

SCIENTIFIC REPORTS



OPEN

A MYB transcription factor, DcMYB6, is involved in regulating anthocyanin biosynthesis in purple carrot taproots

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Received: 28 October 2016

Accepted: 23 February 2017

Published: 27 March 2017

Carrots are widely grown and enjoyed around the world. Purple carrots accumulate rich anthocyanins in the taproots, while orange, yellow, and red carrots accumulate rich carotenoids in the taproots. Our previous studies indicated that variation in the activity of regulatory genes may be responsible for variations in anthocyanin production among various carrot cultivars. In this study, an R2R3-type MYB gene, designated as *DcMYB6*, was isolated from a purple carrot cultivar. In a phylogenetic analysis, *DcMYB6* was grouped into an anthocyanin biosynthesis-related MYB clade. Sequence analyses revealed that *DcMYB6* contained the conserved bHLH-interaction motif and two atypical motifs of anthocyanin regulators. The expression pattern of *DcMYB6* was correlated with anthocyanin production. *DcMYB6* transcripts were detected at high levels in three purple carrot cultivars but at much lower levels in six non-purple carrot cultivars. Overexpression of *DcMYB6* in *Arabidopsis* led to enhanced anthocyanin accumulation in both vegetative and reproductive tissues and upregulated transcript levels of all seven tested anthocyanin-related structural genes. Together, these results show that *DcMYB6* is involved in regulating anthocyanin biosynthesis in purple carrots. Our results provide new insights into the regulation of anthocyanin synthesis in purple carrot cultivars.

Carrots (*Daucus carota* L.; $2n = 2x = 18$) are an economically important root crop worldwide. The taproot of cultivated carrots exhibits a range of colors including orange, yellow, red, white, and purple. Purple carrots contain anthocyanins, whereas the orange, red, and yellow pigmentation of carrot taproots is due to carotenoids¹. White carrots contain very low levels of carotenoids². Thus, cultivated carrots can be divided into two distinct groups: the anthocyanin or eastern group (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.) and the carotene or western group (*Daucus carota* ssp. *sativus* var. *sativus*)³.

Anthocyanins are a group of flavonoids that perform various important functions in plants. They provide pigmentation in vegetative and reproductive tissues^{4–8}, enhance cold, drought, and salt tolerance^{9–11}, and protect against damage from ultraviolet light, insect herbivory, and pathogen attack^{12,13}. Purple carrots accumulate rich anthocyanins in the fleshy taproot. The role of anthocyanins in carrot taproots is unclear; however, it is conceivable that they could protect the taproot from insect and pathogen attack. Anthocyanins are beneficial to human health¹⁴ and are used as natural food colorants in beverages, candies, and ice cream¹⁵.

The genetics of anthocyanin biosynthesis have been extensively studied in many plant species. Regulatory genes encoding transcription factors (TFs) control the transcription of structural genes encoding enzymes involved in anthocyanin biosynthesis. In our previous studies, the transcripts of numerous structural genes in the anthocyanin pathway were undetectable or barely detectable in the taproots of non-purple carrots, but were detected at high levels in those of purple carrots. It seems likely that variation in the activity of regulatory genes is the key factor determining anthocyanin production in carrots¹⁶.

In many species studied to date, the R2R3-MYB TFs, basic helix-loop-helix (bHLH) TFs, and WD-repeat (WDR) proteins form 'MBW' complexes that bind to the promoters of target genes to directly activate the transcription of structural genes in the anthocyanin pathway¹⁷. The MYB proteins in this complex are often the key component determining variation in anthocyanin production^{18–20}. Anthocyanin-related MYBs have been

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identified in many plant species, for example, *Arabidopsis* AtMYB75 (PAP1), AtMYB90 (PAP2), AtMYB113, and AtMYB114²¹, *Vitis vinifera* VvMYB1a²², *Ipomoea batatas* IbMYB1⁸, and *Malus × domestica* MdMYB10, MdMYB1/MdMYBA^{19,23,24}. Overexpression of genes encoding these MYB TFs in heterologous or homologous plant species leads to enhanced anthocyanin accumulation.

In this study, a gene encoding an R2R3-type MYB, designated as *DcMYB6*, was isolated from a purple carrot cultivar. The correlation between its expression with anthocyanin production in purple and non-purple carrots was analyzed. The function of *DcMYB6* was also analyzed by overexpression in *Arabidopsis* plants. These results will further our understanding of how anthocyanin synthesis is regulated in carrots.

Results

Sequence analysis of *DcMYB6*. The amplification products of the genomic DNA sequence and the open reading frame (ORF) sequence of *DcMYB6* from the carrot cultivar ‘Deep purple’ are shown in Supplementary Fig. S1. The genomic DNA sequence of *DcMYB6* was 1,801 bp long while the ORF sequence of *DcMYB6* was 903 bp long, encoding a polypeptide of 300 amino acids (Supplementary Fig. S1A). Alignment analysis of genomic DNA and ORF sequences revealed that the *DcMYB6* gene consisted of two introns and three exons (Supplementary Fig. S1B).

