pigmentation on Honey Crisp apple fruit (Telias et al., 2011).

There are some investigations of color mutants in pear (Pyrus communis; Thomas et al., 2010). For example, Wu et al. (2012) studied the pigmentation of Early Red Doyenne du Comice, which is a red-skin sort of Doyenne du Comice, and found that the PyMADS18 gene is involved in the early stages of anthocyanin biosynthesis. Pierantoni et al. (2010) reported that the red color trait of Max Red Bartlett (MRB) seems to have no direct relation with the MYB10 gene. Thus far, the mechanism behind pigmentation color mutants in pear is still unclear.

MRB is a red-skinned European pear variety (Abu-Qaoud et al., 1990). We recently found many green-skinned fruits on MRB trees (MRB-G; Supplemental Fig. S1). Based on 4 years of observation, the mutation rate of MRB is 21% to 24% (Supplemental Table S1), and the green-skinned fruits occur stably on a whole branch, or together with red-skinned fruits on a single branch, or appeared in one cluster with red-skinned fruits. Such chimerism is caused by a mutation of meristematic cells during the development of plant tissues (Pohlheim, 2003). The common mutant types causing chimera include chromosomal aberrations, transposon activation, point mutations, and DNA methylation (Pohlheim, 2003). Chromosomal aberrations and point mutations do not occur very often under natural conditions, while DNA methylation and transposon activation can happen frequently in plants (Gong et al., 2008).

In this study, we analyzed the mechanism of the formation of MRB-G and found that the methylation of the PcMYB10 promoter might be the factor causing green-skinned MRB-G. The possible reasons behind the methylation of the PcMYB10 promoter are discussed.

RESULTS

The Concentration of Anthocyanin Is Lower in the Skin of MRB-G Fruits

The concentrations of anthocyanin and chlorophyll in the skin of MRB and MRB-G fruits were measured at different development stages (Fig. 1). The concentration of chlorophyll in fruit skin changed similarly in both MRB and MRB-G, and no significant difference was observed between them (Fig. 1B), indicating that chlorophyll was not responsible for MRB-G. In MRB fruit skin, the concentration of anthocyanin started to increase at 9 d after full bloom (DAFB), peaked at 45 to 55 DAFB, and then gradually decreased until 85 DAFB (Fig. 1B). In MRB-G fruit skin, the concentration of anthocyanin increased slightly and then declined during fruit development, and it was significantly lower than that in MRB (Fig. 1B). We also investigated the amount of anthocyanin in the whole fruit. The concentration of fruit skin anthocyanin in whole fruit was paralleled by the concentration by unit weight in MRB and MRB-G (Fig. 1C).

UFGT (for UDP-Glc:flavonoid 3-O-glucosyltransferase) is a key enzyme in anthocyanin biosynthesis. The activity of UFGT was measured in fruit skin at 55 and 115 DAFB of MRB and MRB-G (Supplemental Fig. S2). UFGT enzyme was active in both MRB and MRB-G fruit. Its activity in MRB was 25% higher than that in MRB-G at 55 DAFB and 15% higher at 115 DAFB. From 55 to 115 DAFB, the activity of UFGT did not change in MRB-G, similar to that of anthocyanin concentration.

The Expression of PcUFGT, a Key Structural Gene in Anthocyanin Biosynthesis, Was Much Lower in MRB-G Fruits

To investigate whether structural genes in anthocyanin biosynthesis were involved in the trait differences between MRB and MRB-G, we isolated seven structural genes: PcPAL (for l-Phe ammonia lyase), PcCHS, PcCHI, PcF3H (for flavonone-3-hydroxylase), PcDFR, PcANS, and PcANR (for anthocyanidin reductase). These genes were amplified from 55-DAFB fruit skin of MRB and MRB-G, with primers designed according to the relevant gene sequences as found in GenBank (Supplemental Table S2). Two other structural genes, PcUFGT and PcF3’H (for flavonoid 3’-hydroxylase), were isolated by homology cloning and RACE. The coding regions of PcUFGT and PcF3’H were 1,440 and 1,536 bp, respectively, and showed 89.05% identity with apple MdUFGT (AF117267) and 95.05% identity with MdF3’H (FJ919632). All genes obtained were submitted to GenBank under the accession numbers listed in Supplemental Table S2.

