Plant Cell Physiol. 48(7): 958–970 (2007)
doi:10.1093/pcp/pcm066, available online at www.pcp.oxfordjournals.org

Isolation and Functional Analysis of a MYB Transcription Factor Gene that is a Key Regulator for the Development of Red Coloration in Apple Skin

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Red coloration of apple (*Malus* × *domestica*) skin is an important determinant of consumer preference and marketability. Anthocyanins are responsible for this coloration, and their accumulation is positively correlated with the expression level of anthocyanin biosynthetic genes. Regulation of expression of these genes is believed to be controlled by MYB transcription factors, and the MYB transcription factors involved in the activation of anthocyanin biosynthetic genes have been isolated in various plants. In the present study, we isolated and characterized a *MYB* transcription factor gene (*MdMYBA*) from apple skin. Characterization of *MdMYBA* demonstrated that (i) *MdMYBA* expression was specifically regulated depending on the tissue and cultivar/species; (ii) its expression level was much higher in a deep-red cultivar ('Jonathan') than in a pale-red cultivar ('Tsugaru'); (iii) when cauliflower mosaic virus 35S::MdMYBA was introduced into the cotyledons of apple seedlings by means of a transient assay, reddish-purple spots were induced, and *MdMYBA* also induced anthocyanin accumulation in reproductive tissues of transgenic tobacco; (iv) the expression of *MdMYBA* was induced by UV-B irradiation and low-temperature treatment, both of which are known to be important in the promotion of anthocyanin accumulation in apple skin; (v) MdMYBA bound specifically to an anthocyanidin synthase (*MdANS*) promoter region in a gel-shift assay; and (vi) *MdMYBA* was mapped to the near region of the BC226-STS (a^1) marker for the red skin color locus (R_f). These results suggest that *MdMYBA* is a key regulatory gene in anthocyanin biosynthesis in apple skin.

Keywords: Anthocyanin — Apple — Mapping — MYB transcription factor — Red skin color.

Abbreviations: ANS, anthocyanidin synthase; bHLH, basic helix–loop–helix; CaMV, cauliflower mosaic virus; CHS, chalcone synthase; DAFB, days after full bloom; DFR, dihydroflavonol 4-reductase; DIG, digoxigenin-dUTP; F3H, flavanone 3-hydroxylase; GUS, β-glucuronidase; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT–PCR, reverse transcription–PCR; UFGT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase; UTR, untranslated region; X-gluc, 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid.

The nucleotide sequences reported in this paper have been submitted to DDBJ under accession numbers AB279598 (*MdMYBA*) and AB279599 (*MdANS* promoter).

Introduction

Apple (*Malus* × *domestica*) is one of the most widely cultivated temperate fruits around the world (King 1996). At Japan's National Institute of Fruit Tree Science, systematic apple breeding began in 1939, and more than 10 cultivars (including 'Sansa' and 'Fuji') have been released so far. Improvement of the quality characteristics of the flesh of the apple fruits, such as sugar and acid contents, fruit size and fruit flavor, is a major objective in apple breeding. In addition, red-colored skin is an important target because consumers generally prefer red apples, and the high marketability of these fruits is thus important for farmers. Red coloration in apple skin is derived from anthocyanins. Biochemical studies have demonstrated that these anthocyanins are predominantly glycosylated cyanidins, among which cyanidin 3-galactoside accounts for >80% of the total cyanidin 3-glycosides in red skin (Lancaster 1992).

Considerable effort has been invested in improving the coloration of apple skin and in clarifying the mechanism underlying the red coloration. Based on these efforts, two major factors that affect red coloration have been proposed. First, the formation of anthocyanins is genetically determined. The structural genes encoding enzymes in the anthocyanin biosynthetic pathway have therefore been isolated from apple and their expression during fruit development has been analyzed. The results indicated that most of the genes in the anthocyanin biosynthetic pathway, and particularly those for chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*) and

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UDP-glucose:flavonoid 3-*O*-glucosyltransferase (*UFGT*), are positively induced and increase the accumulation of anthocyanin in apple skin (Honda et al. 2002). This expression pattern in apple differs from that in grape (*Vitis vinifera* and *V. labruscana*), in which *UFGT* induction during anthocyanin accumulation is a key regulatory step in the development of red coloration (Boss et al. 1996, Kobayashi et al. 2001).

Secondly, it has been known for some time that the nutritional conditions of apple trees, including nitrogen and carbohydrate status, and various environmental factors are involved in the development of red coloration. Environmental factors have been intensively investigated, and both UV-B (280–320 nm) irradiation and low temperatures are important factors that stimulate the production of anthocyanins (Saure 1990, Lancaster 1992). Therefore, to maximize color development by improving the light and temperature conditions for apple fruits, farmers are concerned about management of the tree canopies and use silver mulching materials to reflect more light towards the fruits. Furthermore, several lines of biochemical evidence suggest that the activities of anthocyanin biosynthetic enzymes and the expression of their genes are highly induced by both UV-B irradiation and low-temperature treatment. Indeed, Dong et al. (1995) showed that the enzymatic activities of phenylalanine ammonia lyase and chalcone isomerase were increased 10- to 20-fold by UV-B irradiation. In addition, the mRNA levels of *CHS*, *F3H*, *DFR*, *ANS* and *UFGT* were induced by both UV-B irradiation and low temperatures (Ubi et al. 2006, Ban et al. 2007). Thus, most of the genes involved in anthocyanin biosynthesis appear to participate in the development of red coloration in apples under the regulation of UV-B and temperature, but the importance of a possible regulatory mechanism upstream of the anthocyanin biosynthesis cascade in apple skin has been proposed recently (Takos et al. 2006, Espley et al. 2007).

MYB transcription factors are a potent candidate for such a regulatory factor in the upstream cascade because the expression of anthocyanin biosynthetic genes is believed to be under the control of MYB transcription factors (Stracke et al. 2001). MYB transcription factors have been isolated in various plant species. The best-characterized plant *MYB* genes involved in anthocyanin biosynthesis are *C1* and *PL* in maize. In maize, the accumulation of anthocyanin in competent tissues requires the presence of either *C1* in the seed or *PL* in the plant tissue (Cone and Burr 1989). Moreover, *C1* and *PL* induce the expression of multiple anthocyanin biosynthetic genes such as *CHS*, *DFR* and *UFGT* (Cone and Burr 1989).

MYB transcription factors can be classified into three subfamilies based on the number of highly conserved imperfect repeats in the DNA-binding domain (one, two or three; Rosinski and Atchley 1998, Jin and Martin 1999). Accordingly, MYB-like proteins with one repeat have been designated as 'MYB1R', those with two repeats as 'R2R3-MYB' and those with three repeats as 'MYB3R' (Stracke et al. 2001). Among these MYB transcription factors, R2R3-MYB constitutes the largest *MYB* gene family in plants. In *Arabidopsis thaliana*, 125 distinct *R2R3-MYB* genes have been detected within the complete genome sequence (Arabidopsis Genome Initiative 2000), and *AtPAP1* and *AtPAP2* have been shown to regulate phenylpropanoid metabolism, since overexpression of these two genes results in the accumulation of anthocyanins (Borevitz et al. 2000).

In fact, in all species analyzed to date, the common denominators in the regulation of anthocyanin biosynthetic genes are MYB transcription factors, basic helix–loop–helix (bHLH) transcription factors and a WD40 protein (Stracke et al. 2001). Yeast two-hybrid assays indicate that MYB, bHLH and WD40 can interact to form a protein with a complex three-dimensional form. However, MYB transcription factors such as *AtPAP1*, *AtPAP2*, *PhAN2*, *LeANT1* and *GMYB10* are sufficient for ectopic activation of anthocyanin biosynthetic genes in transgenic plants, possibly through the formation of complexes with endogenous bHLH and WD40 (Quattrocchio et al. 1999, Borevitz et al. 2000, Elomaa et al. 2003, Mathews et al. 2003). Moreover, a retrotransposon insertion in *VvMybA1* is the molecular basis of the loss of pigmentation in a white grape cultivar of *V. vinifera* (Kobayashi et al. 2004). These results indicated that modulation of *MYB* gene was enough to cause dramatic changes in anthocyanin accumulation; in other words, MYB transcription factors appear to be a dominant factor in anthocyanin accumulation.

Here we report the isolation of a cDNA, designated as *MdMYBA*, that encodes a putative R2R3-MYB protein. Functional characterization of *MdMYBA*, including expression analysis using different materials and different developmental stages of apple, transformation of apple cotyledons and tobacco, and binding assays with the *MdANS* promoter region, suggested a critical function of *MdMYBA* in the regulation of anthocyanin biosynthesis in apple skin. The linkage map position of *MdMYBA* calculated in our study further strengthens the importance in apple red coloration.

