Short title

DcMYB7 regulates anthocyanin modification

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Changing Carrot Color: Insertions in DcMYB7 Alter the Regulation of Anthocyanin Biosynthesis and Modification

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One-sentence summary: Insertion of a non-functional DcMYB7 duplication or transposons into the promoter region of DcMYB7 in non-purple carrots blocks anthocyanin biosynthesis and modification.

Author contributions
Z.-S.X. and A.-S.X. conceived and designed the experiments; Q.-Q.Y. and K.F. performed most of the experiments; Z.-S.X. analyzed the data; Z.-S.X. and A.-S.X. wrote the article.
Abstract

The original domesticated carrots (Daucus carota L.) are thought to have been purple, accumulating large quantities of anthocyanins in their roots. A quantitative trait locus associated with anthocyanin pigmentation in purple carrot roots has been identified on chromosome 3 and includes two candidate genes, DcMYB6 and DcMYB7. Here, we characterized the functions of DcMYB6 and DcMYB7 in carrots. Overexpression of DcMYB7, but not DcMYB6, in the orange carrot ‘Kurodagosun’ led to anthocyanin accumulation in roots. Knockout of DcMYB7 in the solid purple (purple periderm, phloem and xylem) carrot ‘Deep purple’ using the CRISPR/Cas9 system resulted in carrots with yellow roots. DcMYB7 could activate expression of its DcbHLH3 partner, a homologue of the anthocyanin-related apple bHLH3, and structural genes in the anthocyanin biosynthetic pathway. We determined that the promoter sequence of DcMYB7 in non-purple carrots was interrupted either by DcMYB8, a non-functional tandem duplication of DcMYB7, or by two transposons, leading to the transcriptional inactivation of DcMYB7 in non-purple carrot roots. As a result, non-purple carrots fail to accumulate anthocyanins in their roots. Our study supports the hypothesis that another genetic factor suppresses DcMYB7 expression in the phloem and xylem of purple peridermal carrot root tissues. DcMYB7 also regulated the glycosylation and acylation of anthocyanins by directly activating DcUCGXT1 and DcSAT1. We revealed the genetic factors conditioning anthocyanin pigmentation in purple versus non-purple carrot roots. Our results also provide insights into the...
mechanisms underlying anthocyanin glycosylation and acylation.

**Introduction**

Carrot (*Daucus carota* L. ssp. *sativus*; 2n = 2x = 18) provides rich health-promoting nutrients to humans. Carrots are classified into two groups according to exact botanical determination: the carotene group (var. *sativus*) and the anthocyanin group (var. *atrorubens* Alef.) (Kammerer et al., 2004). Carotene group members, also known as non-purple carrots, accumulate massive amounts of carotenoids in their roots (Clotault et al., 2008; Arscott and Tanumihardjo, 2010); anthocyanin group members, also known as purple carrots, accumulate high levels of anthocyanins in their roots (Kammerer et al., 2004; Montilla et al., 2011). Anthocyanins are water-soluble flavonoid compounds and confer red, blue and purple pigmentation to plants. In addition to their important roles in plants, they are also beneficial human nutrients (He and Giusti, 2010).

Non-purple carrots are considered to have arisen from purple carrots that acquired mutations (Banga, 1963; Arscott and Tanumihardjo, 2010; Iorizzo et al., 2013; Leja et al., 2013; Iorizzo et al., 2016). In addition, purple carrot root pigmentation extensively varies across different carrot genotypes, ranging from the purple peridermal carrot type (purple periderm but non-purple phloem and xylem) to solid purple carrot type (purple periderm, phloem and xylem). The genetic control of anthocyanin pigmentation in purple carrots has been investigated (Simon, 1996; Yildiz et al., 2013; Cavagnaro et al., 2014; Xu et al., 2014; 2016; 2017; Kodama et al., 2018; Iorizzo et
al., 2019). Two genes that condition the anthocyanin pigmentation of carrot roots from different genetic background, \(P_1\) and \(P_3\), have been identified and genetically mapped within 28.2-cM and 12-cM regions, respectively, on chromosome 3, supporting the theory of two independent mutation and human selection events during the domestication of purple carrots (Cavagnaro et al., 2014). \(P_1\) controls anthocyanin pigmentation in carrots with purple roots but non-purple petioles, whereas \(P_3\) is an inherited dominant gene conditioning anthocyanin biosynthesis in carrots with purple roots (both solid purple and purple peridermal carrot types) and petioles (Cavagnaro et al., 2014).

Anthocyanin biosynthesis involves a number of structural and regulatory genes in many plant species. The structural genes, encoding enzymes that directly catalyze the production of anthocyanin, are regulated by transcription factors. The expression levels of the previously tested structural genes correlate with anthocyanin biosynthesis in carrots and none of them mapped to the same location as \(P_1\) or \(P_3\), indicating that these structural genes are not the key genetic factors controlling anthocyanin pigmentation in purple carrots (Yildiz et al., 2013; Cavagnaro et al., 2014; Xu et al., 2014). Thus, genetic mutations that cause pigmentation changes in carrot root may occur in regulatory genes. In all the plant species studied to date, the anthocyanin biosynthetic structural genes are directly regulated by R2R3–MYB, basic helix-loop-helix (bHLH) and WD-repeat proteins, in the form of the ‘MBW’ complex (Espley et al., 2007; Chagne et al., 2013; Jin et al., 2016). Mutations in R2R3–MYB often lead to aberrant anthocyanin biosynthesis and result in color changes. The
mutational types include nucleotide sequence changes, transposon or microsatellite insertion and methylation (Kobayashi et al., 2004; Morita et al., 2006; Espley et al., 2009; Wang et al., 2013).

DcMYB6, corresponding to DCAR_000385 in the carrot genome, encodes a R2R3–MYB that can induce anthocyanin biosynthesis in Arabidopsis (Arabidopsis thaliana; Xu et al., 2017). Very recently, DcMYB6 was anchored to the P3 region (Iorizzo et al., 2019). Using phylogenetic analyses, Iorizzo et al. also identified five additional MYB transcription factors of the anthocyanin biosynthesis-related subgroup of MYBs, DcMYB7 (DCAR_010745), which was designated DcMYB113-like in the carrot genome (Iorizzo et al., 2016), DcMYB8 (DCAR_010746), DcMYB9 (DCAR_010747), DcMYB10 (DCAR_010749) and DcMYB11 (DCAR_010751), within the P3 genomic region (Iorizzo et al., 2019).