We conducted a phylogenetic analysis with the deduced amino acid sequences of *DcMYB6* and other R2R3-MYB TFs involved in the biosynthesis of different secondary metabolites. Supplementary Table S1 lists the GenBank accession numbers of the R2R3-MYBs used to build the phylogenetic tree. In the phylogenetic tree, R2R3-MYB TFs with similar functions clustered together, and *DcMYB6* grouped into an anthocyanin biosynthesis-related MYB clade (Fig. 1), which included tobacco (*Nicotiana tabacum*) NtAN2, petunia (*Petunia hybrida*) PhAn2, tomato (*Lycopersicon esculentum*) LeANT1, sweet potato (*I. batatas*) IbMYB1, morning glory (*I. nil*) InMYB2, grapevine (*V. vinifera*) VvMYBA1 and VvMYBA2, blood orange (*Citrus sinensis*) CsRuby, snapdragon (*Antirrhinum majus*) AmVENOSA, AmROSEA1, and AmROSEA2, *Arabidopsis thaliana* AtPAP1, AtPAP2, and AtMYB114, mangosteen (*Garcinia mangostana*) GmMYB10, Chinese bayberry (*Myrica rubra*) MrMYB1, apple (*Malus × domestica*) MdMYB10a and MdMYB1-1, *Gerbera hybrida* GhMYB10, *Medicago truncatula* MtLAP1, *Lilium hybrid* LhMYB6, and *Epimedium sagittatum* EsMYBA1.

Next, we conducted an alignment analysis of the deduced amino acid sequence of *DcMYB6* with those of other MYB TFs related to anthocyanin biosynthesis. Like other MYB TFs, *DcMYB6* contained the highly conserved R2R3 domain at the N-terminus (Fig. 2). *DcMYB6* showed high sequence homology with other MYB TFs within the R2R3 domain, sharing the highest identity (85%) with LeANT1 and the lowest identity (80%) with AmVENOSA. However, all the MYB TFs showed little homology in the C-terminus sequence to the R2R3 domain. When whole sequences were compared, *DcMYB6* shared the highest identity (44%) with PhAn2 and the lowest identity (32%) with AmROSEA1.

The alignment showed that the [D/E]Lx2[R/K]x3Lx6Lx3R motif, also known as the bHLH motif²⁵, which is required for the interaction with bHLH proteins, was present in the R3 domain of all the analyzed MYB TFs (Fig. 2). The conserved ANDV motif that has been identified in MYB TFs in the anthocyanin pathway in the Rosaceae²⁶ was also present in all of the analyzed MYB TFs and was modified to [A/G]NDV. Besides these motifs, the MYB TFs contained the motif KPRPR[S/T]F defined by Stracke *et al.*²⁷, which was modified to [K/R]Pxx[H/R] [K/S/T][F//L/Y], in the C-terminal region.

Quantitative real-time PCR analysis of *DcMYB6* in purple and non-purple carrot taproots. At the 90-day-old stage, purple carrot cultivars had accumulated rich anthocyanins whereas anthocyanins were barely detectable in, or absent from non-purple carrot cultivars¹⁶. Using specific primer pairs, qRT-PCR analyses were performed to quantify the transcript levels of *DcMYB6* in purple and non-purple carrots at this stage. The transcript levels of *DcMYB6* in the taproots of three purple carrot cultivars (‘Deep purple’, ‘Purple 68’, and ‘Tianzi2hao’) were approximately 10–229-fold higher than those in the taproots of six non-purple carrot cultivars (‘Kuroda’, ‘Sanhongliucun’, ‘Junchuanhong’, ‘Bejo1719’, ‘Qitouhuang’, and ‘Baiyu’). Among the three purple carrot cultivars, ‘Tianzi2hao’ had the highest transcript level of *DcMYB6* and ‘purple 68’ had the lowest. Among the six non-purple carrot cultivars, ‘Baiyu’ had the lowest transcript level of *DcMYB6* and ‘Sanhongliucun’ had the highest (Fig. 3).

Subcellular localization of *DcMYB6* protein. To investigate the subcellular localization of *DcMYB6*, the *DcMYB6* coding sequence was fused in-frame to the 5′ terminus of the gene encoding GFP, and the construct was transiently expressed in onion cells. In onion cells expressing GFP alone, fluorescence was localized in the cytoplasm and nucleus (Fig. 4 up). Onion cells expressing the *DcMYB6*-GFP fusion protein showed a strong signal in the nucleus (Fig. 4 down).

Overexpression of *DcMYB6* in transgenic *Arabidopsis* induced anthocyanin production. The *DcMYB6* gene driven by the CaMV 35S promoter was overexpressed in *Arabidopsis* plants to test its function. *Arabidopsis* seedlings of three homozygous CaMV 35S:*DcMYB6* transgenic lines (*DcMYB6*-1, *DcMYB6*-2, and *DcMYB6*-3) and one control transgenic line, which were selected on MS agar plates containing hygromycin, showed β-glucuronidase (GUS) activity (Fig. 5A). A PCR product of approximately 900 bp corresponding to the *DcMYB6* coding sequence was detected in all three CaMV 35S:*DcMYB6* transgenic *Arabidopsis* lines analyzed, whereas no such PCR product was amplified from control transgenic plants (Fig. 5B).