Results

Molecular cloning of the apple MYB transcription factor gene

Using reverse transcription–PCR (RT–PCR) and 5′-/3′-rapid amplification of cDNA ends (RACE), we

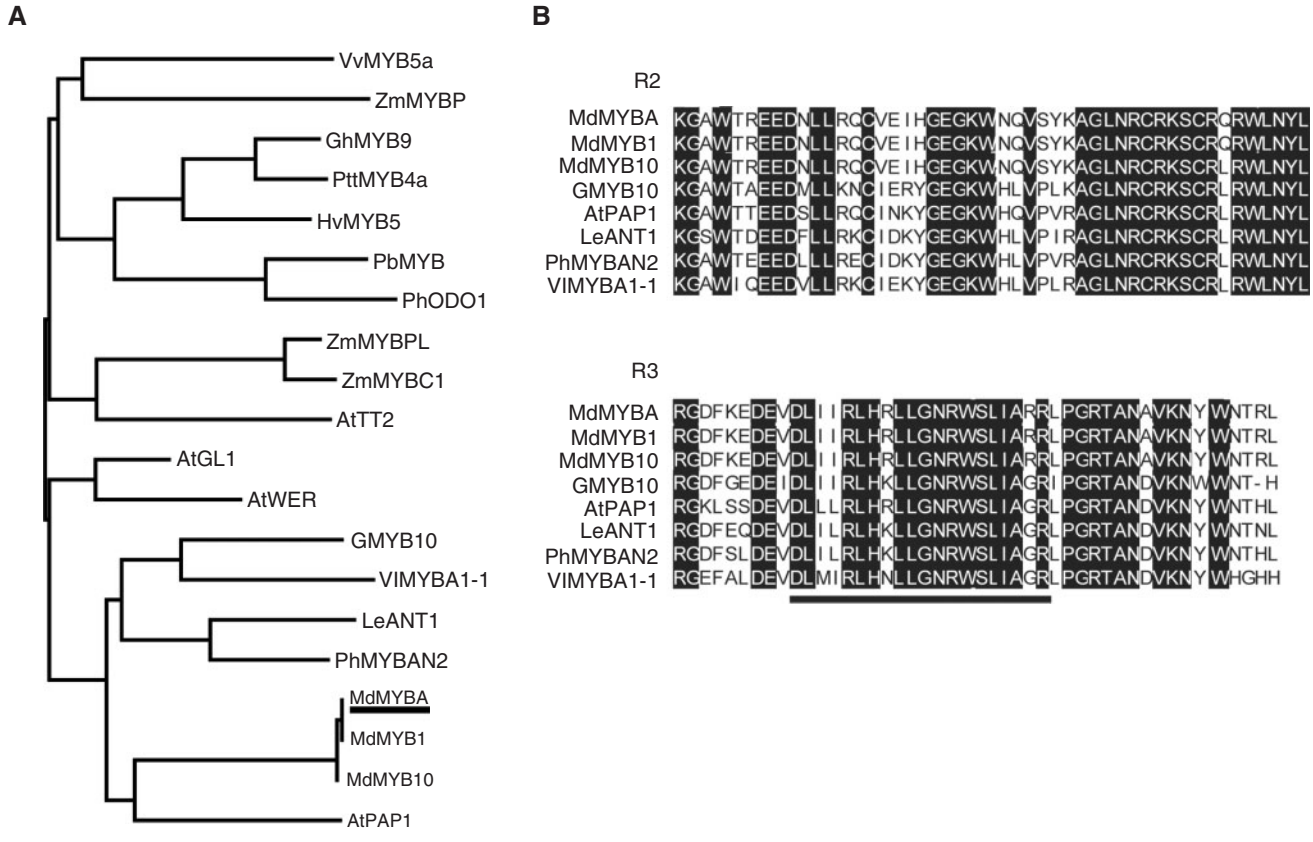

Fig. 1 Comparison of the deduced amino acid sequences of R2R3-type MYB transcription factors from higher plants. (A) A phylogenetic tree for plant R2R3-type MYB transcription factors, including the one isolated from apple in the present study, was constructed by neighbor-joining methods using full-length deduced amino acid sequences. Previously reported MYB transcription factor sequences were retrieved from the EMBL or GenBank databases [*Malus* × *domestica MdMYB1* (ABK58136), *M. domestica MdMYB10* (ABB84754), *Gerbera hybrida GMYB10* (AJ554700), *Vitis labruscana VlMYBA1-1* (AB073010), *Lycopersicon esculentum LeANT1* (AAQ55181), *Petunia hybrida PhMYBAN2* (AF146702), *Arabidopsis thaliana AtPAP1* (AF325123), *A. thaliana AtGL1* (M79448), *A. thaliana AtWER* (AF126399), *Gossypium hirsutum GhMYB9* (AF336286), *Populus tremula* × *tremuloides PttMYB4a* (AJ567346), *Hordeum vulgare HvMYB5* (CAA50221), *Pimpinella brachycarpa PbMYB* (AF161711), *Petunia hybrida PhODO1* (AY705977), *Zea mays ZmMYBC1* (AF320614), *Z. mays ZmMYBPL* (L19496), *Z. mays ZmMYBP* (M73029), *A. thaliana AtTT2* (AJ299452) and *Vitis vinifera VvMYB5a* (AY555190)]. (B) Comparison of the deduced amino acid sequence of the R2 and R3 DNA-binding sites of MdMYBA with anthocyanin-related MYB transcription factors. The R/B-like bHLH binding motif ([D/E]Lx$_2$[R/K]x$_3$Lx$_6$Lx$_3$R) is underlined.

identified one gene, which we named *MdMYBA*, that encodes a putative R2R3-MYB protein. The 859 bp full-length *MdMYBA* cDNA contained an open reading frame (ORF) encoding 243 amino acid residues with the first in-frame ATG at nucleotide position 51 and a stop codon at position 783 (data not shown). A phylogenetic tree for plant R2R3-type MYB transcription factors including the one that we isolated from apple was constructed by means of neighbor-joining methods using the full-length deduced amino acid sequences (Fig. 1A). MdMYBA was identical to MdMYB1 (Takos et al. 2006), and shared 98% homology with MdMYB10 (Espley et al. 2007). In addition, MdMYBA formed a cluster with several previously characterized anthocyanin-related MYB transcription factors in other plants (GMYB10, VlMYBA1-1, LeANT1, PhMYBAN2 and AtPAP1). Comparison of the deduced amino acid sequence of MdMYBA with those of several other plant anthocyanin-related MYB transcription factors revealed a high degree of sequence similarity in the R2 and R3 DNA-binding domains of these proteins (Fig. 1B), whereas the C-terminal region of MdMYBA showed a very low degree of sequence similarity to the proteins shown in Fig. 1B, except for the presence of a motif similar to KPRPR[S/T]F (at positions 132–138 in the deduced amino acid sequence, data not shown), which was defined as a conserved motif in the genes that encode the proteins AN2, AtPAP1, AtPAP2 and AtMYB113 (Stracke et al. 2001). Moreover, the R3 DNA-binding domain contains an R/B-like bHLH-binding motif ([D/E]Lx$_2$[R/K]x$_3$Lx$_6$Lx$_3$R; Zimmermann et al. 2004).

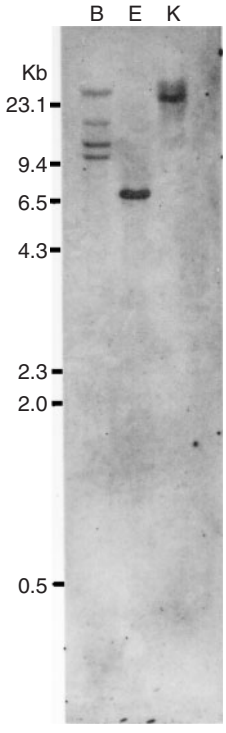

Fig. 2 Southern blot analysis of *MdMYBA* in 'Tsugaru' total DNA. Each lane contained 10 μg of genomic DNA from 'Tsugaru' leaves digested with each restriction enzyme. Lane B, *Bam*HI; E, *Eco*RV; K, *Kpn*I.

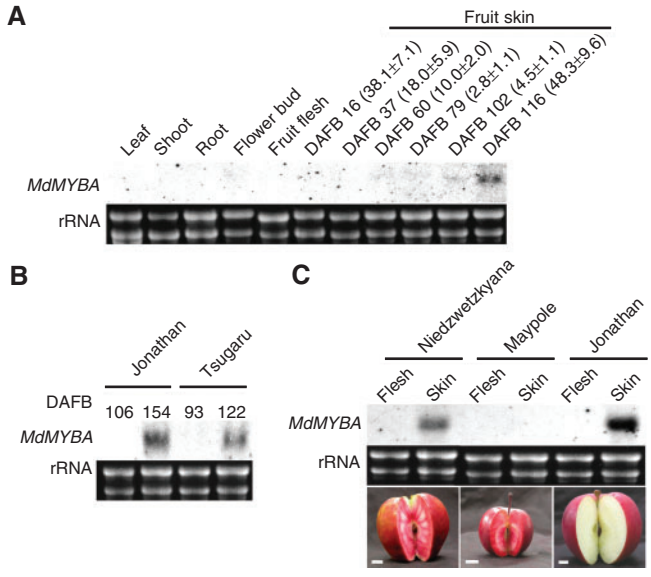

Fig. 3 Northern blot analysis of *MdMYBA* in several tissues and in fruit skins at different ripening stages (A), in ripened apple skins of 'Tsugaru' and 'Jonathan' at two stages (B) and in ripened apple skins and flesh of 'Jonathan', 'Maypole' and *Malus* × *domestica* niedzwetzkyana (C). Each lane contained 10 μg of total RNA from each sample. In the lower panel, the total RNA on the gel was stained with ethidium bromide to confirm equivalent loading. Parentheses after each DAFB indicate the anthocyanin concentration (μg g^{-1} FW). The values are means ± SE ($n = 5$). Bars in (C) = 1 cm.