The complementary DNA (cDNA) sequences of the nine structural genes above were compared between MRB and MRB-G; however, no differences were found. We also compared the expression patterns of these
structural genes in MRB and MRB-G during fruit development (Supplemental Fig. S4). There was no difference in the expression of \textit{PcPAL}, \textit{PcCHS}, \textit{PcCHI}, and \textit{PcF3’H} between MRB and MRB-G. The expression of \textit{PcF3H}, \textit{PcDFR1/2}, \textit{PcANS}, and \textit{PcANR} was higher in MRB than in MRB-G in the early fruit development stage (about 0–55 DAFB) but lower in the middle fruit development stage (about 55–85 DAFB). The expression of \textit{PcUFGT} in MRB fruit increased from 9 DAFB, reached a maximum at 55 DAFB, and then declined to a very low level. In MRB-G fruits, \textit{PcUFGT} expression increased slightly and then dropped at 70 DAFB. Moreover, the expression of \textit{PcUFGT} was substantially higher in MRB fruits than in MRB-G. This expression pattern paralleled anthocyanin accumulation differences between MRB and MRB-G fruits. Interestingly, \textit{PcUFGT} expression in MRB and MRB-G fruit decreased sharply at 70 and 55 DAFB, respectively, suggesting that anthocyanin biosynthesis may have stopped or slowed after this stage. In addition, the expression of all genes increased sharply at 130 DAFB.

To further assess whether \textit{PcUFGT} is a key causal factor behind the different colors of MRB and MRB-G, we analyzed the concentrations of the substrate and product of the UFGT enzyme in the fruits of MRB and MRB-G at 55 DAFB. The concentration of the main product of the UFGT enzyme, cyanidin 3-galactoside, showed a clear difference between MRB and MRB-G, while cyanidin, the substrate of the UFGT enzyme, showed no observable difference (Fig. 2).

The above results indicated that the reduced concentration of anthocyanin in MRB-G could be the result of the low expression of \textit{PcUFGT}.

**Lower Expression of \textit{PcMYB10} Led to Lower Expression of \textit{PcUFGT} in MRB-G Fruits**

To investigate why \textit{PcUFGT} was expressed at lower levels in MRB-G, we amplified the promoter region of \textit{PcUFGT} (1,790 bp from the transcription initiation site) from genomic DNA of MRB and MRB-G, but no nucleotide sequence difference was observed.
The structural genes of anthocyanin biosynthesis are usually regulated by a protein complex formed from three types of transcription factors, MYB, bHLH, and WD40 (Gonzalez et al., 2008). We isolated three transcription factor genes related to anthocyanin synthesis: \textit{PcMYB10} (JX403957), \textit{PcbHLH} (JX403960), and \textit{PcWD40} (JX403961) from MRB. Among these genes, \textit{PcMYB10} has the same sequence as \textit{MYB10} (EU153575) of pear, and \textit{PcbHLH} and \textit{PcWD40} have higher identity with their respective homologs in apple (Supplemental Table S2). The coding regions of \textit{PcMYB10}, \textit{PcbHLH}, and \textit{PcWD40} were the same between MRB and MRB-G, but their expression levels showed some differences. In particular, the expression of \textit{PcMYB10} exactly paralleled the expression of \textit{PcUFGT}, both being much lower in MRB-G fruits than in MRB (Fig. 3). Therefore, we focused on the \textit{PcMYB10} gene in our study.

To investigate the regulation of \textit{PcUFGT} by \textit{PcMYB10}, we isolated the promoter region of \textit{PcUFGT} (1,790 bp from the transcription initiation site), which we named \textit{proUFGT} (JX403963). When \textit{proUFGT} was ligated to pCAMBIA 1305 vector holding a GFP gene and transformed into tobacco \textit{(Nicotiana benthamiana)} leaves, it was able to initiate GFP expression (Supplemental Fig. S5), suggesting that it was an active promoter.

\textit{proUFGT} contained six MYB-binding sites (MBS) related to anthocyanin biosynthesis (Supplemental Table S3). A yeast one-hybrid (Y1H) assay was performed to test the association of \textit{PcMYB10} with \textit{proUFGT}. The results showed that \textit{PcMYB10} could associate with two MBS located at \(-347\) and \(-451\) bp of \textit{proUFGT} (Fig. 4B), and the mutation in these binding sites could break the association (Fig. 4C), supporting the hypothesis that \textit{PcMYB10} was the transcription factor associating with \textit{PcUFGT}. These results indicated that the lower expression of the transcription factor \textit{PcMYB10} resulted in the lower expression of \textit{PcUFGT} in MRB-G.