Overexpression of *DcMYB6* in *Arabidopsis* induced anthocyanin accumulation. Compared with control plants, *DcMYB6*-1, *DcMYB6*-2, and *DcMYB6*-3 plants exhibited dark-purple pigments in the leaves, siliques, and immature and mature seed coats (Fig. 6A–D), and delayed growth. The total anthocyanin content in the whole plants of *DcMYB6*-1, *DcMYB6*-2, and *DcMYB6*-3 plants was approximately 66–228-fold higher than

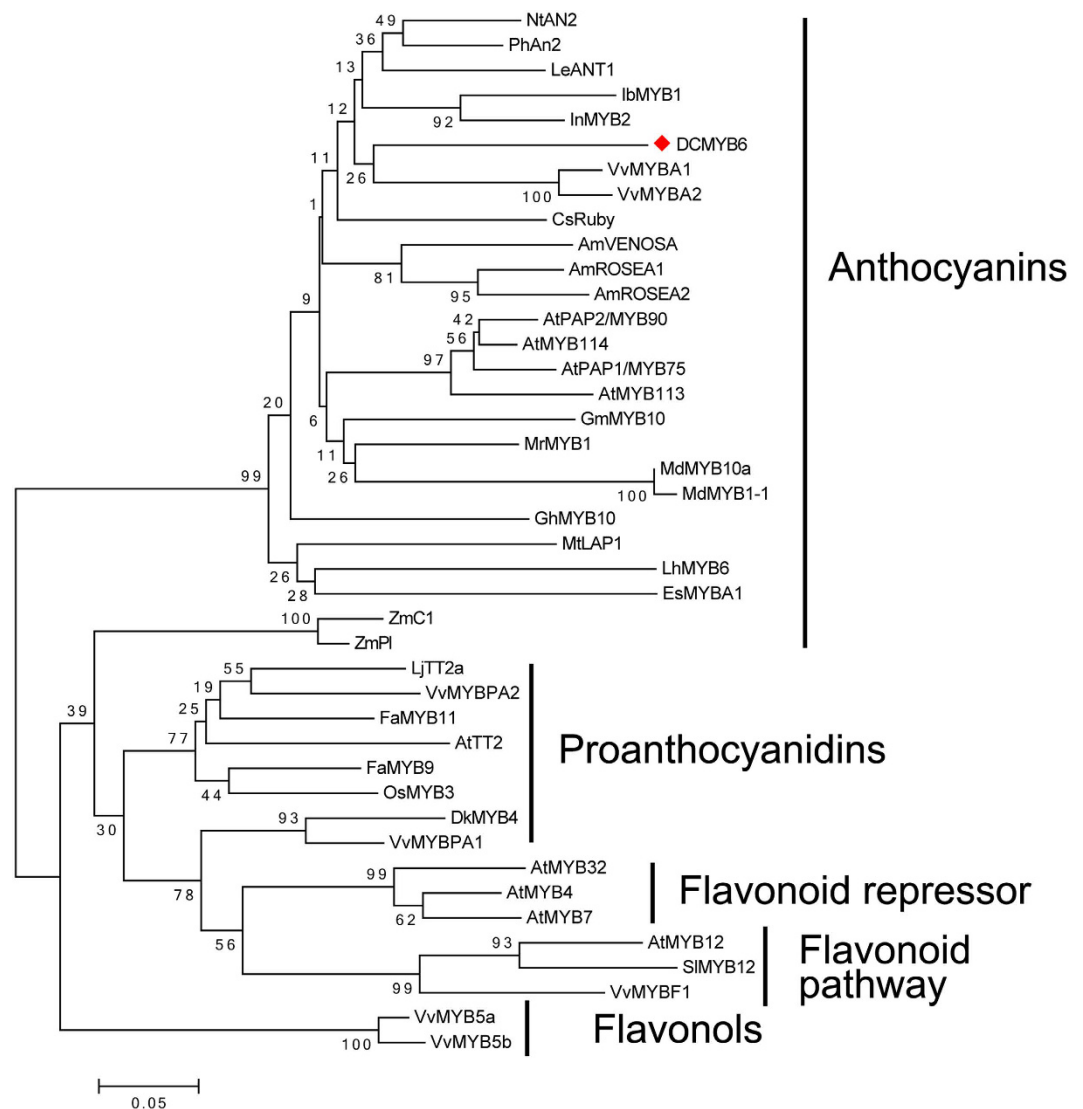


Figure 1. Phylogenetic relationships among DcMYB6 and flavonoid-related R2R3-MYBs from other plant species. Phylogenetic tree was built using the neighbor-joining method using MEGA 5 software; bootstrap value was set to 1000. Red diamond indicates DcMYB6. Putative functions of all R2R3-MYBs are listed on the right.

that in control plants (Fig. 6E). Among the three CaMV 35S:DcMYB6 transgenic *Arabidopsis* lines, DcMYB6-1 showed the lowest total anthocyanin content and DcMYB6-3 showed the highest.

Up-regulation of anthocyanin biosynthetic genes in transgenic *Arabidopsis* overexpressing DcMYB6. Among the three CaMV 35S:DcMYB6 transgenic *Arabidopsis* lines, DcMYB6-1 plants showed the lowest *DcMYB6* transcript levels and DcMYB6-3 plants showed the highest (Fig. 7). As expected, *DcMYB6* transcripts were undetectable in the control *Arabidopsis* plants. The results of the qRT-PCR analyses also determined which endogenous anthocyanin pathway structural genes were up-regulated in the transgenic *Arabidopsis* plants overexpressing DcMYB6. Compared with the control line, the transgenic *Arabidopsis* plants overexpressing DcMYB6 showed significantly increased transcript levels of *AtCHS* (chalcone synthase), *AtCHI* (chalcone isomerase), *AtF3H* (flavanone 3-hydroxylase), *AtF3'H* (flavonoid 3'-hydroxylase), *AtDFR* (dihydroflavonol 4-reductase), *AtLDOX* (leucoanthocyanidin dioxygenase), and *AtUGT78D2* (Fig. 7). Among the three transgenic *Arabidopsis* lines overexpressing DcMYB6, DcMYB6-1 plants showed the lowest transcript levels of these structural genes and DcMYB6-3 plants showed the highest. Transcripts of these structural genes were undetectable or barely detectable in the control *Arabidopsis* plants.