Southern blot analysis

To estimate the copy number of *MdMYBA* in the apple genome, the genomic DNA from 'Tsugaru' leaves was digested with *Bam*HI, *Eco*RV or *Kpn*I, and Southern blot analysis was carried out under high-stringency conditions using a non-conserved region (outside of the R2 and R3 DNA-binding sites) that included the 3′-untranslated region (UTR) of *MdMYBA* (positions 421–859 in the nucleotide sequence) as a probe to avoid cross-hybridization with other members of the *MYB* gene family. In 'Tsugaru', one band was detected in the *Eco*RV and *Kpn*I digestions (lanes E and K in Fig. 2), and four bands were observed in the *Bam*HI digestion (lane B in Fig. 2). These results indicate that a few copies of *MdMYBA* are present in the 'Tsugaru' apple genome, although the possibility that alleles also showed up as separate bands could not be ruled out.

Expression analysis of MdMYBA in tissues at different ripening stages of several cultivars

MdMYBA expression was analyzed by Northern blotting using the same probe as that in the Southern blot analysis. The transcript of *MdMYBA* was detected only in red-colored skin at 116 d after full bloom (DAFB) (Fig. 3A), although high levels of anthocyanin accumulation were observed at both 16 and 116 DAFB when anthocyanin concentrations in the skin samples were measured. Moreover, the expression level of *MdMYBA* in 'Jonathan' (a deep-red cultivar) was much higher than that in 'Tsugaru' (a pale-red cultivar) (Fig. 3B). Anthocyanin concentrations in the two cultivars used in Fig. 3B had previously been measured by Honda et al. (2002) and Ubi et al. (2006). Both studies reported no detectable accumulation of anthocyanins in 'Tsugaru' and 'Jonathan' at 93 and 106 DAFB, respectively, but noticeable anthocyanin accumulations at 122 and 154 DAFB, respectively. In addition, the final anthocyanin concentrations in 'Jonathan' were much higher than those in 'Tsugaru' (about 20 times higher in absorbance at 530 nm). These results indicate that the level of *MdMYBA* expression is positively correlated with the accumulation of anthocyanins in apple skin after a certain age (i.e. not in young fruit skin). When other apples, *Malus* × *domestica* niedzwetzkyana and 'Maypole', were used for analysis, no *MdMYBA* signals were detected in the flesh from ripened fruits of both apples regardless of the red coloration (Fig. 3C). Interestingly, *MdMYBA* was detected in the skin of *Malus* × *domestica* niedzwetzkyana as in

'Jonathan', but not in the skin of 'Maypole' (Fig. 3C). Furthermore, we re-confirmed the specific expression pattern, i.e. only in the skin from ripened fruits of 'Jonathan' (Fig. 3C). These results indicated that the specific expression of *MdMYBA* depends on the tissue and cultivar/species.

Functional analysis of MdMYBA

To ascertain the role of *MdMYBA*, transient gene expression assays were used. *MdMYBA* under the control of the cauliflower mosaic virus (CaMV) 35S promoter was introduced into 'Tsugaru' apple cotyledons by means of particle bombardment. Reddish-purple spots were observed in non-colored apple cotyledons after 2 d of cultivation when *MdMYBA* was delivered (Fig. 4A). In contrast, when the β-glucuronidase (*GUS*) gene was delivered to apple cotyledon, only blue spots were observed after GUS staining (Fig. 4B). Therefore, the reddish-purple spots that we observed in the apple cotyledons were not artifacts, but rather they truly resulted from the induction of anthocyanin accumulation by *MdMYBA*.

We also used tobacco as a model system for analysis of heterologous gene expression. A total of seven of the T_0 generation of transgenic tobacco plants that constitutively expressed *MdMYBA* under the control of the CaMV 35S promoter showed no significant differences in growth compared with the wild-type lines (data not shown). However, conspicuous anthocyanin accumulation was observed in the reproductive tissues of all the transgenic plants (Fig. 4D, F, H, J and L) but not in those of the wild type (Fig. 4C, E, G, I and K). Reddish coloration of the sepals, petals, anthers, filaments and ovary walls in the flowers of transgenic plants increased compared with that in the wild type (Fig. 4C–J). No clear changes in stigma and style coloration were observed. Conspicuous levels of such coloration were also observed in the epidermis of the capsule (Fig. 4L). Other noticeable changes caused by the introduction of *MdMYBA* appeared in the petal shapes. The shape of each petal of all the transgenic plants became

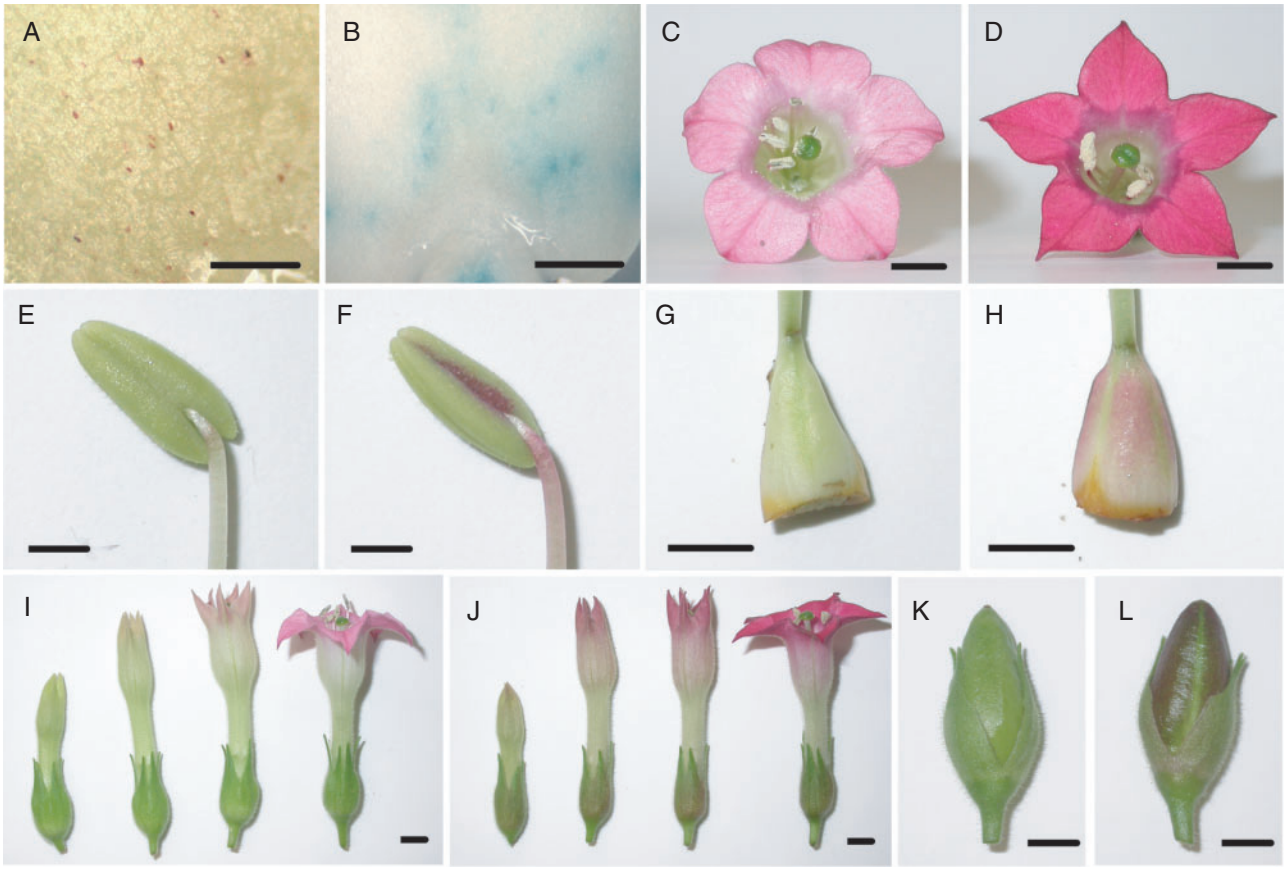

Fig. 4 Assay of transient gene expression and phenotypes of transformed tobacco constitutively expressing *MdMYBA*. *MdMYBA* under control of the 35S promoter was introduced into apple cotyledons by means of particle bombardment (A). The β-glucuronidase (GUS) gene was used as a negative control (B). Flowers of transgenic plants (D, F, H, J and L) showed a phenotypic change in flower organ pigmentation compared with the wild-type plants (C, E, G, I and K). Bars in (A) and (B) = 0.5 mm; (E) and (F) = 1 mm; others = 5 mm.

more triangular than that of the wild type, and, consequently, lobation of the flower petals was more apparent in all seven transformants (Fig. 4D) than in the wild type (Fig. 4C). The reason for this change is unknown.