Among these R2R3–MYBs, the expression of DcMYB7 was consistently associated with purple root pigmentation in all the purple carrots tested, while the expression of DcMYB6 was only associated with anthocyanin pigmentation in the solid purple carrots. Iorizzo et al. speculated that DcMYB6 controls anthocyanin pigmentation in the inner root tissues, while DcMYB7 determines anthocyanin pigmentation in the root outer-phloem. The expression levels of the other four R2R3–MYBs were not associated with anthocyanin pigmentation in the roots of purple carrots according to the transcriptome analysis. Previously, we also obtained DcMYB7-knockout (originally DcMYB113-like-knockout) ‘Deep purple’ (DPP) carrot plantlets (Xu et al., 2019). To date, however, the functions of DcMYB6 and DcMYB7 have still not been...
systematically studied in carrots, and the genetic mechanism behind pigmentation-related mutations in purple carrot versus non-purple carrot roots is still unclear. In purple carrots, cyanidin-based anthocyanins are almost exclusively responsible for the purple pigment, while trace amounts of derivatives of peonidin- and pelargonidin-based anthocyanins are also present in some purple carrot cultivars (Kammerer et al., 2004; Montilla et al., 2011). In carrot, UCGalT1 catalyzes the first glycosylation step of anthocyanidins, generating stable anthocyanins (Xu et al., 2016). Anthocyanins further undergo several glycosylation and acylation steps (Glassgen et al., 1998; Cavagnaro et al., 2014). These changes increase their stability and water solubility. Owing to their stable and water-soluble characteristics, as well as their health-promoting properties, anthocyanins from purple carrots serve as excellent natural colors in beverages, candies and ice cream (Netzel et al., 2007). Progress in understanding the genetic control of anthocyanin glycosylation and acylation in purple carrot has been reported (Cavagnaro et al., 2014). Several quantitative trait loci (QTLs), distributed across six chromosomes, were proposed by Cavagnaro et al. as being associated with anthocyanin glycosylation and acylation (Cavagnaro et al., 2014). However, the genes conditioning anthocyanin glycosylation and acylation in purple carrots have still not been identified and characterized. In this study, we characterized the functions of DcMYB6 and DcMYB7 using stable plant transformations and CRISPR/Cas9-based genome-editing. The mechanism of purple pigment loss in non-purple carrot roots and the roles of DcMYB7 in
anthocyanins glycosylation and acylation were also analyzed. Our results not only improve our understanding of the molecular mechanism behind the origin of the non-purple root phenotype during carrot domestication but are also valuable for breeding programs aimed at manipulating and modifying anthocyanin biosynthesis in carrot and other plant species.

**Results**

**Differential expression profiles of DcMYB6 and DcMYB7 in purple and non-purple carrot roots**

The transcript levels of DcMYB6 and DcMYB7 were detected in different root tissues of six purple carrot and five non-purple carrot cultivars (Fig. 1A). DPP, ‘Purple 68’ (PP68) and ‘Tianzi2hao’ (TZ2H) are solid purple carrot cultivars, accumulating anthocyanins in root periderm, phloem and xylem tissues. ‘Zibacun’ (ZBC) is a carrot cultivar with two types of roots: ZBC-S is a solid purple carrot, accumulating anthocyanins across the entire root section, while ZBC-P is a purple peridermal carrot, accumulating anthocyanins in root peridermal tissue. ‘Cosmic purple’ (CPP) is also a carrot cultivar with purple root peridermal tissue. ‘Kurodagosun’ (KRD), ‘Sanhongliucun’ (SHLC) and ‘Junchuanhong’ (JCH) are orange carrot cultivars, while ‘Qitouhuang’ (QTH) and ‘Baiyu’ (BY) are yellow carrot cultivars.

RT-qPCR analyses indicated that DcMYB6 and DcMYB7 were co-expressed in purple carrot roots (Fig. 1B). DcMYB6 showed high transcript levels in the
anthocyanin-pigmented tissues of purple carrot roots. The transcript levels of

*DcMYB6* were relatively lower but still detectable in the non-purple root tissues of purple and non-purple carrot cultivars. *DcMYB7* was positively correlated with anthocyanin pigmentation in carrot roots, showing high transcript levels in the anthocyanin-pigmented root tissues of the purple carrot cultivars but almost undetectable transcript levels in those non-purple root tissues of purple and non-purple carrot cultivars. Compared with *DcMYB7*, *DcMYB6* displayed higher transcript levels in all the root tissues of purple and non-purple carrot cultivars.

**DcMYB6 and DcMYB7 functional assays**

In our previous study, the heterologous overexpression of *DcMYB6* in Arabidopsis induced anthocyanin accumulation (Xu et al., 2017). Here, *DcMYB6* was introduced into the orange carrot KRD under the control of the CaMV 35S promoter to investigate its function in the homologous system. No purple pigmentation was observed at either the callus or plantlet stages after transformation (Fig. 2A). The 4-month-old 35S:*DcMYB6* transgenic carrot lines had no purple pigmentation in their roots or petioles. After bolting, 35S:*DcMYB6* transgenic carrots generated no purple-pigmented inflorescences or seeds (Fig. 2A).

*DcMYB7* from the solid purple carrot DPP was also introduced into Arabidopsis plants and the orange carrot KRD under the control of the CaMV 35S promoter to investigate its functions. The heterologous overexpression of *DcMYB7* in Arabidopsis induced anthocyanin accumulation in both vegetative and reproductive organs.
When the orange carrot KRD explants were transformed with 35S:DcMYB7, deep purple calli were produced and regenerated to produce purple plantlets (Fig. 2B). The 35S:DcMYB7 transgenic KRD carrots accumulated high levels of anthocyanins across the entire root section (Fig. 2B and Fig. 2C). After bolting, 35S:DcMYB7 transgenic KRD carrots generated purple pigmented ovaries and seeds but no purple pigmented petals or stamens.