Discussion

Anthocyanins are water-soluble pigments responsible for purple colors in carrots. In plants, TFs such as MYB, bHLH, and WD40 upregulate the expression of structural genes in the anthocyanin biosynthesis pathway. Two previous studies showed that the expression levels of all anthocyanin pathway structural genes were significantly

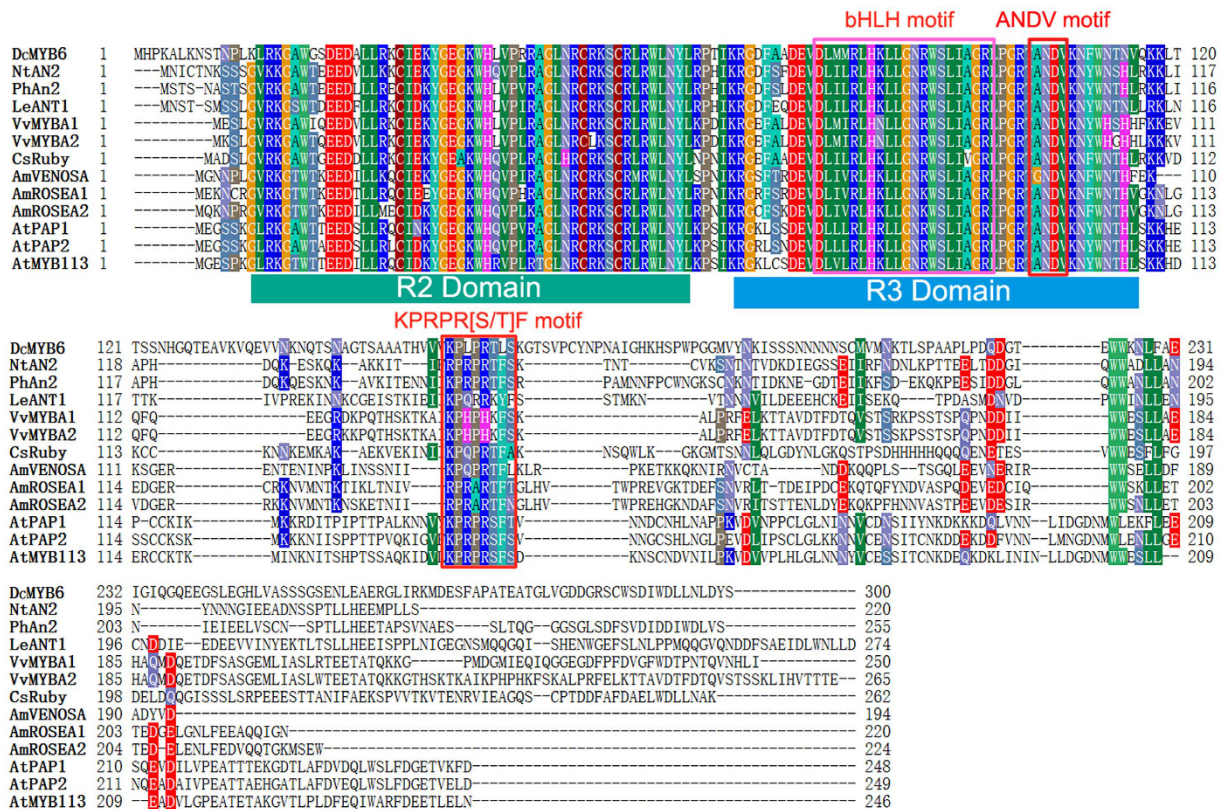


Figure 2. Alignment of deduced amino acid sequence of DcMYB6 and R2R3-MYB proteins from other plant species. Alignment was conducted using BioEdit (Version 7.0.1). Identical amino acid residues are shaded as per color table (threshold for shading was set to 60%). R2 and R3 domains are indicated. Boxes show BHLH, ANDV and KPRPR[S/T]F motifs.

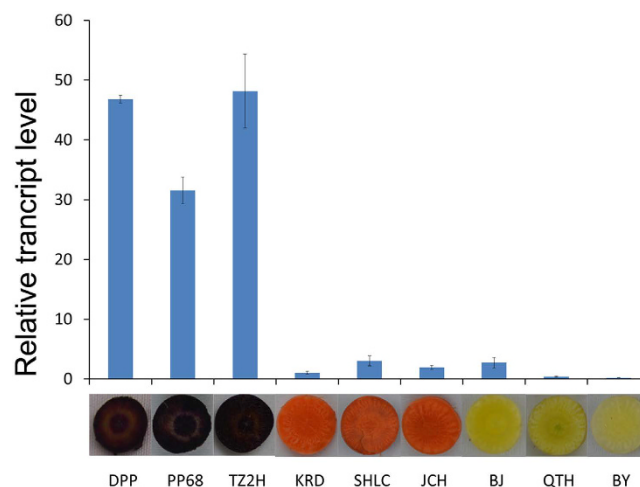


Figure 3. Transcript profiles of DcMYB6 in 90-day-old taproots of three purple and six non-purple carrot cultivars. Data represent means of three biological replicates \pm SD. Cultivar abbreviations: DPP, Deep purple; PP68, Purple 68; TZ2H, Tianzi2hao; KR, Kuroda; SHLC, Sanhongliucun; JCH, Junchuanhong; BJ, Bejo1719; QTH, Qitouhuang; BY, Baiyu.

lower in non-purple carrot cultivars than in purple carrot cultivars, which possibly resulted from the inactivation of regulator genes^{16,28}. In other plant species, many R2R3-MYB TFs are known to control anthocyanin biosynthesis by regulating structural genes in the anthocyanin pathway^{4,16,17,22,25}. However, little is known about the R2R3-MYB TFs involved in regulating the anthocyanin pathway in carrot. A previous study reported that DcMYB3 and DcMYB5 might upregulate the activity of the *DcPAL3* promoter²⁹. In the present study, a gene encoding R2R3-MYB, namely *DcMYB6*, was isolated from ‘Deep purple’, a purple carrot cultivar.