Expression of MdMYBA in apple skin in response to UV-B irradiation and temperature

Changes in anthocyanin concentrations in the 'Tsugaru' fruit skins in response to the UV-B and temperature treatments were analyzed (Fig. 5A). The UV-B irradiation and low temperature both enhanced anthocyanin accumulation. UV-B irradiation significantly stimulated anthocyanin synthesis regardless of the temperature conditions, but the effect was greater at the low (17°C) temperature. However, there were no significant differences in anthocyanin accumulation in the absence of UV-B irradiation, irrespective of temperature.

The expression of *MdMYBA* was also analyzed using the same samples used for determining the anthocyanin concentration (Fig. 5B). The expression of *MdMYBA* was induced more strongly by UV-B irradiation, regardless of the temperature conditions, whereas its expression in the skins was inhibited by high temperature with or without UV-B irradiation, although a faint signal was still observed in the skins treated with UV-B at the higher temperature.

Binding of MdMYBA to an MdANS promoter in vitro

To assess whether MdMYBA can bind to the promoters of anthocyanin biosynthetic genes, we carried out a gel-shift assay (Fig. 6A). We used the 138 bp fragment of the *MdANS* promoter (the F2 fragment) as a probe. The F2 fragment contains four types of MYB-binding sites and one bHLH-binding site (Fig. 6B). The MdMYBA protein was synthesized by in vitro translation. A strong signal of MdMYBA binding to the apple *ANS* promoter was observed in the presence of 100 ng of poly-L-lysine and 125 ng of poly[d(A–T)] (Fig. 6A, lane 2). In addition, the binding signal was reduced by adding high concentrations of the non-specific competitor poly[d(A–T)] and was inhibited by the non-labeled F2 fragment (Fig. 6A, lanes 3–7). These results indicated that MdMYBA can bind specifically to the *MdANS* promoter F2 fragment.

The position of the MdMYBA gene in the molecular linkage map and further characterization of the MdMYBA marker

A molecular marker linkage map from the 'Delicious' × Mitsubakaido cross was used for gene mapping. Polymorphic DNA bands were detected in two parents by means of PCR using primers created for

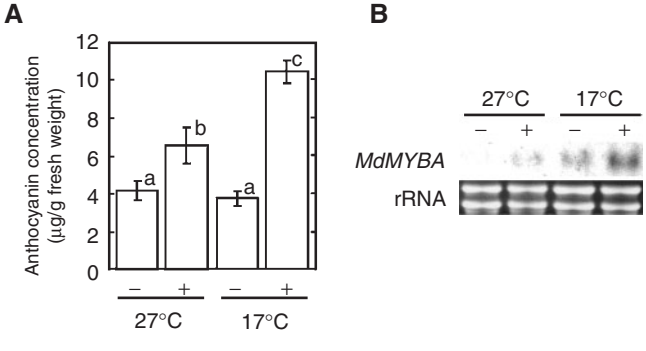

Fig. 5 Levels of anthocyanin accumulation (A) and Northern analysis of *MdMYBA* in apple fruit skins under UV-B and low-temperature treatments (B). The treatment conditions are indicated in each panel: with UV-B (+) or without UV-B (–), and at 17 or 27°C. (A) The values represent averages of five replications. Error bars indicate the SE. Bars labeled with different letters differ significantly ($P<0.01$, Tukey's LSD test). (B) Each lane contained 10 μg of total RNA from each sample. In the lower panel, the total RNA on the gel was stained with ethidium bromide to confirm equivalent loading.

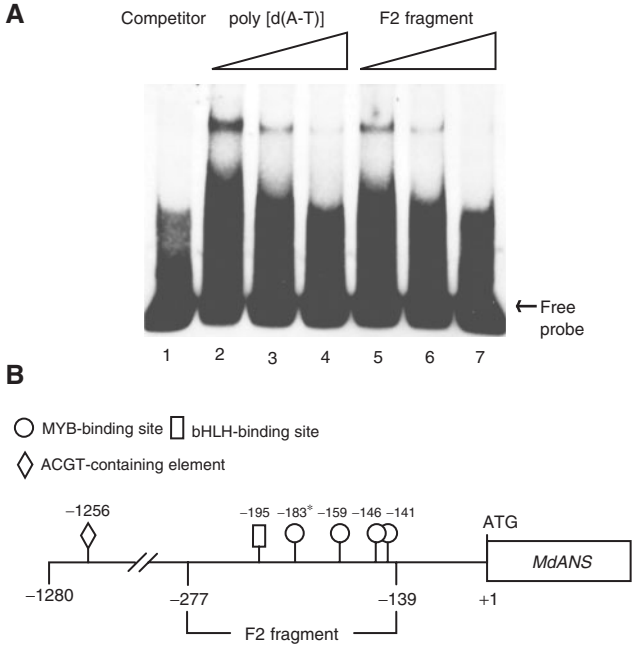

Fig. 6 In vitro DNA-binding activity of MdMYBA and features of the *MdANS* promoter. (A) Gel-shift assays were carried out using the 138 bp fragment (F2 fragment) of the apple *ANS* promoter as the probe and reaction solutions of cell-free protein synthesis. Lane 1 contained no pIVEX 1.3 WG-*MdMYBA* in the reaction solution of protein synthesis, whereas lanes 2–7 did. All reactions were carried out in the presence of 100 ng of poly-L-lysine and 125 ng of poly[d(A–T)], except lanes 3 and 4. Lanes 3 and 4 contained 225 and 375 ng of poly[d(A–T)], respectively. Lanes 5–7 contained 25 ng (lane 5), 75 ng (lane 6) and 250 ng (lane 7) of the F2 fragment, which was used for probe synthesis. (B) Schematic structure of the *MdANS* promoter. Numbers above the symbols indicate site or element positions (bp) counted from the start codon. The MYB-binding site at position −183 (indicated with an asterisk) shows high similarly to the MYB-recognition element in *Arabidopsis* that was proposed by Hartmann et al. (2005).

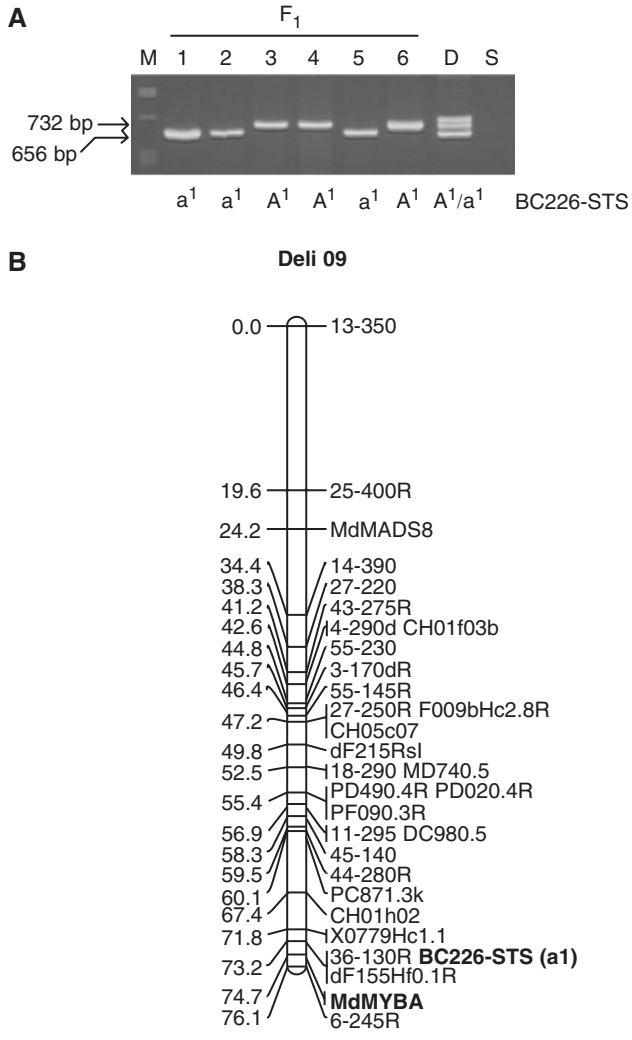

Fig. 7 (A) Segregation of amplified fragments using primers for the *MdMYBA* gene in the 'Delicious' × Mitsubakaido F_1 population. F_1 (1–6), F_1 individual; D, 'Delicious'; S, Mitsubakaido; M, DNA size marker. Letters below the panel indicate segregation of the BC226-STS marker from 'Delicious'. The BC226-STS marker is an apple skin color marker developed by Cheng et al. (1996). (B) Map position of *MdMYBA* in linkage group Deli 09 in the 'Delicious' map.

MdMYBA amplification. Three DNA bands were amplified from the genomic DNA template of 'Delicious', whereas no DNA bands were amplified from Mitsubakaido (Fig. 7A), indicating that Mitsubakaido has null alleles for the *MdMYBA* marker using the developed primers. Two different length bands (723 and 656 bp) in 'Delicious' were segregated in the 'Delicious' × Mitsubakaido F_1 population (Fig. 7A). The third band (upper band) was not detected in the F_1 population. The result of sequencing of this third band shows that it contains recombinant molecules between the 723 and 656 bp bands. Recombination is known to occur between similar sequences during PCR amplification (Meyerhans et al. 1990, Judo et al. 1998). We therefore concluded that the third band is an artificial band created from the other two DNA bands. By counting these segregated DNA bands, we mapped *MdMYBA* to the lower part of linkage group Deli 09 [near the region of the BC226-STS (a^1) marker] in the 'Delicious' map (Fig. 7B).