The High Degree of Methylation in the \textit{PcMYB10} Promoter Resulted in Its Lower Expression in MRB-G Fruits

To investigate why \textit{PcMYB10} was expressed at lower levels in MRB-G, the promoter of \textit{PcMYB10} (1,725 bp from the transcription initiation site) was isolated from MRB and MRB-G by chromosome walking and assigned the name \textit{proPcMYB10} (JX403962). This contained a core promoter at position \(-60\) bp and an enhancer promoter at position \(-675\) bp (as analyzed by TSSP software on softberry; http://linux1.softberry.com/berry.phtml). Besides the promoter elements, the sequence also contained many cis-acting elements related to light, temperature, and other environmental factors (Supplemental Fig. S6A; Supplemental Table S4). To test the activation of \textit{proPcMYB10}, the 1,725-bp \textit{proPcMYB10} was cloned into a pCAMBIA 1305 vector and transformed into tobacco leaves. The result showed that it could activate the expression of \textit{GFP} (Supplemental Fig. S5), indicating that \textit{proPcMYB10} was an active promoter.

The nucleotide sequence of \textit{proPcMYB10} was compared between MRB and MRB-G, but no difference was found. This indicated that the methylation of \textit{proPcMYB10} might cause the differential \textit{PcMYB10} expression between MRB and MRB-G. Therefore, the methylation of \textit{proPcMYB10} was predicted by a methylation-specific endonuclease (McrBC)-PCR. The degree of methylation...
in the regions +1 to −377 bp and −378 to −707 bp showed no difference between MRB and MRB-G fruit skin. However, the regions −707 to −1,208 bp and −1,209 to −1,847 bp were quite different in methylation (Supplemental Fig. S6B). To verify this result, we also analyzed the methylation level of cytosine in proPcMYB10 of MRB and MRB-G fruit skin by bisulfite sequencing (BSP)-PCR. The regions −604 to −911 bp, −1,218 to −1,649 bp, and −1,804 to −1,965 bp were highly methylated, and the methylation level of regions −604 to −911 bp and −1,218 to −1,649 bp showed the most difference between MRB and MRB-G (Fig. 5A). The methylation level of the region −604 to −911 bp in proPcMYB10 of MRB fruit skin was 45.6% and showed no significant difference with MRB-G. Moreover, the methylation levels of CG and CHG (where H is A, C, or T) cytosines were 28.9% and 23.2% lower in MRB than those in MRB-G (Fig. 5B). Methylation of the −1,218 to −1,649 bp region of proPcMYB10 in MRB fruit skin was 5.5% lower than that in MRB-G. In this region, only the CHH-type cytosine methylation was 7.3% lower in MRB than that in MRB-G (Fig. 5B). Interestingly, the CHH-type cytosine methylation inside this region (−1,557 to 1,628 bp) in MRB was 27% lower than that in MRB-G (Supplemental Fig. S7C).

We also analyzed the methylation level of the −1,557 to −1,628 bp region of proPcMYB10 in young fruit skin (55 DAFB) and mature fruit skin (115 DAFB) of MRB and MRB-G. The methylation of this region in young and mature MRB fruit skin was 48.7% (Supplemental Fig. S7, A and C) and 46.1% (Supplemental Fig. S7, B and C), respectively, which was 27.0% and 32.4% lower than that in MRB-G. These data indicated that the methylation level of −1,557 to −1,628 bp proPcMYB10 did not change much during fruit development.

Virus-Induced Gene Silencing Led to Abnormal Coloring of Young MRB Fruit Peel

Virus-induced gene silencing (VIGS) potentially induces two types of gene silencing: posttranscriptional gene silencing and transcriptional gene silencing. Viral vectors containing target gene fragments transformed into the plant can inhibit the expression of homologous genes and lead to posttranscriptional gene silencing (Brodersen et al., 2008). If DNA sequences such as promoter sequence are ligated into viral vector and transformed into the plant, this causes transcriptional repression through the cytosine methylation of corresponding DNA sequences, which leads to transcriptional gene silencing (Bai et al., 2011). In this study, we used two VIGS plasmids to induce transcriptional
gene silencing of \textit{PcMYB10} in young MRB fruit: pTRV2-69 containing the DNA fragment \textendash 604 to \textendash 911 bp and pTRV2-1216 containing the DNA fragment \textendash 1,218 to \textendash 1,649 bp of pro\textit{PcMYB10}. MRB inflorescences were bagged through full bloom to 10 DAFB to keep the fruit unpigmented. After removing the bags, the fruits (on trees) were immediately infiltrated with \textit{Agrobacterium tumefaciens} strain AH105 holding plasmids pTRV2-69 and pTRV2-1216 and exposed to natural light. Fruits infiltrated with empty pTRV2 vector were used as a control. Four days after infiltration, the control fruits turned red, while fruits infiltrated with pTRV2-69 or pTRV2-1216 remained green (Fig. 6A).