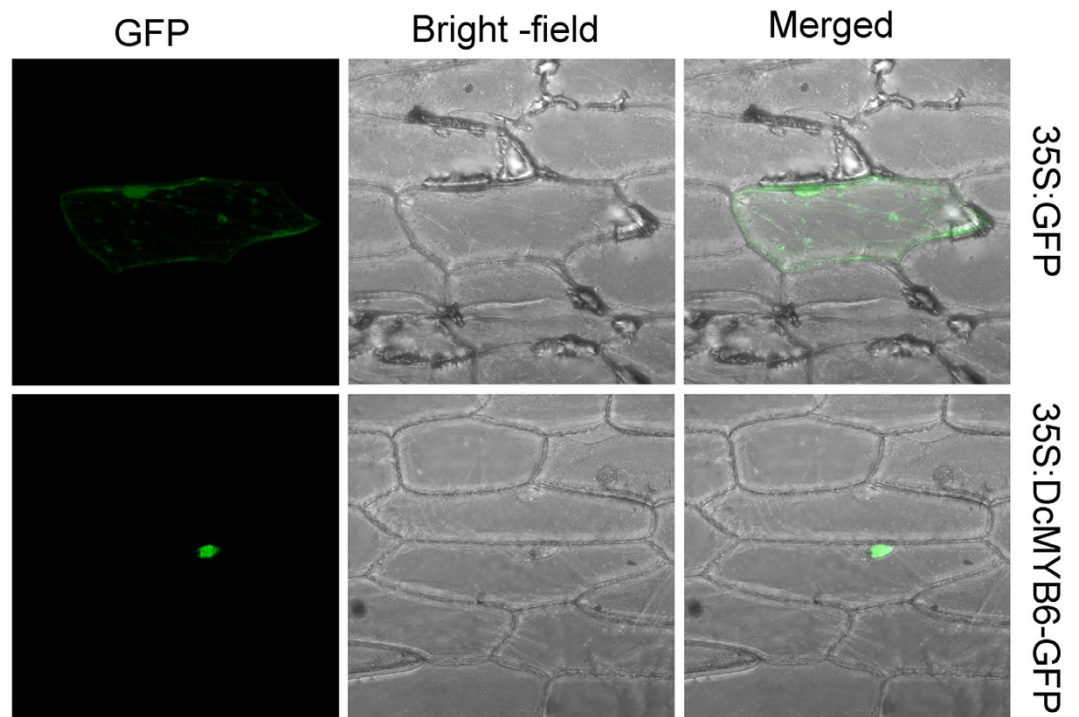


Figure 4. Subcellular localization of GFP fusions of DcMYB6. Onion epidermal cells transiently expressing GFP and DcMYB6-GFP under the control of the CaMV 35S promoter.

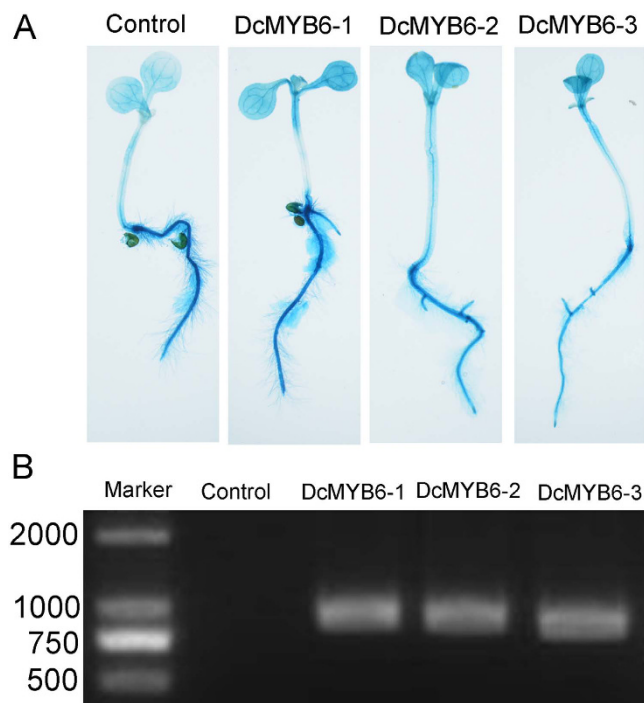


Figure 5. Identification of transgenic *Arabidopsis* plants with histochemical GUS activity and PCR analyses. (A) Histochemical GUS activity analysis of transgenic *Arabidopsis* plants overexpressing empty vector (control) and *DcMYB6* (DcMYB6-1, DcMYB6-2, and DcMYB6-3). (B) PCR-amplified *DcMYB6* fragments from the same transgenic *Arabidopsis* plants as above.