Table 1 Segregation of the *MdMYBA* marker and BC226-STS marker in the 'Delicious' × Mitsubakaido F_1 population

BC226-STS	MdMYBA	
	723 bp	656 bp
A^1 (red allele)	29	0
a^1 (no red allele)	1	42

To confirm the linkage relationships between the *MdMYBA* marker and the BC226-STS marker, the segregation ratio was counted in the 'Delicious' × Mitsubakaido F_1 population (Table 1). The red allele (A^1) perfectly matched the 723 bp band amplified by the *MdMYBA* marker, whereas the no red allele (a^1) matched the 656 bp band, indicating that each A^1 and a^1 allele is tightly linked with a 723 and 656 bp band, respectively. One progeny, however, possessed a 723 bp *MdMYBA* band regardless of the no red allele, which may be due to the recombination between the *MdMYBA* marker and the BC226-STS marker.

Because F_1 progeny between the 'Delicious' × Mitsubakaido cross have not yet borne fruits, we could not compare the *MdMYBA* marker and their skin colors at present. Therefore, 14 apple cultivars with known skin colors were used for confirmation of the *MdMYBA* marker (Fig. 8). In these 14 apple cultivars, six types of BC226-STS genotypes (A^1A^1, A^1a^2, A^1a^1, a^1a^1, a^1a^2 and a^2a^2) were detected. It was apparent that the amplified band size (723 bp) of the a^2a^2 genotype (yellow or green skin color) was the same as that of the A^1A^1 genotype (red skin color) regardless of the different skin color. Moreover, the amplified bands in A^1a^1 and a^1a^2 genotypes showed the same patterns, which also did not correspond to their skin color. These results indicate that the discrimination of the A^1 and a^2 genotype was impossible by the *MdMYBA* marker. Meanwhile, the a^1a^1 genotype (five cultivars) stably showed the 656 bp band of the *MdMYBA* marker, which was consistent with a yellow or green skin color. In addition to these five cultivars, nine breeding lines that were homozygotes of the a^1 allele also produced a 656 bp band of the *MdMYBA* marker, and these breeding lines showed yellow or green skin color phenotypes (data not shown).

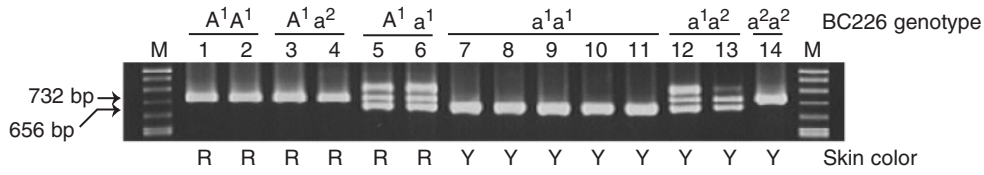

Fig. 8 Amplification of the *MdMYBA* marker from 14 apple cultivars. Lane 1, 'Ralls Janet'; 2, 'Jonathan'; 3, 'Tsugaru'; 4, 'Gala'; 5, 'Fuji'; 6, 'Delicious'; 7, 'Kinsei'; 8, 'Gunmameigetsu'; 9, 'Ohsyu'; 10, 'Narihoko'; 11, 'Ambitious'; 12, 'Golden Delicious'; 13, 'Orin'; 14, 'Indo'; M, DNA size marker. The BC226 genotype and skin color (R, red skin; Y, yellow or green skin) of each apple cultivar are shown.

Discussion

Comparison of anthocyanin-related MYB transcription factor genes in apple

Our study showed that *MdMYBA*, a cDNA encoding a putative R2R3-MYB protein, regulated anthocyanin biosynthesis in apple skin. Recently, Takos et al. (2006) isolated the *MYB* (*MdMYB1*) gene, whose nucleotide sequence in the ORF was the same as that of *MdMYBA* (Fig. 1A). Therefore, the results for *MdMYBA* and *MdMYB1* (Takos et al. 2006) partly overlapped. However, a discrepancy between *MdMYBA* and *MdMYB1* was observed in the expression in the skin at the young fruitlet stage. In fruitlets of 'Tsugaru', we could not detect any *MdMYBA* transcripts despite apparent anthocyanin accumulation (Fig. 3A), whereas the *MdMYB1* transcript could be detected in the red skin of young fruit from 'Cripps Red' at 48 DAFB. Although this discrepancy may be due to the sensitivity of the methods used or different environmental cues at this early point in the season, another possibility that the different cultivars used for *MdMYBA* ('Tsugaru') and *MdMYB1* ('Cripps Red') might be ascribed to this result cannot be ruled out. If this is the case, anthocyanin biosynthesis in fruitlets and ripened fruits is under different regulatory mechanisms in 'Tsugaru', and *MdMYBA* could contribute to red coloration only in the skin from ripened 'Tsugaru' fruits. In grape, two kinds of MYB transcription factors that regulate phenylpropanoid biosynthesis and show different expression patterns have been isolated. One is encoded by *VlMybA1-1*, whose expression was detected in ripening grape (Kobayashi et al. 2002). The other is encoded by *VvMYB5a*, whose expression was observed in the early fruit stage (Deluc et al. 2006). VvMYB5a is involved in controlling the flavonoid pathway for the synthesis of tannins and flavonols in grape, but anthocyanin accumulation occurs in the whole plant when *VvMYB5a* is constitutively expressed in tobacco (Deluc et al. 2006), implying that VvMYB5a has the potential to induce the accumulation of anthocyanins. These results in grape showed that different types of MYB transcription factors differentially regulated the biosynthesis of phenylpropanoid in the early and ripening stages. Thus, in 'Tsugaru' apple fruitlets, other MYB transcription factors might also regulate the biosynthesis of phenylpropanoids, including anthocyanin. Since an analysis of the genomic sequences including the promoter regions has not yet been carried out for *MdMYBA*, sequence comparisons of the promoter regions of *MdMYBA* ('Tsugaru') and *MdMYB1* ('Cripps Red') would help to address this issue.

More recently, Espley et al. (2007) isolated the *MYB* (*MdMYB10*) gene. Although the nucleotide sequences in the ORF of *MdMYBA* and *MdMYB10* shared 98% homology (Fig. 1A) and the deduced amino acid sequences were different in only three amino acid residues, different features were apparent, especially in their expression patterns. *MdMYB10* was highly expressed in the cortex and skin from the red-fleshed cultivar 'Red Field', while *MdMYBA* expression was not detected in the cortex and skin from the red-fleshed cultivar 'Maypole'. Contrary to our expectations, *MdMYBA* expression was not detected in the cortex but in the skin of *Malus* × *domestica* niedzwetzkyana, a parental material used in the study by Espley et al. (2007) (Fig. 3C). Moreover, high expression levels of *MdMYB10* were detected in the fruit skin of the white-fleshed cultivar Pacific Rose™ ('Sciros') even at mid-season (102 DAFB) (Espley et al. 2007), while *MdMYBA* was below the level of detection in the skins collected from mid-season fruits and was detected only in the ripened skin from the white-fleshed cultivar 'Tsugaru' (Fig. 3A). When tobacco leaves were transformed with *MdMYB10* under the control of the CaMV 35S promoter, anthocyanin accumulation was observed in leaf epidermal cells. In contrast, *MdMYBA* could not induce anthocyanin accumulation in leaf epidermal cells of transgenic tobacco (data not shown). These results indicated that *MdMYB10* and *MdMYBA* could be different genes derived from different loci and that the preferable expression of either *MdMYBA* or *MdMYB10* is genetically regulated depending on the apple cultivar/species.

Based on these results on the MYB transcription factors in apple, there could be at least two loci of anthocyanin-related MYB transcription factors in apple, one corresponding to the coloration of the cortex of red-fleshed cultivars (*MdMYB10*) and the other to the skin

color of white-fleshed cultivars (*MdMYB1* and *MdMYBA*). However, the expression of *MdMYBA* in the skins of *Malus* × *domestica* niedzwetzkyana and 'Maypole' was different; therefore, further study is needed. Recently, Walker et al. (2006) suggested that the red allele possessed two *MYB* genes, *VvMYBA1* and *VvMYBA2*, and that these two genes were located in the same chromosome in grape. Taking this fact into consideration, we hypothesized the tandem (or close) arrangement of these two loci in the same chromosome in apple. Comprehensive genomic information about *MdMYBA*, *MdMYB1* and *MdMYB10* would validate our hypothesis.