DcMYB7 was knocked out in the solid purple carrot DPP using the CRISPR/Cas9 system (Xu et al., 2019). Four DcMYB7-knockout plantlet lines with two or three edited target sites were previously generated. After growing for 4 months, three DcMYB7-knockout DPP carrot lines (line 1, line 2 and line 3) were chosen for study (Fig. 2D). The roots of these DcMYB7-knockout DPP carrot lines were yellow across the entire section. In contrast to normal DPP carrots, which accumulate high levels of anthocyanins in their roots, the three DcMYB7-knockout DPP carrot lines accumulated undetectable or trace amounts of anthocyanins in their roots (Fig. 2E). These results together suggest that DcMYB7 is the P3 gene that conditions anthocyanin biosynthesis in purple carrot roots. However, petioles of the three DcMYB7-knockout DPP carrot lines retained purple pigmented, supporting the hypothesis that, in addition to DcMYB7, another genetic factor also controls anthocyanin production in purple carrot petioles.

DcMYB7 increased the expression levels of DcbHLH3 and anthocyanin biosynthesis-related structural genes in carrots
MYBs often interact with bHLHs to co-regulate anthocyanin biosynthesis in many plant species. MYBs control corresponding bHLH interactor expression in Arabidopsis (Tohge et al., 2005). Iorizzo et al. identified several differentially expressed genes within the $P_3$ genomic regions of chromosome 3, but none grouped with anthocyanin-related bHLHs from other species (Iorizzo et al., 2019). Two carrot bHLHs, both located on chromosome 1, corresponding to the predicted carrot genes DCAR_002739 and DCAR_004632, were identified by referring to orthologous apple ($Malus \times domestica$) bHLH3 and through phylogenetic analyses. MdbHLH3 is a bHLH that can enhance anthocyanins accumulation (Espley et al., 2007).

DCAR_002739 and DCAR_004632 clustered together with anthocyanin-related bHLHs from other plant species in phylogenetic analyses (Supplemental Fig. S2). However, RT-qPCR analyses indicated that DCAR_004632 was not co-expressed with anthocyanin pigmentation in carrots and showed much lower transcript levels than DCAR_002739 (hereafter DcbHLH3) in the purple pigmented tissues of all the tested purple carrot roots. DcbHLH3, which shared higher amino acid sequence identity with MdbHLH3 than DCAR_004632, was positively correlated with anthocyanin biosynthesis in carrot roots, displaying high transcript levels in the purple pigmented tissues of all the tested purple carrot roots, but very low or undetectable transcript levels in the non-purple root tissues of purple and non-purple carrot cultivars (Supplemental Fig. S3). Yeast ($Saccharomyces cerevisiae$) two-hybrid assays indicated that DcMYB7 could interact with DcbHLH3 (Fig. 3A).

To determine whether $DcMYB7$ regulates $DcbHLH3$, RT-qPCR analyses were...
performed in roots of untransformed KRD carrots and three 35S:DcMYB7 transgenic KRD carrot lines at 4 months of age. Compared with the untransformed KRD carrots, DcbHLH3 transcript levels were greatly increased in the three 35S:DcMYB7 KRD carrot lines (Fig. 3B). Transcriptional analysis of anthocyanin biosynthetic structural genes (DcCHS1, DcCHI1, DcF3H1, DcF3'H1, DcDFR1, DcLDOX1 and DcUCGalT1) indicated that these genes were all up-regulated in the roots of the three 35S:DcMYB7 transgenic KRD carrot lines compared with untransformed KRD carrots (Fig. 3B). In addition, we analyzed the transcript levels of DcbHLH3 and the structural genes in the untransformed DPP carrot and the three DcMYB7-knockout DPP carrot lines. Compared with the untransformed DPP carrot, DcbHLH3 and all the tested structural genes were down-regulated in the roots of the three DcMYB7-knockout DPP carrots (Fig. 3C). These results together suggest that DcMYB7 controls the expression of its partner DcbHLH3 and the anthocyanin biosynthetic structural genes.

**Functional tests of DcMYB7 from different purple and non-purple carrot cultivars**

DcMYB7 was cloned from genomic DNA (gDNA) and cDNA libraries constructed using 11 different purple and non-purple carrot cultivars. DcMYB7 gDNA sequences were different lengths among the carrot cultivars (Fig. 4A). On the basis of the sequencing results, four variant gDNA sequences were identified among the 11 different purple and non-purple carrot cultivars (Fig. 4B). The DcMYB7 gDNA sequences from three solid purple carrot cultivars (DPP, PP68 and TZ2H) are
identical and 3,039-bp long, which is much longer than the lengths of the gene in the
other 8 carrot cultivars. The *DcMYB7* gDNA sequences from solid purple carrot
ZBC-S and purple peridermal carrots ZBC-P and CPP are identical and 1,947-bp long.
The *DcMYB7* gDNA sequences from the orange carrot KRD and yellow carrots QTH
and BY are 2,193-bp long and share 100% identity to *DcMYB7* sequence from the
DH1 (orange) carrot genome (Iorizzo et al., 2016). The *DcMYB7* gDNA sequences
from the orange carrot SHLC and JCH are identical and 1,926-bp long.
The cDNA sequence of *DcMYB7* was also cloned from the 11 different purple or
non-purple carrot cultivars. Four variant cDNA sequences were identified among the
11 different purple and non-purple carrot cultivars. Based on the sequencing and
alignment analyses, *DcMYB7* cDNA from the 11 tested carrot cultivars were 903-bp
long. The alignment analysis of *DcMYB7* cDNA and gDNA sequences indicated that
the genomic structure consisted of three exons and two introns. The different lengths
of *DcMYB7* gDNA products from various carrot cultivars were caused by variation of
intron II (Fig. 4B).
The gDNA sequences of *DcMYB7* from the 11 different purple and non-purple
carrot cultivars differed. To determine whether the variation among *DcMYB7* gDNA
sequences affected their function in inducing anthocyanin biosynthesis, the four
variants gDNAs were amplified from the solid purple carrot DPP, purple peridermal
carrot CPP and orange carrots KRD and SHLC. They were then independently
introduced into the orange carrot KRD for expression under the control of the CaMV
35S promoter. Deep purple calli were induced from explants transformed with
DcMYB7 gDNAs from the solid purple carrot DPP (35S:DcMYB7<sub>DPP</sub>), purple peridermal carrot CPP (35S:DcMYB7<sub>CPP</sub>) and orange carrot KRD (35S:DcMYB7<sub>KRD</sub>) and SHLC (35S:DcMYB7<sub>SHLC</sub>), whereas non-purple pigmented calli were produced from orange carrot KRD explants transformed with the pCAMBIA 1301 vector (control) (Fig. 4C). Thus, gDNA sequences of DcMYB7 from different non-purple carrot cultivars appear to have retained their anthocyanin-induction function.