We then measured the concentration of anthocyanin in the peel of infiltrated fruits. It was 7.46 and 3.99 times lower in fruits infiltrated with pTRV2-69 and pTRV2-1216 than in control fruits (Fig. 6B). In fruit infiltrated with pTRV2-69 and pTRV2-1216, the expression of \textit{PcUFGT} was 37.2 and 12.1 times lower than in control fruits, respectively. And the expression of \textit{PcMYB10} was 44.6 and 19.7 times lower than in control fruits (Fig. 6, C and D). Then, we analyzed the methylation level of the \textendash 2826 to \textendash 2610 bp and \textendash 21,628 to \textendash 21,424 bp regions of pro\textit{PcMYB10} in infiltrated fruits. The methylation level increased 28.5\% in the \textendash 2826 to \textendash 2610 bp region and 26.7\% in the \textendash 21,628 to \textendash 21,424 bp region after infiltration (Fig. 6E). At the same time, the methylation levels of all three cytosine types (CHH, CHG, and CG) were increased significantly (Supplemental Fig. S10).

These results demonstrated that the methylation of pro\textit{PcMYB10} repressed the expression of \textit{PcMYB10} and \textit{PcUFGT} and subsequently inhibited the biosynthesis of anthocyanin.
of anthocyanin, which probably caused the formation of a green-skin chimera sport of MRB.

DISCUSSION

Plant diversity is enhanced by the process of mutation. The color change of plant tissue is one of the commonly observed phenotypes of natural mutation. The mechanisms of color change in some plants have been studied extensively (Tegopati, 2001; Li et al., 2005; Yamaguchi et al., 2009; Palai and Rout, 2011), but not in the sport varieties of the European pear. In this paper, we studied the mechanism of the formation of green-skinned MRB-G fruit and demonstrated that hypermethylation of the PcMYB10 promoter was probably the causative factor. To our knowledge, this is the first report on the mechanism of fruit color change in the European pear.

The Change of Anthocyanin Concentration in MRB and MRB-G

Chlorophyll and anthocyanin are the most important pigments in the color formation of plant tissues, including fruit. In this study, we measured the concentrations of chlorophyll and anthocyanin in fruit skin of MRB and MRB-G at different developmental stages (Fig. 1A). The concentration of chlorophyll showed no difference between MRB and MRB-G fruit skin (Fig. 1B), suggesting that the formation of green skin of MRB-G is not related to chlorophyll. A very low concentration of anthocyanin was observed in MRB-G fruit (from 0 to 55 DAFB; Fig. 1B), which was not unexpected due to its green color. In addition, unlike the anthocyanin biosynthesis in apple fruit, which starts immediately prior to fruit maturation (Honda et al., 2002), the concentration of anthocyanin
in MRB fruits increased from 9 DAFB and started to decline after 55 DAFB (Fig. 1B). This change paralleled the change of fruit pigmentation (Fig. 1A), indicating that the degradation of anthocyanin might be faster than its biosynthesis after 55 DAFB. On the other hand, UFGT is a key enzyme in plant anthocyanin biosynthesis (Kobayashi et al., 2002; Ubi et al., 2006). In MRB, the UFGT enzyme activity in the peel of mature fruit was lower than that in the peel of young fruit (Supplemental Fig. S2), as was UFGT expression. Therefore, the biosynthesis of anthocyanin declined at the later developmental stages of MRB fruit. Steyn et al. (2004) have reported that the anthocyanin content in Rosemarie pear declines at late development stages, due to an increase of the anthocyanin degradation rate. In the case of MRB and MRB-G, further study is needed to confirm whether the degradation of anthocyanin is increased at later stages.

Expression of Key Genes Involved in the Biosynthesis of Anthocyanin

The biosynthesis of anthocyanin involves at least nine enzymes (Supplemental Fig. S3). We compared the expression of these genes during the development of MRB and MRB-G fruits (Fig. 3; Supplemental Fig. S4). Several genes, such as PcF3H, PcANS, PcANR, and PcUFGT, were expressed differentially, leading us to speculate that these genes contributed to the difference between MRB and MRB-G fruits. Furthermore, the expression of PcUFGT showed obvious consistency with the changing pattern of anthocyanin. This result is consistent with the results in other species, such as apple and grape (Kobayashi et al., 2002; Ubi et al., 2006), suggesting an essential role of UFGT in the biosynthesis of anthocyanin. In our study, we measured the concentration of the UFGT enzyme product, which was significantly higher in MRB than in MRB-G fruit, again suggesting its important role (Supplemental Fig. S2). As a result, our study focused on the function of PcUFGT in the difference of anthocyanin biosynthesis between MRB and MRB-G fruits. However, no difference was observed between MRB and MRB-G by analyzing the coding region and promoter region of PcUFGT, suggesting that other factors upstream may contribute to the difference between MRB and MRB-G.