Functional features of MdMYBA in transgenic plants

MdMYBA induced anthocyanin accumulation in homologous plants (apple cotyledons) (Fig. 4), providing further evidence that *MdMYBA* is involved in anthocyanin biosynthesis in apple skin. When tobacco was transformed with *MdMYBA*, anthocyanin accumulation was restricted to the reproductive tissues. The same result was observed in *Arabidopsis* transformed with *MdMYB1* (Takos et al. 2006). On the other hand, when *VlMybA1-1* from grape (Kobayashi et al. 2002) was constitutively expressed in tobacco, anthocyanin accumulation occurred in the whole plant, including both vegetative and reproductive tissues (data not shown). The same phenotypic features as those found in grape were observed in transgenic tobacco plants in which *GMYB10* from *Gerbera hybrida*, *LeANT1* from tomato and *AtPAP1* from *Arabidopsis* were constitutively expressed (Borevitz et al. 2000, Elomaa et al. 2003, Mathews et al. 2003). These phenotypic differences between the constitutive expression of *MdMYBA* and other anthocyanin-related MYB transcription factors may be ascribed to whether MYB can induce a bHLH transcription factor or not. Indeed, previous observations indicated that MYB transcription factors functioned as an activator for bHLH transcription, therefore MYB efficiently formed a complex with bHLH and the resultant complex could successively induce anthocyanin biosynthesis (Koes et al. 2005). This scenario is confirmed by the fact that MYB transcription factors are dominant factors in anthocyanin accumulation, possibly through the activation of bHLH. Therefore, we assumed that bHLH in tobacco could be present earlier in flowers due to their potent ability to accumulate anthocyanins, thus MdMYBA could form a complex with this endogenous bHLH; in contrast, bHLH could not be present in other tissues or organs, in which bHLH should be activated by MYB factors for anthocyanin accumulation, thus no accumulation occurred. It can therefore be hypothesized that MdMYBA may lack this ability for bHLH activation, unlike other anthocyanin-related MYB transcription factors in tobacco. As a result, anthocyanin accumulation took place only in the reproductive tissues, not in other tissues, regardless of the constitutive expression of *MdMYBA*.

Expression of MdMYBA and its possible regulatory mechanism

Takos et al. (2006) showed the induction of *MdMYB1* expression by light, but they did not confirm the effects of a specific lightwave length (UV-B) and low temperature on the expression of *MdMYB1*. We therefore analyzed the changes in *MdMYBA* expression in apple skins treated with UV-B and different temperatures. The expression of *MdMYBA* was enhanced by both UV-B irradiation and low temperature, and its expression was positively correlated with the accumulation of anthocyanin in apple skins treated with UV-B and low temperature (Fig. 5). Ubi et al. (2006) and Ban et al. (2007) reported that the expression of anthocyanin biosynthetic genes, including *MdCHS*, *MdF3H*, *MdANS* and *MdUFGT*, was enhanced by UV-B irradiation and low-temperature treatment. The expression pattern of *MdMYBA* is the same as that of these three anthocyanin biosynthetic genes. Therefore, the increase in anthocyanin concentrations induced by UV-B irradiation and low-temperature treatment could be attributed to the activation of *MdMYBA*. This activation of *MdMYBA* expression might sequentially cause the induction of the genes involved in the anthocyanin pathway.

We also showed direct evidence for interactions between MdMYBA and the F2 fragment in the promoter region of *MdANS* by gel-shift assay (Fig. 6A). The F2 fragment, located between positions −139 and −277 of the *MdANS* promoter region sequence, contained four possible sequences for MYB-binding sites: at positions −141 to −146, −146 to −151, −159 to −168 and −183 to −189 in the nucleotide sequence of the *MdANS* promoter region (Fig. 6B). When compared with previous reports on MYB-binding sites (Urao et al. 1993, Solano et al. 1995, Abe et al. 2003, Hartmann et al. 2005), two sequences in the *MdANS* promoter region (positions −159 to −168 and −183 to −189) showed high similarity to the MYB-binding site that is related to the activation of phenylpropanoid biosynthesis (Solano et al. 1995, Hartmann et al. 2005). Thus, MdMYBA might bind to one or both of these sequences. In addition to the MYB-binding site, one bHLH-binding site was located near the MYB-binding sites (positions −195 to −200 in the sequence of the F2 fragment; Fig. 6B). Recently, Hartmann et al. (2005) identified light regulatory units that consisted of a MYB recognition element and an ACGT-containing element in *Arabidopsis*, and they suggested that a bZIP transcription factor or bHLH might bind to the ACGT-containing element. Takos et al. (2006) reported that the promoters of *MdDFR* and *MdUFGT* contained light regulatory units. In the apple *ANS* promoter region, putative MYB recognition and ACGT-containing

elements were also located at positions −183 to −189 (in the F2 fragment) and −1256 to −1260, respectively, but these two elements are much farther apart than they are in *Arabidopsis* (Fig. 6B). Thus, further investigation of the other promoters of anthocyanin biosynthetic genes and the inter-relationships among MdMYBA and other factors (e.g. bHLH and bZIP), as shown in the preliminary experiment by Espley et al. (2007), will shed light on the regulatory mechanisms underlying anthocyanin biosynthesis in apple skin.

Mapping of MdMYBA

MdMYBA was mapped at the lower part of the Deli 09 linkage group in the 'Delicious' map (Fig. 7B). The BC226-STS (a^1) marker for the red skin color locus (R_f) in apple, developed by Cheng et al. (1996), was mapped to the near region of Deli 09 (Fig. 7B). The distance between *MdMYBA* and BC226-STS (a^1) was estimated to be 1.5 cM. Cheng et al. (1996) showed that the recombination frequency between skin colors for the color marker was approximately 1.7%, indicating that a distance of 1.5 cM between *MdMYBA* and BC226-STS (a^1) may be within the allowable range of error. These results supported the hypothesis that *MdMYBA* is an important gene for controlling skin color.

Because F_1 progeny between 'Delicious' × Mitsubakaido had not yet borne fruits, we used 14 apple cultivars with known skin colors to compare *MdMYBA* markers with apple skin color (Fig. 8). When genomic DNA from the 14 apple cultivars was amplified by the *MdMYBA* marker, this marker could not discriminate the a^2 genotype because it had the same amplification pattern as A^1 (Fig. 8). We detected two types of *MdMYBA* alleles: one of 656 bp (no red allele: corresponding to a^1) and one of 723 bp (red allele: corresponding to A^1). On the other hand, Takos et al. (2006) isolated three types of *MdMYB1* alleles: *MdMYB1-1* (red allele), *MdMYB1-2* (no red allele) and *MdMYB1-3* (no red allele). A comparison of the genomic sequences of *MdMYB1-1* and *MdMYB1-3* showed a 67 bp shortage in the first intron of *MdMYB1-3* (Takos et al. 2006). Since the fragments of the *MdMYBA* marker included the first intron, the 67 bp difference between A^1 and a^1 in our *MdMYBA* marker could be truly comparable with the differences between the *MdMYB1-1* and *MdMYB1-3* alleles. Meanwhile, no differences between *MdMYB1-1* and *MdMYB1-2* were detected in the first intron (Takos et al. 2006), and we supposed that *MdMYB1-2* could correspond to a^2, which was why the *MdMYBA* marker could not discriminate A^1 and a^2. In this study, the F_1 population from 'Delicious' × Mitsubakaido did not possess the BC226 allele a^2 (Fig. 7A); therefore, the *MdMYBA* marker could be successfully mapped in the F_1 population from 'Delicious' × Mitsubakaido regardless of the incomplete co-segregation of the *MdMYBA* marker with skin color (Fig. 7B). Furthermore, a homozygote with 656 bp *MdMYBA* alleles (a^1a^1) was completely co-segregated with the yellow or green skin color character. Consequently, we proposed that the A^1, a^1 and a^2 alleles developed by Cheng et al. (1996) correspond to *MdMYB1-1*, *MdMYB1-3* and *MdMYB1-2*, respectively.

Materials and Methods

Plant materials

For isolation of the gene for the MYB transcription factor, we used the 'Tsugaru' cultivar of *Malus* × *domestica* (a pale-red cultivar) and, for functional analysis, we used both 'Tsugaru' and 'Jonathan' (deep-red cultivars). All fruits were obtained from the orchard of the National Institute of Fruit Tree Science at Morioka, Japan, in 2004, 2005 and 2006. Fruits from 'Tsugaru' were harvested at 93 and 122 DAFB in 2004 and at 16, 37, 60, 79, 102 and 116 DAFB in 2005. Fruits from 'Jonathan' were harvested at 106 and 154 DAFB in 2004. For further characterization of *MdMYBA* expression, the flesh and skin of mature fruits of 'Maypole' (119 DAFB), *M.* × *domestica* niedzwetzkyana and 'Jonathan' were also used. The entire skin was collected, including 1 mm of the cortical tissue, and then was immediately frozen in liquid nitrogen and stored at −80°C until it could be used for RNA isolation.

UV-B and temperature treatments were performed as described by Ubi et al. (2006). 'Tsugaru' fruits were bagged on the trees about 1 month before commercial harvest (116 DAFB). The treatments were (i) 17°C with UV-B; (ii) 17°C without UV-B; (iii) 27°C with UV-B; and (iv) 27°C without UV-B. In this report, we designated 17°C as the low-temperature treatment and 27°C as the high-temperature treatment. These treatments were maintained for 48 h, then the skin samples (including 1 mm of the cortical tissue) were collected from the half of each fruit sample that had been most directly exposed to the light source (i.e., the upper half). Collected skin samples were immediately frozen in liquid nitrogen, and then were stored at −80°C until they could be used for RNA isolation.