Insertion of a non-functional DcMYB7 duplication or transposons into the promoter region of DcMYB7 in non-purple carrots

To identify the molecular mechanism underlying the differential expression patterns of DcMYB7 in 11 different purple and non-purple carrots, we cloned the upstream sequence of DcMYB7 from the 11 carrot cultivars. According to the carrot genome sequence (Iorizzo et al., 2016) and a recent report (Iorizzo et al., 2019), DcMYB7, DcMYB8 and DcMYB6 are organized in tandem within the 27,816,911 –27,833,545-bp region of chromosome 3 (Fig. 5A). The P7-F1 and P7-R1 primers were designed based on the DNA sequences of DcMYB7 and DcMYB8 to amplify the sequence upstream of DcMYB7 (Fig. 5A). The upstream sequence of DcMYB7 was successfully amplified from the orange carrot KRD and yellow carrots QTH and BY but not from the other 8 carrot cultivars (Fig. 5B). Sequencing results showed that the sequences upstream of DcMYB7 from KRD, QTH and BY are identical to that from the DH1 orange carrot genome (Supplemental Fig. S4). We further analyzed the region containing DcMYB7, DcMYB8 and DcMYB6 on chromosome 3 of the DH1
orange carrot genome. The full-length predicted protein sequences of DcMYB7 (300 aa) and DcMYB8 (292 aa) were highly similar (over 90%) (Supplemental Fig. S5).

However, DcMYB8 expression was undetectable in all the purple carrots tested in a previous report (Iorizzo et al., 2019). Thus DcMYB8 was regarded as a non-functional duplication of DcMYB7 and inserted between DcMYB7 and DcMYB6 at 403-bp upstream of the putative DcMYB7 start codon (Fig. 5C and Supplemental Fig. S4).

The sequence upstream of DcMYB7 was successfully amplified from the other 8 carrot cultivars using the P7-F1 and P7-R2 primers designed based on the DNA sequences of DcMYB7 and DcMYB6 (Fig. 5B). Sequencing results indicated that DcMYB8 was absent in the regions between DcMYB7 and DcMYB6 in these 8 carrot cultivars. Sequencing results also showed that the sequence upstream of DcMYB7 from DPP, PP68 and TZ2H carrots are identical (MK637849), those from ZBC-S, ZBC-P and CPP carrots are identical (MK637850), and those from SHLC and JCH are identical (MK637851) (Fig. 5C).

To investigate why the promoters of DcMYB7 were not functional in non-purple carrots, an alignment of the DcMYB7 promoter regions from DPP, CPP, SHLC and KRD, was conducted. The promoter region of DcMYB7 from KRD (Pro-DcMYB7-KRD) shared a high sequence identity with those from the other carrot cultivars within the 1- to 281-bp upstream region of the putative start codon, but there were no common sequences in the region 403-bp upstream of the putative start codon owing to the insertion of DcMYB8 (Supplemental Fig. S6). The DcMYB7 promoter sequence from the DPP (Pro-DcMYB7-DPP) shared a high identity with those from
CPP (Pro-DcMYB7-CPP) and SHLC (Pro-DcMYB7-SHLC) within the 1- to 1,392-bp upstream region of the putative start codon, designated as the consensus sequences region. A poly (dA-dT) element and a tandem repeat element were found in the consensus sequences region of DPP. The latter element was not repeated in the DcMYB7 promoters from CPP and SHLC, and it was interrupted by another unknown element in SHLC (Fig. 5C and Supplemental Fig. S6). This unknown element was widely present in the carrot genome on the basis of BLAST-search analysis (E value ≤10^{-120}) against the carrot genome sequence. On the basis of BLAST-search analysis against the GIRI repeat database (score=236) (http://www.girinst.org/) (Kohany et al., 2006), it belongs to Tc1/Mariner transposon family. Another transposon belonging to the hAT family was also identified in DcMYB7 promoter sequences of SHLC on the basis of BLAST-search analysis against the GIRI repeat database (score=480). This transposon was also widely present in carrot genome on the basis of BLAST-search analysis (E value ≤10^{-150}) against the carrot genome sequence. In addition, the DcMYB7 promoter sequences from SHLC and CPP were nearly identical, except that the former was interrupted by Tc1/Mariner and hAT transposons.

The entire coding DNA sequence (CDS) and promoter region of DcMYB7 were cloned from DPP and CPP to construct Pro_{DPP}:DcMYB7 and Pro_{CPP}:DcMYB7, respectively. These constructs and the pCAMBIA 1301 vector were then independently genetically transformed into the orange carrot KRD. After transformation, purple pigmented calli were produced from explants transformed with Pro_{DPP}:DcMYB7 and Pro_{CPP}:DcMYB7 (Fig. 5D). However, non-purple pigmented
calli were produced from explants transformed with pCAMBIA 1301 vector (Fig. 5D). These results suggested that inactivation of \( \text{DcMYB7} \) from KRD, QTH and BY resulted from the insertion of \( \text{DcMYB8} \), a non-functional duplication of \( \text{DcMYB7} \), in its promoter region. The \( \text{Pro}_{\text{CPP}}:\text{DcMYB7} \) and pCAMBIA 1301 vector were also independently genetically transformed into orange carrot SHLC. After transformation, purple pigmented calli were produced from explants transformed with \( \text{Pro}_{\text{CPP}}:\text{DcMYB7} \), but non-purple pigmented calli were produced from explants transformed with the pCAMBIA 1301 vector (Fig. 5D). The promoter sequences of SHLC and CPP were nearly identical except for the region interrupted by transposons, supporting the idea that the transposon insertions in the \( \text{DcMYB7} \) promoter of the former have resulted in the loss of function.