Transcription factors such as MYB are important regulators in the biosynthesis of anthocyanin in plants (Kobayashi et al., 2002; Ban et al., 2007; Gonzalez et al., 2008; Espley et al., 2009). The promoter region and expression pattern of PcMYB10 were analyzed in developing fruit of MRB and MRB-G. The nucleotide sequence of the PcMYB10 promoter was the same in MRB as in MRB-G, while PcMYB10 expression was different between MRB and MRB-G and showed the same trend as PcUFGT during fruit development (Fig. 3). Interestingly, the expression patterns of both PcUFGT and PcMYB10 paralleled the change of anthocyanin concentration in MRB and MRB-G fruits (Figs. 1B and 3). Our study also showed that PcMYB10 could associate with the promoter of PcUFGT (Fig. 4), indicating that PcMYB10 might be a key gene contributing to the difference between MRB and MRB-G.

In addition, the expression of anthocyanin biosynthesis genes and the regulatory gene PcMYB10 increased rapidly at 130 DAFB in MRB and MRB-G (Fig. 3; Supplemental Fig. S4). Because the fruits at 130 DAFB are fully mature, the contents of secondary metabolites such as a variety of flavonoids have changed greatly (Bain, 1961); we presume that the expression increase in anthocyanin biosynthesis genes at 130 DAFB is an adaptation to secondary metabolite variation at fruit maturity.

Methylation of the PcMYB10 Promoter

The aberrant expression of MYB could be caused by promoter sequence variations, such as insertion of transposons (Kobayashi et al., 2004) and other sequences (Espley et al., 2009) into the promoter region, and additionally by promoter methylation (Cocciolone and Cone, 1993; Telias et al., 2011). In this study, the expression of PcMYB10 was much lower in MRB-G fruit than in MRB fruit (Fig. 3). To ascertain the reason for the expression difference of PcMYB10, we cloned the promoter of PcMYB10 from MRB and MRB-G fruits and compared their sequences, but no differences were observed. This suggests that the decreased expression of PcMYB10 in MRB-G might result from the methylation of its promoter region. Therefore, we analyzed the methylation of the PcMYB10 promoter. In addition to some very small changes in methylation between MRB and MRB-G that may be caused by multiple cell types, methylation of the −604 to −911 bp and −1,218 to −1,649 bp regions in the PcMYB10 promoter was much higher in MRB-G fruit skin than that from MRB fruit skin. A similar result was obtained in apple, in which the methylation of the promoter of MdMYB10 caused a striped phenotype in Honey Crisp (Telias et al., 2011). To better understand the different methylation between MRB and MRB-G fruit, we also analyzed the methylation of three types of cytosine (CHH, CHG, and CG) in the two regions mentioned above. CG and CHG cytosine types in the −604 to −911 bp region of the PcMYB10 promoter have higher methylation levels in MRB-G fruit peel than in MRB. In region −1,218 to −1,649 bp of the PcMYB10 promoter, only CHH-type cytosine showed a higher methylation level in MRB-G fruit peel than in MRB. The type of DNA methylation that induced plant color mutation was largely CG/CHG cytosine methylation, such as the CG/CHG cytosine methylation that occurs in the promoter of the Pi-wr gene, inducing color mutation of maize (Sekhon and Chopra, 2009). However, there are no previous reports showing that CHH cytosine hypermethylation and CG/CHG cytosine hypermethylation exist in different regions of a single promoter. We speculate that the DNA methylation-based
regulatory mechanism of woody plants (e.g. pear) is more complicated than that occurring in herbaceous plants (e.g. maize), and the existence of both types of CHH and CG/CHG cytosine methylation in the promoter of *PcMYB10* increases the stability of the green mutant of MRB through the generations.

In region −1,557 to −1,628 bp, CHH-type cytosine methylation level in MRB fruit peel was 27% lower than in MRB-G. This region may play an important role in the regulation of the *PcMYB10* promoter, but with no strong cis-acting elements in this region. We speculate that methylation is not directly blocking the binding of the MYB but acting through an alternative mechanism. We also tested the methylation of −1,557 to −1,628 bp of the *PcMYB10* promoter in leaf of MRB and MRB-G and found that methylation level in MRB-G leaves was only 15.6% higher than in MRB (Supplemental Fig. S9), which is different from the results in fruit. We suspect that this variation is due to the different tissues.

To clarify whether *PcMYB10* promoter methylation induced the aberrant expression of *PcMYB10* in MRB-G, we used a VIGS system in which the endogenous *PcMYB10* promoter was methylated. This suppressed its expression, which indicated that the methylation level of the *PcMYB10* promoter was increased in fruits infiltrated with plasmid pTRV2-69 and pTRV2-1216 (Fig. 6E; Supplemental Fig. S10), and the anthocyanin synthesized in these fruits decreased to trace levels (Fig. 6B). Meanwhile, the expression of *PcUFTG* and *PcMYB10* also decreased in the infiltrated fruits (Fig. 6C and D). These data indicate that DNA methylation in the promoter of *PcMYB10* was associated with reduced transcript accumulation, which repressed the expression of *PcUFTG* and the biosynthesis of anthocyanin and, consequently, led to green-skinned sport.