Isolation of the apple MYB transcription factor gene

Partial cDNA fragments of the *MYB* transcription factor gene were amplified by RT–PCR. Total RNA was isolated from 'Tsugaru' fruit skin 122 DAFB using the hot borate method (Wan and Wilkins 1994). First-strand cDNA was synthesized with a SMART RACE cDNA amplification kit (Clontech, Palo Alto, CA, USA), and was used as a template for amplification of fragments. Forward (5′-CTGGACTGAACAGATGCAGAAARWSHTGCMG-3′) and reverse (5′-CCAGCGATCAGGGACCANCKRTTDCC-3′) degenerate primers were designed from highly conserved regions of plant MYB transcription factors [*Petunia hybrida MYBAN2* (AF146702), *Lycopersicon esculentum ANT1* (AAQ55181), *A. thaliana PAP1* (AF325123] and *V. labruscana MYBA1-1* (AB073010)] using the CODEHOP program (Rose et al. 1998). The PCR was performed in a total volume of 20 µl containing 50 ng of cDNA, 200 µM dNTPs, 150 µM $MgCl_2$, 2.5 µM of each primer, 0.5 U of AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA) and GeneAmp PCR buffer II (Applied Biosystems). After pre-PCR heating at 95°C for 12 min, a reaction cycle of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min was repeated 40 times.

The PCR product was cloned into the pCR2.1 vector using a TA cloning kit (Invitrogen, San Diego, CA, USA) and sequenced. To isolate the full-length cDNA of the apple *MYB* transcription factor gene, 5′-RACE and 3′-RACE were used. A 5 μg aliquot of total RNA from 'Tsugaru' apple skin was used to prepare RACE-Ready cDNAs with a SMART RACE cDNA Amplification Kit (Clontech) following the manufacturer's instructions. The 5′-RACE and 3′-RACE were carried out with 5′ and 3′ gene-specific primers which were designed based on the partial sequence of the *MYB* transcription factor gene. The cDNA ends were ligated into pCR 2.1 (Invitrogen) and sequenced. The resultant sequences were aligned to obtain full-length cDNA. The full-length gene was designated as *MdMYBA*.

Southern and Northern blot analysis

For Southern blot analysis, genomic DNA was isolated from mature leaves of 'Tsugaru' trees according to a modified cetyl trimethyl ammonium bromide (CTAB)-based method (Hasebe and Iwatsuki 1990). A 10 μg aliquot of genomic DNA was digested with *Bam*HI, *Eco*RV or *Kpn*I, and electrophoresis was carried out in a 0.8% (w/v) agarose gel and the products transferred to a nylon Hybond N membrane (Amersham Biosciences, Piscataway, NJ, USA) by means of capillary transfer. The partial cDNA including the 3′-UTR of *MdMYBA* was labeled with digoxigenin (DIG)-dUTP (Roche Diagnostics, Mannheim, Germany) and used as a probe. Pre-hybridization (1 h) and hybridization (overnight) were carried out in a high-SDS hybridization buffer containing 50% formamide, 5× SSC, 2% blocking solution (Roche Diagnostics), 0.1% lauroylsarcosine and 7% SDS at 42°C. After hybridization, the membranes were washed with 2× SSC (75 mM NaCl and 7.5 mM tri-sodium citrate, pH 7.0) and 0.1% SDS at room temperature for 15 min, followed by washing twice with 0.1× SSC and 0.1% SDS at 68°C for 15 min. The detection was performed according to the manufacturer's instructions using a DIG-CSPD system (Roche Diagnostics), and the membranes were exposed to X-ray film (Fuji Photo Film, Tokyo, Japan).

For Northern blot analysis, total RNA was isolated from various tissues and from the fruit skins of 'Tsugaru', 'Jonathan', 'Maypole' and *Malus* × *domestica* niedzwetzkyana using the same method as described above. Total RNA (10 μg) was electrophoresed on a 1.2% agarose–formaldehyde gel and blotted onto a nylon Hybond-N membrane (Amersham Biosciences). Hybridization, washing and detection were carried out under the same conditions as described for Southern blot analysis.

Transient gene expression assays and plant transformation

The plasmid used for the transient expression assay was constructed by ligating full-length *MdMYBA* amplified with *Taq* polymerase to pBI221 (Clontech). The constructed vector (pBI221-MdMYBA) was delivered to the cotyledons of 2 day-old seedlings of 'Tsugaru' using a PDS-1000/He particle gun (Bio-Rad, Hercules, CA, USA). Gold microcarriers (1.1 μm) coated with plasmids according to the manufacturer's instructions were used for delivery of the vector, and bombardment was carried out with a vacuum of 28 inches of Hg, a helium pressure of 1,350 p.s.i. and a 6 cm target distance. After bombardment, apple cotyledons were incubated for 2 d in a growth chamber (24 h in the dark, 22°C). For GUS staining, apple cotyledons were incubated in phosphate buffer containing 0.1% Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-gluc), 10 mM EDTA, 0.1% sodium lauroyl sarcosinate and 10 mM 2-mercaptoethanol for 24 h at 37°C (Jefferson 1987). The stained tissues were treated with pure ethanol several times for bleaching.

For *Agrobacterium*-mediated transformation, the *Eco*RI fragment (CaMV 35S::MdMYBA) was excised from pBI221-MdMYBA and ligated into the cassette Ti-vector pSMAK312-Blue (kindly provided by Dr. Hiroaki Ichikawa, National Institute of Agrobiological Sciences). The resultant construct (pSMAK-35S::MYBA) was introduced into *Agrobacterium tumefaciens* strain LBA4404. These recombinant strains were used to transform *Nicotiana tabacum* 'Petit Havana SR1' according to the leaf disk method (Horsch et al. 1989). Transgenic plants were selected based on kanamycin resistance. CaMV 35S::MdMYBA T_0 lines were selected and propagated on MS-HF medium under an illumination of ~2,500 lux in a 16 h light/8 h dark cycle at 25°C. Phenotypic features, including color changes, of the transgenic tobacco were monitored to allow comparison with the wild-type line.

Measurement of the total anthocyanin concentration

Total anthocyanin was extracted according to the method of Dong et al. (1995). A 1 g aliquot of peel disks randomly chosen from each sample was placed in 5 ml of 12 N hydrochloric acid : methanol (1 : 99, v/v) at 4°C for at least 6 h. The absorbance of each extract (100 μl) was measured at 530, 620 and 650 nm with a spectrophotometer (UV-1600, Shimadzu, Kyoto, Japan). The concentration of anthocyanin was calculated according to the methods of Siegelman and Hendriks (1958).

Gel-shift assay

For protein synthesis, *MdMYBA* was amplified with *Taq* polymerase and ligated into the pIVEX 1.3 WG vector (Roche Diagnostics). The resultant construct (pIVEX 1.3 WG-MdMYBA) was used for MdMYBA protein synthesis using the RTS 100 Wheat Germ CECF Kit (Roche Diagnostics) according to the manufacturer's instructions. The promoter region of the anthocyanidin synthase gene (*MdANS*) (1,280 bp) was screened from the 'Tsugaru' genomic library. A 138 bp fragment of the *MdANS* promoter (the F2 fragment) was amplified with *Taq* polymerase using primers (forward, 5′-TCACTCTCCAACACGCTCAC-3′; reverse, 5′-AATGGTTTAACGGGCATTGA-3′) and the fragment was used for labeling. Gel-shift assay was carried out using the DIG Gel Shift Kit, 2nd Generation (Roche Diagnostics), according to the manufacturer's instructions.

Mapping of MdMYBA on the linkage map

The molecular marker linkage map from the cross 'Delicious' × Mitsubakaido (*Malus sieboldii*) (M. Igarashi of the Aomori Green BioCenter, unpublished data) was used to map *MdMYBA*. To search for polymorphisms, the intron region of *MYBA* was amplified from 'Delicious' genomic DNA and the sequence was analyzed. The primer set (forward, 5′-ATTCTAGGTGTCTTTCTGGAGTGTA-3′ for the 5′ non-coding region; reverse, 5′-AGGTCCAATTTCCGTACAATG-3′ for the second intron region) was designed based on the sequences of 'Tsugaru' and used to amplify the polymorphic fragments. The PCR was performed according to the instructions provided by the supplier of the *Taq* DNA polymerase (*r-Taq* or *LA-Taq*, TAKARA SHUZO CO. LTD, Siga, Japan). The amplification conditions were as follows: initial denaturation at 94°C for 4 min; 30 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 1 min; and a final extension of 5 min at 72°C. The PCR products were separated on a 2.0% agarose gel and stained with ethidium bromide. The two different bands (723 or 656 bp) that segregated in 72 individuals of the 'Delicious' × Mitsubakaido F_1 population were counted and mapped in the 'Delicious' linkage group using

the markers developed by M. Igarashi et al. (unpublished data) and the BC226-STS marker, which is an apple skin color marker developed by Cheng et al. (1996). The grouping of the markers and the map calculations were performed using the JOINMAP 3.0 software (Van Ooijen and Voorrips 2001). A LOD score of 5.0 was applied for each subset to identify markers belonging to the same linkage group.

Fourteen apple cultivars differing in their skin colors were used for the further characterization of the *MdMYBA* marker. The PCR was carried out as described above. The BC226 genotype and skin color of the 14 apple cultivars are shown in the legend of Fig. 8.

Acknowledgments

We thank Mutsuko Kimura and Kaoru Monma (National Institute of Fruit Tree Science) for technical assistance, and Dr. Shozo Kobayashi (National Institute of Fruit Tree Science) for valuable suggestions throughout the experiments.