\( \text{DcMYB7} \) regulates further anthocyanin modifications by activating \( \text{DcUCGXT1} \) and \( \text{DcSAT1} \) in carrot

In purple carrots, anthocyanins are usually stored in glycosylated and acylated forms (Kammerer et al., 2004; Montilla et al., 2011; Cavagnaro et al., 2014). In this study, the anthocyanin compositions of DPP and \( \text{35S:DcMYB7} \) transgenic KRD carrot (line 1) roots were measured using high-performance liquid chromatography–mass spectrometry. DPP carrot produced seven peaks that were confirmed to be those of anthocyanins (Fig. 6A). The major peak, 4, was identified as cyanidin 3-xylosyl (feruloylglucosyl) galactoside (Cy3XFGG). The other six peaks, 1, 2, 3, 5, 6 and 7, were identified as cyanidin 3-xylosyl (glucosyl) galactoside (Cy3XGG), cyanidin...
3-xylosylgalactoside (Cy3XG), cyanidin 3-xylosyl (sinapoylglucosyl) galactoside (Cy3XSGG), cyanidin 3-xylosyl (coumaroylglucosyl) galactoside, pelargonidin 3-xylosyl (feruloylglucosyl) galactoside and peonidin 3-xylosyl (feruloylglucosyl) galactoside. The 35S:DcMYB7 transgenic KRD carrot only produced four peaks, 1-4, corresponding to Cy3XGG, Cy3XG, Cy3XSGG and Cy3XF GG, respectively (Fig. 6A). Through observation of the peak areas from the HPLC measurements, Cy3XSGG was determined to be the predominant anthocyanin in 35S:DcMYB7 transgenic KRD carrot roots. A reaction scheme for the biosynthesis of Cy3XSGG from cyanidin-3-galactoside (Cy3G) in carrot has been proposed (Glassgen et al., 1998). Cy3G undergoes two glycosylation steps and one acylation step in the formation of Cy3XSGG (Fig. 6B). UDP-xylose:cyanidin 3-galactoside xylosyltransferase (UCGXT), UDP-glucose:cyanidin 3-xylosylgalactoside glucosyltransferase (UCGXGT), and sinapoyl-Glc:anthocyanin acyltransferase (SAT) participate in Cy3XSGG biosynthesis.

In a previous report, Cavagnaro et al. identified three QTLs (Q1, Q2 and Q3) associated with Cy3XSGG accumulation in carrots (Cavagnaro et al., 2014). Q1 was genetically mapped within the same locus on chromosome 3 as P3 (Cavagnaro et al., 2014), a gene confirmed to be DcMYB7 in this study. Q2 and Q3 were genetically mapped to chromosomes 6 and 3, respectively (Cavagnaro et al., 2014). Within the Q3 locus associated with Cy3XSGG, a gene encoding UCGXT, DcUCGXT1 (DCAR_021269), was identified by referring to orthologous F3GGT1 (FG404013) from kiwifruit (Actinidia chinensis) and UGT79B1 (At5g54060) from Arabidopsis.
The F3GGT1 and UGT7291 proteins catalyze the glycosylation of Cy3G to produce Cy3XG. Within the Q2 locus, a gene encoding SAT, DcSAT1 (no corresponding predicted gene in the carrot genome), was identified by referring to the orthologous SAT (At2g23000) from Arabidopsis. At2g23000 encodes a protein capable of adding sinapoylglucose to anthocyanins to synthesize sinapoylated anthocyanins (Fraser et al., 2007).

An RT-qPCR analysis indicated that DcUCGXT1 and DcSAT1 showed high transcript levels in roots of DPP but visually undetectable transcript levels in roots of KRD (Fig. 7A). Compared with untransformed KRD carrot, DcUCGXT1 and DcSAT1 showed greatly increased transcript levels in the three 35S:DcMYB7 KRD carrot lines.

In addition, transcript levels of DcUCGXT1 and DcSAT1 in the roots of the three DcMYB7-knockout DPP carrot lines were obviously decreased compared with untransformed DPP carrots. Thus, DcMYB7 probably conditions anthocyanin glycosylation and acylation by regulating DcUCGXT1 and DcSAT1 in carrots.

DcMYB7 directly regulates the expression levels of DcUCGXT1 and DcSAT1

Yeast one-hybrid assays were performed to detect the binding of DcMYB7 to the promoters of DcUCGXT1 and DcSAT1. The yeast cells co-transformed with pGADT7-DcMYB7 and ProDcUCGXT1-HIS or ProDcSAT1-HIS showed greater resistances to 50 mM 3-amino-1,2,4-triazole (3-AT) than the negative control (Fig. 7B), indicating that DcMYB7 could bind the promoters of DcUCGXT1 and DcSAT1.

A dual luciferase (LUC) reporter system was used to further confirm the interaction...
of DcMYB7 with the DcUCGXT1 and DcSAT1 promoters. DcMYB7 was cloned into the pGreenII 62-SK vector to generate DcMYB7-62SK, and DcUCGXT1 and DcSAT1 promoter sequences were independently fused to LUC of the pGreenII 0800 vector to generate ProDcUCGXT1:LUC and ProDcSAT1:LUC, respectively. DcMYB7-62SK was co-infiltrated with ProDcUCGXT1:LUC or ProDcSAT1:LUC into Nicotiana benthamiana leaves for expression. As a result, the co-expression of DcMYB7-62SK with either ProDcUCGXT1:LUC or ProDcSAT1:LUC exhibited strong a luminescence intensity, whereas the co-expression of pGreenII 62-SK with ProDcUCGXT1:LUC or ProDcSAT1:LUC exhibited no visible or a weak luminescence signals (Fig. 7C).