Cytosine methylation variations have the potential to play a role in phenotypic plasticity as a response to environmental stress. For example, the mangrove trees (Rhizophoraceae) growing on riverside microenvironments are larger and display more cytosine methylation than stunted trees from nearby salt marshes (Lira-Medeiros et al., 2010). This finding is consistent with the view that epigenetic variation among individuals with similar genotypes can drive phenotypic variation in response to varying physical environments (Richards, 2011). Actually, MRB is a red-pear sport of Bartlett found in the United States, and MRB-G is found in an orchard near Beijing. The difference in methylation level between MRB and MRB-G might be caused by the adaption to new environments. Further study is needed to determine this.

MRB is a red-skin sport of the green-skinned variety Bartlett. In our study, the methylation level of −1,557 to −1,628 bp in the *PcMYB10* promoter of MRB fruit peel was not significantly different from that of Bartlett (Supplemental Fig. S8), which indicated that MRB-G was not a reversion of Bartlett.

The expression of F3H, DFR, ANS, and ANR was increased during the mid development stage of MRB-G fruit. While other elements such as transcription factors might be available to regulate their expression, more work needs to be done to examine whether they contribute to the formation of green-skinned sport.

**CONCLUSION**

In MRB-G fruits, the expression of *PcMYB10* was reduced by the methylation of regions −604 to −911 bp and −1,218 to −1,649 bp in its promoter. As a result, the expression of *PcUFTG*, a key gene involved in anthocyanin biosynthesis and regulated by *PcMYB10*, was also reduced. This might be associated with the inhibition of anthocyanin biosynthesis and the formation of green-skinned sport (Fig. 7).
MATERIALS AND METHODS

Plant Materials and Nucleic Acid Isolation

MRB and MRB-G branches of pear (Pyrus communis) were found in an orchard located in the suburbs of Beijing, China. Fruits of full bloom and 3, 6, 9, 12, 15, 20, 25, 30, 35, 40, 45, 50, 55, 70, 85, 100, 115, and 130 DAFB were collected from both clones, and the skin was carefully shaved off and collected for the measurement of anthocyanin and chlorophyll contents and nucleic acid isolation. DNA isolation was conducted using the cetyl-trimethyl-ammonium bromide method (Ma and Zhang, 2009). Total RNA was extracted according to Gong et al. (2011). RNA isolated from sample of 55 DAFB was used for gene isolation, and samples of 55 and 115 DAFB were used for methylation analysis of the PcmYB10 gene promoter.

Measurements of Anthocyanin, Chlorophyll, and UFGT Activity

Measurement of total anthocyanin was according to Rabino and Mancinelli (1986), with slight modifications. Briefly, frozen ground peel tissue (0.5 g) was homogenized in 2 mL of methanol with 1% HCl. The homogenate was left 8 h in dark conditions and then centrifuged for 5 min at 8,000g. Absorbance of the supernatant was measured at 530 and 600 nm using a UV-visible spectrophotometer (UNICO UV-2000). Three biological replicates were performed. Relative anthocyanin content was calculated according to the following formula: anthocyanin content (units g\(^{-1}\) fresh weight) = \(2 \times (\text{OD}_{530nm} - \text{OD}_{600nm}) / 100\), where OD equals optical density. Chlorophyll content determination was performed as described by Bruinisma (1963).

UFGT activity was measured according to the method of Lester et al. (1996) with minor modifications. One gram of fruit skin was ground into fine powder in liquid nitrogen, adding 7.5 mL of extraction buffer (0.2 mol L\(^{-1}\) borate buffer, pH 8.5, 0.005 mol L\(^{-1}\) EDTA, and 0.001 mol L\(^{-1}\) diethiothreitol) and 10% polyvinylpolypyrrolidone homogenate. After centrifugation at 8,000g for 5 min, the supernatant was collected as enzyme solution and used for UFGT activity measurement. A total of 100 \(\mu\)L of enzyme solution was added to the reaction mixture containing 100 \(\mu\)L of 50 mmol L\(^{-1}\) Gly buffer (pH 8.5), 15 \(\mu\)L of 2 mg mL\(^{-1}\) substrate quercetin (Sigma Chemical), and 10 \(\mu\)L of 15 mg mL\(^{-1}\) UDP-Gal (Sigma Chemical). The mixture was incubated in a 30°C water bath for 30 min, followed by the addition of 75 \(\mu\)L of 20% TCA in methanol to terminate the reaction, then centrifuged at 8,000g for 5 min. The supernatant was collected and used for HPLC (Beckman) analysis. Five microliters of sample was injected into a C18 column, with mobile phase A (acetic acid; 10%, v/v) and mobile phase B (acetonitrile) at A:B = 85:15. The flow rate was 1 mL min\(^{-1}\), with elution for 15 min, 350-nm UV detector assay quercetin content, and UFGT activity was calculated according to a decrease of quercetin with units of mmol g\(^{-1}\) fresh weight h\(^{-1}\). Three biological replicates were performed.