References

Abe, H., Urao, T., Ito, T., Seki, M., Shinozaki, K. and Yamaguchi-Shinozaki, K. (2003) Arabidopsis AtMYC2 (bHLH) and AtMYB2 (MYB) function as transcriptional activators in abscisic acid signaling. *Plant Cell* 15: 63–78.

Arabidopsis Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* 408: 796–815.

Ban, Y., Honda, C., Bessho, H., Pang, X.M. and Moriguchi, T. (2007) Suppression subtractive hybridization identifies genes induced in response to UV-B irradiation in apple skin: isolation of a putative *UDP-glucose 4-epimerase*. *J. Exp. Bot*. 58: 1825–1834.

Borevitz, J.O., Xia, Y.J., Blount, J., Dixon, R.A. and Lamb, C. (2000) Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. *Plant Cell* 12: 2383–2393.

Boss, P.K., Davies, C. and Robinson, S.P. (1996) Expression of anthocyanin biosynthesis pathway genes in red and white grapes. *Plant Mol. Biol*. 32: 565–569.

Cheng, F.S., Weeden, N.F. and Brown, S.K. (1996) Identification of co-dominant RAPD markers tightly linked to fruit skin color in apple. *Theor. Appl. Genet*. 93: 222–227.

Cone, K.C. and Burr, B. (1989) Molecular and genetic analysis of light requirement for anthocyanin synthesis in maize. *In* The Genetics of Flavonoids. Edited by Styles, D.E., Gavazzi, G.A. and Racchi, M.L. pp. 143–146. Edizioni Unicopli, Milan, Italy.

Deluc, L., Barrieu, F., Marchive, C., Lauvergeat, V., Decendit, A., Richard, T., Carde, J.P., Merillon, J.M. and Hamdi, S. (2006) Characterization of a grapevine R2R3-MYB transcription factor that regulates the phenylpropanoid pathway. *Plant Physiol*. 140: 499–511.

Dong, Y., Mitra, D. and Kootstra, A. (1995) Postharvest stimulation of skin color in Royal Gala apple. *J. Am. Soc. Hortic. Sci*. 120: 95–100.

Elomaa, P., Uimari, A., Mehto, M., Albert, V.A., Laitinen, R.A.E. and Teeri, T.H. (2003) Activation of anthocyanin biosynthesis in *Gerbera hybrida* (Asteraceae) suggests conserved protein–protein and protein–promoter interactions between the anciently diverged monocots and eudicots. *Plant Physiol*. 133: 1831–1842.

Espley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E., Kutty-Amma, S. and Allan, A.C. (2007) Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. *Plant J*. 49: 414–427.

Hartmann, U., Sagasser, M., Mehrtens, F., Stracke, R. and Weisshaar, B. (2005) Differential combinatorial interactions of cis-acting elements recognized by R2R3-MYB, BZIP, and BHLH factors control light-responsive and tissue specific activation of phenylpropanoid biosynthesis genes. *Plant Mol. Biol*. 57: 155–171.

Hasebe, M. and Iwatsuki, K. (1990) *Adiantum capillus-veneris* chloroplast DNA clone bank: as useful heterologous probes in systematics of the leptosporangiate ferns. *Amer. Fern J*. 80: 20–25.

Honda, C., Kotoda, N., Wada, M., Kondo, S., Kobayashi, S., Soejima, J., Zhang, Z., Tsuda, T. and Moriguchi, T. (2002) Anthocyanin biosynthetic genes are coordinately expressed during red coloration in apple skin. *Plant Physiol. Biochem*. 40: 955–962.

Horsch, R.B., Fry, J., Hoffmann, N., Neidermeyer, J., Rogers, S.G. and Fraley, R.T. (1989) Leaf disc transformation. *In* Plant Molecular Biology Manual. Edited by Gelvin, S.B. and Schilperoort, R.A. pp. A5/1–A5/9. Kluwer Academic Publishers, Dordrecht, The Netherlands.

Jefferson, R.A. (1987) Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Mol. Biol. Rep*. 5: 387–405.

Jin, H. and Martin, C. (1999) Multifunctionality and diversity within the plant MYB-gene family. *Plant Mol. Biol*. 41: 577–585.

Judo, M.S.B., Wedel, A.B. and Wilson, C. (1998) Stimulation and suppression of PCR-mediated recombination. *Nucleic Acids Res*. 26: 1819–1825.

King, G.J. (1996) Progress of apple genetic mapping in Europe. *HortScience* 31: 1108–1111.

Kobayashi, S., Goto-Yamamoto, N. and Hirochika, H. (2004) Retrotransposon-induced mutations in grape skin color. *Science* 304: 982.

Kobayashi, S., Ishimaru, M., Ding, C.K., Yakushiji, H. and Goto, N. (2001) Comparison of UDP-glucose:flavonoid 3-*O*-glucosyltransferase (UFGT) gene sequences between white grape (*Vitis vinifera*) and their sports with red skin. *Plant Sci*. 160: 543–550.

Kobayashi, S., Ishimaru, M., Hiraoka, K. and Honda, C. (2002) Myb-related genes of the Kyoho grape (*Vitis labruscana*) regulate anthocyanin biosynthesis. *Planta* 215: 924–933.

Koes, R., Verweij, W. and Quattrocchio, F. (2005) Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci*. 10: 236–242.

Lancaster, J.E. (1992) Regulation of skin color in apples. *Crit. Rev. Plant Sci*. 10: 487–502.

Mathews, H., Clendennen, S.K., Caldwell, C.G., Liu, X.L., Connors, K., Matheis, N., Schuster, D.K., Menasco, D.J., Wagoner, W., Lightner, J. and Wagner, D.R. (2003) Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. *Plant Cell* 15: 1689–1703.

Meyerhans, A., Vartanian, J. and Wain-Hobson, S. (1990) DNA recombination during PCR. *Nucleic Acids Res*. 18: 1687–1691.

Quattrocchio, F., Wing, J., van der Woude, K., Souer, E., de Vetten, N., Mol, J. and Koes, R. (1999) Molecular analysis of the *anthocyanin2* gene of petunia and its role in the evolution of flower color. *Plant Cell* 11: 1433–1444.

Rose, T.M., Schultz, E.R., Henikoff, J.G., Pietrokovski, S., McCallum, C.M. and Henikoff, S. (1998) Consensus-degenerate hybrid oligonucleotide primers for amplification of distantly related sequences. *Nucleic Acids Res*. 26: 1628–1635.

Rosinski, J.A. and Atchley, W.R. (1998) Molecular evolution of the Myb family of transcription factors: evidence for polyphyletic origin. *J. Mol. Evol*. 46: 74–83.

Saure, M.C. (1990) External control of anthocyanin formation in apple. *Sci. Hortic*. 42: 181–218.

Siegelman, H.W. and Hendricks, S.B. (1958) Photocontrol of anthocyanin synthesis in apple skin. *Plant Physiol*. 33: 185–190.

Solano, R., Nieto, C., Avila, J., Canas, L., Diaz, I. and Paz-Ares, J. (1995) Dual DNA binding specificity of a petal epidermis-specific MYB transcription factor (MYB.Ph3) from *Petunia hybrida*. *EMBO J*. 14: 1773–1784.

Stracke, R., Werber, M. and Weisshaar, B. (2001) The *R2R3-MYB* gene family in *Arabidopsis thaliana*. *Curr. Opin. Plant Biol*. 4: 447–456.

Takos, A.M., Jaffe, F.W., Jacob, S.R., Bogs, J., Robinson, S.P. and Walker, A.R. (2006) Light induced expression of a *MYB* gene regulates anthocyanin biosynthesis in red apples. *Plant Physiol*. 142: 1216–1232.

Ubi, B.E., Honda, C., Bessho, H., Kondo, S., Wada, M., Kobayashi, S. and Moriguchi, T. (2006) Expression analysis of anthocyanin biosynthetic genes in apple skin: effect of UV-B and temperature. *Plant Sci*. 170: 571–578.

Urao, T., Yamaguchi-Shinozaki, K., Urao, S. and Shinozaki, K. (1993) An *Arabidopsis* myb homolog is induced by dehydration stress and its gene product binds to the conserved MYB recognition sequence. *Plant Cell* 5: 1529–1539.

Van Ooijen, J.W. and Voorrips, R.E. (2001) JoinMap® Version 3.0, Software for the Calculation of Genetic Linkage Maps. Plant Research International, Wageningen, The Netherlands.

Walker, A.R., Lee, E. and Robinson, S.P. (2006) Two new grape cultivars, bud sports of Cabernet Sauvignon bearing pale-coloured berries, are the result of deletion of two regulatory genes of the berry colour locus. *Plant Mol. Biol.* 62: 623–635.

Wan, C. and Wilkins, T.A. (1994) A modified hot borate method significantly enhances the yield of high-quality RNA from cotton (*Gossypium hirsutum* L.). *Anal. Biochem.* 223: 7–12.

Zimmermann, I.M., Heim, M.A., Weisshaar, B. and Uhrig, J.F. (2004) Comprehensive identification of *Arabidopsis thaliana* MYB transcription factors interacting with R/B-like BHLH proteins. *Plant J.* 40: 22–34.

(Received March 15, 2007; Accepted May 20, 2007)