The ratio of luminescence produced by ProDcUCGXT1:LUC or ProDcSAT1:LUC to that produced by 35S:Renilla was calculated to determine the transactivation activity (Fig. 7D). There was greater than 100-fold increase in the transactivation activity of DcMYB7-62SK on ProDcUCGXT1:LUC compared with pGreenII 62-SK on ProDcUCGXT1:LUC. Additionally, the transactivation activity of DcMYB7-62SK on ProDcSAT1:LUC increased more than 4-fold compared with that of pGreenII 62-SK on ProDcSAT1:LUC. Thus, DcMYB7 appears to directly activate the promoters of DcUCGXT1 and DcSAT1.

**Discussion**

Carrots with purple or yellow root were first cultivated as root crop in Central Asia region ~ 1,100 years ago and spread to the East and West (Iorizzo et al., 2013).
yellow carrot was concluded to be a color mutant of the purple carrot (Banga, 1963). The color of carrot roots has significantly changed during the domestication processes. White and red carrots originated in Europe and China, respectively, between the 11th and 15th centuries (Arscott and Tanumihardjo, 2010). The Europeans preferred yellow carrot over purple and white carrot until orange carrot arrived in the 16th century (Simon, 2000). Then, the latter gradually spread throughout Europe and other continents worldwide. Nowadays, cultivated carrot roots are purple, orange, yellow, red and white, resulting from anthocyanin, carotene, lutein and lycopene accumulations, and the lack of pigments, respectively (Surles et al., 2004; Clotault et al., 2008; Montilla et al., 2011).

Anthocyanins have protective functions against biotic and abiotic stresses in plants, such as insects attack in Arabidopsis (Johnson and Dowd, 2004), blast fungus infection in rice (Oryza sativa L.) (Gandikota et al., 2001) and cold stress in sweet potato (Ipomoea batatas L.) storage roots (Wang et al., 2013). We speculate that anthocyanins in purple carrot roots may also have protective functions. In addition, anthocyanin can protect humans against diseases (Netzel et al., 2007; He and Giusti, 2010; Tsutsumi et al., 2019). The intake of anthocyanin-rich food decreases the risks of several kinds of diseases. Purple carrots are still popular and cultivated in China, Japan, India and other Asiatic countries. With the human health benefits of anthocyanins, purple carrots have been “re-discovered” by breeders that are aiming to improve the nutrient content. However, to date, the molecular basis of the mutation leading to anthocyanin
pigment loss in carrot has still not been revealed. Two inherited genes, $P_1$ and $P_3$, controlling anthocyanin pigmentation in purple carrot roots were genetically mapped within 28.2-cM and 12-cM regions, respectively, on chromosome 3 in carrots from different genetic backgrounds (Simon, 1996; Cavagnaro et al., 2014). $P_1$ is responsible for anthocyanin pigmentation in carrots with purple roots but green petioles, whereas $P_3$ controls anthocyanin pigmentation in carrots with purple roots and petioles (Cavagnaro et al., 2014). Previously, we found that the expression level of $DcMYB6$, a gene anchored in the $P_3$ region (Iorizzo et al., 2019), was associated with anthocyanin pigmentation in purple carrot roots (Xu et al., 2017). Iorizzo et al. also identified another MYB gene, $DcMYB7$, in the $P_3$ region (Iorizzo et al., 2019). Because the expression of $DcMYB7$ was correlated with anthocyanin pigmentation in all the tested purple carrot roots and $DcMYB6$ was only expressed in solid purple carrots, Iorizzo et al. speculated that $DcMYB6$ and $DcMYB7$ control anthocyanin biosynthesis in the inner root tissues and the root outer-phloem, respectively. Here, the expression levels of $DcMYB6$ and $DcMYB7$ were both consistent with anthocyanin pigmentation in different purple carrot roots. However, the expression of $DcMYB6$, which could lead to anthocyanin accumulation in Arabidopsis plants (Xu et al., 2017), was unable to induce anthocyanin biosynthesis in the orange carrot KRD. The overexpression of $DcMYB7$ in the orange carrot KRD resulted in the reconstitution of anthocyanin accumulation in vegetative and reproductive tissues, including roots, by activating its partner $DcbHLH3$ and all the tested anthocyanin biosynthetic structural genes. The $DcMYB7$-knockout DPP carrots produced yellow
roots, which suggests that \textit{DcMYB7} is the \textit{P}_3 gene that controls anthocyanin
biosynthesis both in inner and outer root tissues of purple carrots. The petioles of
\textit{DcMYB7}-knockout DPP carrots retain their purple pigmentation, indicating that
another dominant gene, probably \textit{DcMYB11} identified in a previous study (Iorizzo et
al., 2019), conditions anthocyanin pigmentation in carrot petioles.

R2R3–MYB mutations can lead to anthocyanin pigment losses in some plant
species (Kobayashi et al., 2004; Ban et al., 2007; Espley et al., 2009; Wang et al.,
2013). Although the gDNA sequence of \textit{DcMYB7} varies among different purple and
non-purple carrots, the CDS regions of \textit{DcMYB7} gDNAs from orange and yellow
carrots retained their abilities to induce anthocyanin biosynthesis. Our data indicated
that the promoter sequence of \textit{DcMYB7} was interrupted either by \textit{DcMYB8}, a
non-functional tandem duplication of \textit{DcMYB7}, or by transposons, leading to the
transcriptional inactivation of \textit{DcMYB7} in non-purple carrot roots. As a result,
non-purple carrots were unable to accumulate anthocyanins in their roots.