DcMYB6 grouped into the same clade as the MYB TF family of the anthocyanin pathway, and shared high identity with anthocyanin-regulating MYB TFs from other species within the R2R3 domain. DcMYB6 was found to contain the conserved bHLH interaction motif [D/E]Lx2[R/K]x3Lx6Lx3 R in the R3 domain, and an

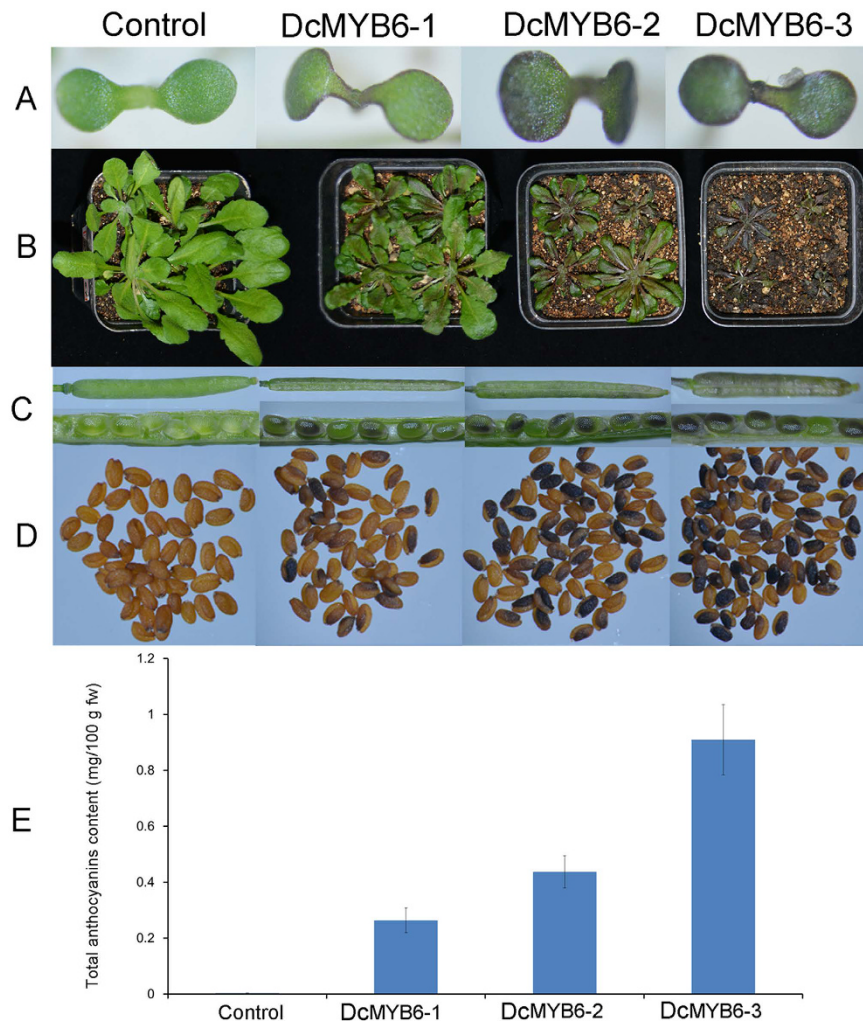


Figure 6. Functional analysis of DcMYB6 in *Arabidopsis*. (A and B) Ten-day-old and 40-day-old transgenic *Arabidopsis* plants overexpressing empty vector (control) and *DcMYB6* (DcMYB6-1, DcMYB6-2, and DcMYB6-3). (C) Immature capsules and seeds from control and three lines of transgenic *Arabidopsis* plants overexpressing *DcMYB6*. (D) Mature seeds from control and three lines of *DcMYB6*-overexpressing transgenic *Arabidopsis* plants. (E) Total anthocyanin contents of 40-day-old transgenic *Arabidopsis* plants overexpressing empty vector (control) and *DcMYB6* (DcMYB6-1, DcMYB6-2, and DcMYB6-3).

atypical anthocyanin regulator motif KPRPR[S/T]F at the C-terminus. Another conserved motif, [A/G]NDV, which distinguishes anthocyanin and non-anthocyanin MYB TFs in the Rosaceae, was also found in DcMYB6. The presence of these motifs suggested that DcMYB6 may be involved in regulating anthocyanin biosynthesis. In several other plant species, the expression of many *R2R3-MYB* genes in the anthocyanin pathway is strongly correlated with anthocyanin accumulation. For example, *MdMYB10* was found to be highly expressed in red-fleshed apple and in the colored skin of white-fleshed apple, but was virtually undetectable in the white cortex of white-fleshed apple¹⁹. In the present study, *DcMYB6* transcript levels corresponded well with anthocyanin pigmentation; there were much higher transcript levels in all three 90-day-old purple carrot taproots than in 90-day-old taproots of the six non-purple carrot cultivars. Therefore, DcMYB6 is probably involved in regulating anthocyanin biosynthesis in purple carrot taproots.

The reason why *DcMYB6* transcript levels were much lower in non-purple carrots than in purple carrots is still unknown. In peach (*Prunus persica*), the heterodimer of BL and PpNAC1 was shown to activate transcription of the anthocyanin-related MYB, *PpMYB10.1*³⁰. In European pear (*Pyrus communis*), methylation of the *PcMYB10* promoter reduced *PcMYB10* expression levels and resulted in a peel color change from red to green³¹. Insertions and deletions in the promoter region have been shown to affect the expression levels of anthocyanin-related MYBs in some species, such as apple (*Malus × domestica*) and grapevine (*V. vinifera*)^{20,22}. In future work, we will attempt to establish the reason for the different transcript level of *DcMYB6* in purple and non-purple carrots.