Analysis of the Concentrations of Anthocyanin and Anthocyanin Precursor

Fruit peel was ground into powder in liquid nitrogen. In the direct analysis, samples were dispersed in a 1:1 water:methanol solution containing 2% HCl and sonicated for 30 min. A portion of each sample so-

Isolation of Structure and Regulatory Genes Related to Anthocyanin Biosynthesis

One microgram of total RNA was DNase I treated and used for CDNA synthesis with oligo(dT) primers and the SMARTer cDNA Synthesis Kit (Clontech). Full-length cDNAs of pear genes PAL, CHS, CHI, F3H, DFR, ANR, and ANK were amplified by gene-specific primers based on the available sequences in GenBank (DQ230992, AY786998, EF446163, AF497633, AY227731, DQ230994, and DQ251189). Fragments of pear genes F3’14, UFGT, MYB, bHLH, and WD40 were cloned, using degenerate primers according to the sequence information in grape (Vitis vinifera; DQ786632, AF003372, AB097923, ABM92332, and DQ517914) and apple (Malus domestica; FJ919632, DQ880413, DQ267897, HM122458, and GU173814), and their full-length cDNAs were obtained by using the SMARTer RACE cDNA Amplification Kit (Clontech). All genes obtained were submitted to GenBank with the accession numbers given in Supplemental Table S2. Primer pairs used in this study are listed in Supplemental Table S5.

Real-Time Quantitative PCR Analysis

One microgram of total RNA was used for CDNA synthesis with the primer script Master Mix Kit (TaKaRa). Quantitative reverse transcription-PCR was performed with the SYBR Premix ExTag II Kit (TaKaRa), and amplification was real-time monitored on an Option Continuous Fluorescence Detection System (Bio-Rad). Actin (AB190376) was used for normalization. Primer information is given in Supplemental Table S5.

Isolation and Identification of PcmUFGT and PcmMYB10 Gene Promoters

The promoter regions of PcmYB10 and PcmUFGT were isolated using the Genome Walking Kit (TaKaRa) according to the manufacturer’s protocols. The analysis of these two promoters was performed by the online software PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/). The primer sequences used are given in Supplemental Table S5. Transient expression was employed to test the activity of promoters as described by Bai et al. (2011). Briefly, the promoters of PcmYB10 and PcmUFGT were ligated to pCAMBIA 1305 vector with the GFP gene, generating constructs pLcMYB10-GFP and pLcUFGT-GFP, respectively. The GFP gene was inserted into pCAMBIA 1305 vector as a negative control (GFP control vector). The constructs were introduced into Agrobacterium tumefaciens strain EHA105, and the A. tumefaciens strain was infiltrated into tobacco (Nicotiana benthamiana) leaves. GFP fluorescence was photographed using a stereomicroscope (SZX16-DP72, Olympus).

YIH Assay

YIH assays were performed using the Matchmaker Gold Yeast One-Hybrid System Kit (Clontech) according to the manufacturer’s protocols. The PcmYB10 gene was ligated to pGADT7 to generate the construct AD-MYB. The fragments of the promoter of UFGT were ligated to pAbAi vector to generate pAbAi-bait plasmids, which were then linearized and transformed into yeast strain YIHGold and selected with a selective synthetic dextrose medium lacking uracil plate. The constructs of AD-MYB were transformed into strain YIHGold holding pAbAi-bait and screened on an SD-/Ura/AbA plate. All transformations and screenings were performed three times.

Methylation Analysis

McrBC-PCR was used to analyze the methylation of relative sequences. A 1-μg DNA sample isolated from 55-DAFB fruit skin was digested with McrBC (New England Biolabs), according to the manufacturer’s instructions, with three biological replicates. For the negative control, GFP was replaced by water. The digested DNA was used as a template for semi quantitative PCR analysis. The PcmYB10 gene promoter sequence was divided into four fragments, 1 to −577 bp, −377 to −707 bp, −707 to −1,208 bp, and −1,208 to −1,847 bp, and amplified with their respective primers (Supplemental Table S5). The amount of amplification product was used to estimate the degree of methylation of the corresponding region. BSP-PCR analysis was performed as described by Telias et al. (2011). Briefly, 500 ng of genomic DNA from 55- and 115-DAFB fruit skin was treated
by the EZ DNA Methylation-Gold Kit (Zymo Research). Using the treated DNA as template, PcMYB10 promoter fragments were amplified by Zymo Taq DNA Polymerase (Zymo Research), ligated to PMD18-T vector (TaKaRa), and then sequenced. Sequences of 36 independent clones of each fragment were analyzed with the online software Kismeth (Grunstman et al., 2008), and the methylation level of each fragment was calculated. PcMYB10 promoter was amplified by five pairs of degenerate primers (Supplemental Table S5).