In this study, we unraveled the genetic factors conditioning anthocyanin
pigmentation in purple versus non-purple carrot roots, providing new insight into
carrot domestication. However, the CDS and promoter region of \textit{DcMYB7} from the
solid purple carrot ZBC-S and the purple peridermal carrots ZBC-P and CPP are
identical. \textit{DcMYB7} showed high expression levels across the entire root section in
ZBC-S but was only specifically expressed in the root peridermal tissue of ZBC-P and
CPP carrots, suggesting that there is another genetic factor that suppresses the
expression of \textit{DcMYB7} in phloem and xylem tissues of ZBC-P and CPP carrot roots.
In future work, we will focus on determining the genetic factors contributing to the differential expression pattern of *DcMYB7* in solid purple and purple peridermal carrots.

In plants, anthocyanin usually undergoes modifications that increases its stability and water solubility (Cheng et al., 2014). Anthocyanins from purple carrots undergo a series of glycosylation and acylation events, resulting in relatively higher temperature and pH stability levels than other plant species (Kirca et al., 2007). In purple carrots, anthocyanins are dominated by glycosylated and acylated cyanidin, with Cy3XSGG and Cy3XFGG being the predominant anthocyanin compositions (Kammerer et al., 2004; Montilla et al., 2011). In this study, the overexpression of *DcMYB7* in the orange carrot KRD induced anthocyanin accumulation in their roots, with Cy3XSGG being detected as the predominant anthocyanin.

Three QTLs (Q1, Q2 and Q3) were detected as associated with Cy3XSGG in chromosomes 3 and 6 by Cavagnaro et al. (Cavagnaro et al., 2014). Q1 was anchored to the same region on chromosome 3 as *P3*, which was confirmed to be *DcMYB7* in this study. Within the other two QTLs (Q2 and Q3), *DcUCGXT1* and *DcSAT1* involved in anthocyanin glycosylation and acylation were identified. *UCGXT* and *SAT* had been identified previously in Arabidopsis plants (Fraser et al., 2007; Yonekura-Sakakibara et al., 2012). In carrot, *DcUCGXT1* and *DcSAT1* are co-expressed with *DcMYB7* in anthocyanin pigmented roots, supporting the hypothesis that *DcMYB7* could trigger the expression of both *DcUCGXT1* and *DcSAT1*, which was shown in this study. These data suggest that *DcMYB7* conditions...
the route from Cy3G to acylated Cy3XSGG in the anthocyanin modification pathways of carrot.

Acylation increases anthocyanin stability but reduces it bioavailability (Charron et al., 2009). Carrot breeders can determine breeding objective for anthocyanin composition based on market demand, with preferences for acylated anthocyanins as stable food colorants and non-acylated anthocyanins as bioavailable nutraceuticals. Our study will aid attempts to manipulate anthocyanin composition in carrots and other root crops.

Conclusions

Functional analysis confirmed that DcMYB7 is the P3 gene that controls purple pigmentation in carrot roots by regulating its DcbHLH3 partner and the tested anthocyanin biosynthetic structural genes. The promoter sequence of DcMYB7 in non-purple carrots has been interrupted by a tandem duplication event or transposons insertion, leading to undetectable levels of anthocyanins in their roots. Our study suggests that there is another genetic factor that suppresses DcMYB7 expression in phloem and xylem of purple peridermal carrot root tissues. DcMYB7 also conditions anthocyanin modifications by directly activating DcUCGXT1 and DcSAT1. Our determination of these genetic factors involved in anthocyanin biosynthesis and modification in carrots will aid in the breeding of carrot as well as that of other root crops.
Materials and Methods

Plant materials and growth conditions

All the carrots (Daucus carota) used in the present study were grown in artificial climatic chamber as previously described (Xu et al., 2014). DPP, PP68 and TZ2H are carrot cultivars with solid purple roots (purple periderm, phloem and xylem). ZBC is a carrot cultivar with two types of roots: solid purple (ZBC-S) and purple periderm but non-purple phloem and xylem (ZBC-P). CPP is a carrot cultivar with purple peridermal root tissue. KRD, SHLC and JCH are orange carrot cultivars. QTH and BY are yellow cultivars. Young leaves of carrots were used for gDNA extractions. Different tissues of 4-month-old carrot roots were used for anthocyanin and total RNA extractions. Periderm, phloem and xylem tissues of roots from CPP and ZBC-P were collected separately as samples. The ability to distinguish and excise the periderm from the phloem was impossible for roots of the other carrot cultivars. For these carrot cultivars, root periderm and phloem tissues were collected together as ‘periderm & phloem’ samples, and xylem tissue was individually collected. All the samples were immediately frozen in liquid nitrogen after harvest and then stored at −80 °C before further analyses.

Anthocyanin measurement

Total anthocyanin was extracted from carrot roots using the methanol–HCl method.
(Li et al., 2012), with a modified extraction buffer (50% methanol, v/v; 49.9% ddH$_2$O, v/v; and 0.1% HCl, v/v), and was then quantitatively analyzed as described previously (Li et al., 2012). The anthocyanin composition was analyzed using high-performance liquid chromatography–mass spectrometry with the method described by Feng et al. (Feng et al., 2018).

**Genomic DNA and total RNA extractions, gene cloning and RT-qPCR assays**

The gDNA and total RNA were isolated using a DNAsecure Plant Kit or RNAsimple Total RNA Kit (TIANGEN, Beijing, China) according to the manufacturer’s instructions. First-strand cDNA was synthesized using the HiScript II Q RT SuperMix for qPCR kit (Vazyme Biotech Co. Ltd., Nanjing, China) following the manufacturer’s protocol. Gene cloning was performed using PrimeSTAR HS DNA polymerase (Takara, Dalian, China). RT-qPCR assays were conducted as described previously (Xu et al., 2017). The relative gene transcript levels were normalized to *DcActin1* (Wang et al., 2016) and calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). The primers used for RT-qPCR assays of *DcCHS1*, *DcCHI1*, *DcF3H1*, *DcF3’H1*, *DcDFR1*, *DcLDOX1*, *DcUCGalT1* and *DcActin1* are the same as described in our previous report (Xu et al., 2014). All the other primers used are listed in Supplemental Table S1.

**Sequence alignment and phylogenetic analyses**
Sequence alignments were performed using ClustalW (Chenna et al., 2003).