In several studies, overexpression of anthocyanin-related MYB TFs in heterologous plant species led to enhanced anthocyanin accumulation^{32,33}. In this study, transgenic *Arabidopsis* plants overexpressing *DcMYB6* exhibited a clearly darker color and accumulated higher levels of anthocyanins in both vegetative and

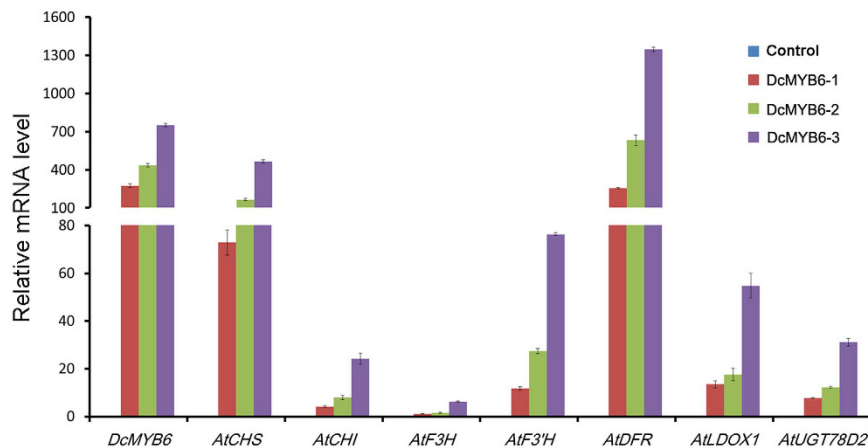


Figure 7. Transcript levels of *DcMYB6* and seven anthocyanin pathway structural genes in transgenic *Arabidopsis* plants. Gene transcript levels in *Arabidopsis* plants overexpressing empty vector (control) and *DcMYB6* (*DcMYB6*-1, *DcMYB6*-2, and *DcMYB6*-3) were detected by quantitative RT-PCR. Data represent means of three biological replicates \pm SD.

reproductive tissues, compared with those in control *Arabidopsis* plants. Furthermore, qRT-PCR analyses of the three transgenic *Arabidopsis* lines with different transcript levels of *DcMYB6* and different total anthocyanin levels showed that higher transcript levels of *DcMYB6* led to greater anthocyanin accumulation. Also, the transcript levels of all seven tested anthocyanin-related structural genes were much higher in transgenic *Arabidopsis* plants overexpressing *DcMYB6* than in control *Arabidopsis* plants. Together, these results indicate that *DcMYB6* could enhance anthocyanin accumulation in *Arabidopsis* by upregulating anthocyanin-related structural genes, and suggest that *DcMYB6* regulates anthocyanin biosynthesis in purple carrots.

In conclusion, an R2R3-MYB TF, *DcMYB6*, was isolated from a purple carrot cultivar and was found to be involved in regulating the anthocyanin biosynthetic pathway. The results of this study provide important information on the pigmentation of purple carrots. Other TFs such as bHLH and WD40 that form complexes with MYB proteins and together regulate anthocyanin biosynthesis have not yet been identified in carrots. In future work, we will test whether overexpression of *DcMYB6* in non-purple carrot cultivars leads to anthocyanin accumulation.

Methods

Plant materials. Three purple carrot cultivars ('Deep purple', 'Purple 68', and 'Tianzi2hao'), three orange carrot cultivars ('Kuroda', 'Sanhongliucun', and 'Junchuanhong'), and three yellow carrot cultivars ('Bejo1719', 'Qitouhuang', and 'Baiyu'), which are widely cultivated in China, were chosen for this work. Seeds were grown in a controlled artificial climatic chamber under the same conditions as previously described¹⁶. *Arabidopsis thaliana* ecotype Columbia was grown under the same conditions.

RNA and DNA extraction from carrots and cDNA preparation. Total RNA was extracted from taproots of 90-day-old carrot plants using an RNAsimple Total RNA Kit (Tiagen, Beijing, China). First-strand cDNA was synthesized using the PrimeScript[™] RT reagent kit with gDNA Eraser (Perfect Real Time; Takara, Dalian, China). cDNA was diluted 20-fold for gene cloning and qRT-PCR analyses. Genomic DNA was isolated from young leaves with a DNAsure plant kit (Tiangen).

Isolation of genomic DNA and cDNA sequence of *DcMYB6*. AtPAP1 (AAG42001) was BLASTed against our CarrotDB: a genomic and transcriptomic database for carrot³⁴ and the high-quality carrot genome which spans 421.5 Mb and accounts for ~90% of the estimated genome size (473 Mb)³⁵. Two transcript contigs showing high sequence identity with AtPAP1 and with higher FPKM values in purple carrots than in non-purple carrots were identified in the transcriptomic database of CarrotDB. After assembling these two transcript contigs, an ORF of 903 bp was identified and predicted to be a MYB TF using Pfam (<http://pfam.xfam.org/>). This MYB TF was designated as *DcMYB6* in this study. Two genomic sequence scaffolds (scaffold 016995 and scaffold 029424) that matched the ORF sequence were identified in the genomic database of CarrotDB. However, no genomic sequence matching the ORF sequence was found in the high-quality carrot genome.

The ORF sequence of *DcMYB6* was amplified from cDNA produced from 90-day-old 'Deep purple' carrot taproots using PrimeSTAR HS DNA polymerase (Takara, Otsu, Japan) with the forward primer (5'-CGCGCGGATCTTCCAGAGATTATGCATCCAAAGGCTTTGAAGAAT-3') and reverse primer (5'-CACGCTGCCGTTCGACGATTTTAACTATAATCCAAGTTAAGAAGGTCCC-3'). The ORF sequence was then cloned into the pMD19-T simple vector (Takara, Otsu, Japan) using the ClonExpress II One Step Cloning Kit (Vazyme Biotech Co. Ltd., Nanjing, China) before sequencing (Genscript, Nanjing, China). The same pairs of primers were also used to amplify the genomic clone of *DcMYB6* from genomic DNA extracted from carrot leaves. The full-length ORF and DNA sequences of *DcMYB6* have been deposited in the GenBank database under the accession numbers KY020445 and KY020446, respectively.