**VIGS in Pear**

Regions –604 to –911 bp and –1,218 to –1,649 bp in the PcMYB10 promoter were amplified by HS Taq DNA polymerase (TaKaRa) and ligated to the pMD18-T vector. The plasmids were digested by EcoRI and Xhol and ligated to the EcoRI-Xhol-cut pTRV2 vector to obtain two constructs: pTRV2-69 (containing the –604 to –911 bp fragment) and pTRV2-1216 (containing the –1,218 to –1,649 bp fragment). pTRV1, pTRV2 (empty plasmid), pTRV2-69, and pTRV2-1216 were transformed into A. tumefaciens EHA105.

The inferences of MRB were bagged at the full-bloom stage. The bags were removed from fruits at 10 DAFB, and these fruits (on the tree) were subjected to A. tumefaciens-mediated tobacco rattle virus vector infiltration. The A. tumefaciens-mediated tobacco rattle virus infiltration was performed according to Fu et al. (2005) and Bai et al. (2011) with some modifications. A. tumefaciens strains containing pTRV1 and pTRV2 or their derivative vectors, pTRV2-69 and pTRV2-1216, were mixed in a 1:1 ratio and infiltrated into 10-DAFB pear fruit flesh near the petiole. After 4 d of exposure to natural light on the tree, anthocyanin content, 

\[ \text{PcUFGT} \] and 

\[ \text{PcMYB10} \] gene expression, and cytosine methylation of the corresponding regions in the PcMYB10 promoter were tested by the method described above. Fruits coinfected with pTRV1 and pTRV2 were used as controls. Primer sequences used for methylation analysis are given in Supplemental Table S5.

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: PcPAL, JX403947; PcCHS, JX403948; PcCHI, JX403949; PcF3H, JX403950; PcF3H, JX403951; PdDFR1-2, JX403952/3; PcANS, JX403954; PcANR, JX403955; PcUFGT, JX403956; PcMYB10, JX403957; PchHLI, JX403960; and PcdW40, JX403961.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Phenotypes of MRB (left) and its green-pear chimeric mutant MRB-G (right).

**Supplemental Figure S2.** Activity of PcUFGT in MRB and MRB-G fruit.

**Supplemental Figure S3.** The pathway of anthocyanin biosynthesis in plants.

**Supplemental Figure S4.** Relative expression of genes related to anthocyanin biosynthesis during MRB and MRB-G fruit development.

**Supplemental Figure S5.** Identification of proPcMYB10 and proPcUFGT.

**Supplemental Figure S6.** Identification of the regions of proPcMYB10 with different methylation levels between MRB and MRB-G by McrBC-PCR.

**Supplemental Figure S7.** Methylation levels of the –1,628 to –1,557 bp region of proPcMYB10 in 55-DAFB and 115-DAFB fruit peel of MRB and MRB-G.

**Supplemental Figure S8.** Methylation levels of the proPcMYB10 –1,628 to –1,557 bp region in fruit peel of MRB and Bartlett.

**Supplemental Figure S9.** Methylation levels of the proPcMYB10 –1,628 to –1,557 bp region in leaves of MRB and MRB-G.

**Supplemental Figure S10.** Methylation analysis of proPcMYB10 in the peel of MRB fruit infiltrated with pTRV-69 and pTRV-1216 by BSP-PCR.

**Supplemental Table S1.** Mutant rate of MRB to MRB-G.

**Supplemental Table S2.** Information of genes isolated.

**Supplemental Table S3.** Parts of cis-acting elements in proPcUFGT of MRB fruit peel.

**Supplemental Table S4.** Parts of cis-acting elements in proPcMYB10 of MRB fruit peel.

**Supplemental Table S5.** Sequence information of PCR primers used in this study.

**ACKNOWLEDGMENTS**

We thank Dr. Susan Brown from the Department of Horticulture, Cornell University, for giving helpful comments on the manuscript.

Received January 17, 2013; accepted April 26, 2013; published April 29, 2013.

**LITERATURE CITED**


Methylation Is Associated with a Skin Mutant of Pear
Wang et al.