Subcellular localization analysis. The protein-coding region of *DcMYB6* was amplified with the forward primer (5'-CACCATCACCATCACGCCATGATGATCAAGAGCACTGGTAATCC-3') and the reverse primer (5'-CACTAGTACGTGACCATGGCACTATAGTCCCTGGTTGAGAAGATCCC-3'), and was subcloned into the pA7-GFP vector at the *Nco* I site to create the CaMV 35S:*DcMYB6*-GFP fusion construct. This construct and the pA7-GFP empty vector (as control) were both bombarded into onion epidermal cells using a Biolistic PDS-1000 instrument (Bio-Rad, Hercules, CA, USA). After incubation at 25 °C for at least 16 h in the dark, samples were observed under a confocal laser scanning microscope.

Overexpression vector construct preparation and *Arabidopsis* transformation. The coding sequence of *DcMYB6* was amplified with the forward primer (5'-TTTACAATTACCATGGGATCCATGCATCCAAAGGCTTTGAAGAAT-3') and the reverse primer (5'-ACCGATGATACGAACGAGCTCTTAATAATCCAAGTTAAGAAGGTCCC-3'), and then subcloned into the binary vector pCAMBIA-1301 under the control of the CaMV 35S promoter and the pea *rbcSE9* terminator to create the CaMV 35S:*DcMYB6* construct. This construct was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation and then transformed into *Arabidopsis* using the floral-dip method³⁶. Transgenic *Arabidopsis* plants carrying the *DcMYB6* gene were identified by selection on half-strength Murashige and Skoog (MS) agar plates containing 35 mg/L hygromycin, assaying for GUS activity, and detecting the presence of the transgene by reverse transcription PCR with the forward primer (5'-ATGCATCCAAAGGCTTTGAAGAAT-3') and the reverse primer (5'-AAGCACAACAAATGGTACAAG-3'), which were designed according to the sequence of *DcMYB6* and the pea *rbcSE9* terminator, respectively. Three transgenic *Arabidopsis* lines (*DcMYB6*-1, *DcMYB6*-2, and *DcMYB6*-3) with black leaves were used for further experiments. *Arabidopsis* plants transformed with the pCAMBIA-1301 empty vector served as controls.

Determination of total anthocyanin content. Total anthocyanins were extracted from 40-day-old transgenic *Arabidopsis* plants (T3) as described previously³⁷. Total anthocyanin quantities are presented in mg cyanidin 3-*O*-glycoside equivalents per 100 g fw (mg/100 g fw). Three biological replicates were analyzed for each sample.

Quantitative real-time PCR expression analysis. The mRNA levels of the *DcMYB6* gene in 90-day-old carrot taproots and in 40-day-old transgenic *Arabidopsis* plants were determined by qRT-PCR with the forward primer (5'-GCCATAGGGACAAGCACTCT-3') and the reverse primer (5'-GATCCCAATTCCGCAAACAA-3'). Total RNA was extracted from 40-day-old transgenic *Arabidopsis* plants and used to synthesize cDNA using the method described above. To determine the transcript levels of anthocyanin pathway structural genes in transgenic *Arabidopsis*, qRT-PCR assays were performed with the primers listed in Supplementary Table S2. The *DcActin1* gene was used as an internal standard in carrot with the same primers as described previously^{16,38}, while the *AtActin2* gene was used as an internal standard for normalization in *Arabidopsis* and was amplified using the primers listed in Supplementary Table S2. Experiments were conducted using three biological replicates for each sample. The relative gene transcript level was calculated with the $2^{-\Delta\Delta C_T}$ method³⁹. To compare *DcMYB6* expression patterns among purple and non-purple carrots at the 90-day-old stage, the $\Delta\Delta C_T$ was calculated by subtracting ΔC_T of 'Kuroda' from ΔC_T of all carrot cultivars. To compare the transcript levels of *DcMYB6* and anthocyanin pathway structural genes among transgenic *Arabidopsis* plants, the $\Delta\Delta C_T$ was calculated by subtracting ΔC_T of the *AtF3H* (flavanone 3-hydroxylase) gene in *DcMYB6*-1 *Arabidopsis* plants from the ΔC_T of all tested genes in transgenic *Arabidopsis* plants.

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Acknowledgements

The research was supported by the National Natural Science Foundation of China (31501775) and the Fundamental Research Funds for the Central Universities (KJQN201657).

Author Contributions

Z. S. X. and A. S. X. initiated and designed the research. Z. S. X., K. F. and F. Q. performed the experiments. Z. S. X. analyzed the data. A. S. X. and W.F. contributed reagents/materials/analysis tools. Z. S. X. wrote the paper. Z. S. X. and A. S. X. revised the paper.

Additional Information

Supplementary information accompanies this paper at <http://www.nature.com/srep>

Competing Interests: The authors declare no competing financial interests.

How to cite this article: Xu, Z.-S. *et al.* A MYB transcription factor, DcMYB6, is involved in regulating anthocyanin biosynthesis in purple carrot taproots. *Sci. Rep.* **7**, 45324; doi: 10.1038/srep45324 (2017).

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