Phylogenetic analysis was conducted with the deduced amino acid sequences of bHLHs from carrot and other plant species. The phylogenetic tree was constructed using MEGA version 5.0 software with the neighbor-joining method (bootstrap value =1000) (Tamura et al., 2011).

**Generation of transgenic carrot and Arabidopsis**

The CDS regions of *DcMYB6* and *DcMYB7* were amplified from a cDNA library of DPP and inserted independently into the pCAMBIA 1301 vector between the CaMV 35S promoter and pea rbcSE9 terminator to create 35S:*DcMYB6* and 35S:*DcMYB7* constructs, respectively. *DcMYB7* was also cloned from gDNA libraries of DPP, CPP, KRD and SHLC to create 35S:*DcMYB7*DPP, 35S:*DcMYB7*CPP, 35S:*DcMYB7*KRD and 35S:*DcMYB7*SHLC constructs, respectively. The gDNA fragments of *DcMYB7*, including the promoter region and entire CDS region, were amplified from DNA libraries of DPP and CPP to prepare Pro*DPP:*DcMYB7* and Pro*CPP:*DcMYB7* constructs, respectively. The recombined vectors were independently transformed into *Agrobacterium tumefaciens* (GV3101). The transformation of carrot was performed using a previously described method (Xu et al., 2019). The orange carrot KRD and SHLC were chosen for transformation. The 35S:*DcMYB7* construct was also transformed into *Arabidopsis thaliana* using the *A. tumefaciens*-mediated floral dip method (Clough and Bent, 1998). All of the primers used in this procedure are listed in Supplemental Table S1.
Generation of \textit{DcMYB7}-knockout mutant plants

\textit{DcMYB7}, which was designated \textit{DcMYB113-like} gene previously (Xu et al., 2019), was successfully knocked out in DPP carrot using the CRISPR/Cas9 system. After growing in an artificial climatic chamber for 4 months, three \textit{DcMYB7}-knockout plant lines (line 1, line 2 and line 3) were chosen for study.

\textbf{Yeast two-hybrid assay}

The CDS regions of \textit{DcMYB7} and \textit{DcbHLH3} from DPP and \textit{35S:DcMYB7} transgenic KRD carrots, respectively, were separately cloned into the pGADT7 and pGBK7 vectors (Clontech, Palo Alto, CA) to generate pGADT7-DcMYB7 and pGBK7-DcbHLH3, respectively. These two recombined constructs were co-transformed into yeast strain Y2HGold cells following the manufacturer’s manual (Clontech, Palo Alto, CA). The pGADT7 and pGBK7 vectors were used as negative controls. The transformants were selected on SD/–Leu/–Trp medium at 30 °C for 3–4 d. The interactions were tested on SD/–Ade/–His/–Leu/–Trp medium with or without X-α-Gal.

\textbf{Yeast one-hybrid assay}

The yeast one-hybrid assay was performed using the method described previously (Feng et al., 2018). The promoter fragments of \textit{DcUCGXT1} and \textit{DcSAT1} corresponding to the regions −1,349 to −1 and −1,662 to −1, respectively, relative to
the translation initiation site were cloned from gDNA of KRD and were then fused to HIS to generate Pro\textsubscript{DcUCGXT1}-HIS and Pro\textsubscript{DcSAT1}-HIS, respectively. These two constructs were separately co-transformed with pGADT7-DcMYB7 into Y1H Gold yeast strains. The pGADT7-T vector was used as a negative control. The interactions were detected on medium supplemented with 50 mM 3-AT.

**Dual LUC reporter assay of transient expression**

The CDS of *DcMYB7* was cloned into the pGreenII 62-SK vector to generate the DcMYB7-62SK effector. The promoters of *DcUCGXT1* (2,431-bp upstream of the putative start codon) and *DcSAT1* (2,568-bp upstream of the putative start codon) were introduced into the pGreenII 0800-LUC vector to generate the Pro\textsubscript{DcUCGXT1}:LUC and Pro\textsubscript{DcSAT1}:LUC reporter constructs, respectively. The constructs were transformed into *A. tumefaciens* strain GV3101 (pMP90). *A. tumefaciens* was mixed and co-infiltrated into *N. benthamiana* leaves for transient expression. The luminescence of firefly LUC was detected using a live imaging system (Tanon-5500Multi) according to the method described by Li et al. (Li et al., 2017). A Dual-Luciferase Reporter Assay System (Promega, USA, Cat.#E1910) was used to measure the ratio of luminescence of firefly LUC to *Renilla* LUC according to the manufacturer’s instructions.

**Accession numbers**
Sequence data from this article can be found in GenBank under the following accession numbers: *DcbHLH3* (MK572822), *DcMYB7* gDNA and cDNA sequence from DPP, CPP, KRD and SHLC (MK572814-572817 and MK572818-572821, respectively), *DcUCGXT1* (MK572822), *DcSAT1* (MK572823), promoter sequences of *DcUCGXT1* and *DcSAT1* (MK572825 and MK572826, respectively), and sequences upstream of *DcMYB7* from DPP, CPP and SHLC (MK637849–MK637851, respectively).

**Supplemental Data**

**Supplemental Figure S1.** Overexpression of *DcMYB7* in Arabidopsis plants.

**Supplemental Figure S2.** Phylogenetic relationships among DCAR_002739 (DcbHLH3), DCAR_004632 and bHLHs involved in anthocyanin biosynthesis in other plant species.

**Supplemental Figure S3.** Expression of DCAR_002739 (*DcbHLH3*) and DCAR_004632 in different root tissues of 11 purple and non-purple carrot cultivars.

**Supplemental Figure S4.** Region containing *DcMYB6*, *DcMYB8* and *DcMYB7* on chromosome 3 of the DH1 orange carrot genome.

**Supplemental Figure S5.** Alignment analysis of the predicted protein sequences of DcMYB7 and DcMYB8 from the carrot genome.

**Supplemental Figure S6.** Alignment analysis of the promoter sequences of *DcMYB7* from ‘Deep purple’ (3,000 bp), ‘Cosmic purple’ (2,775 bp), ‘Sanhongliucun’ (3,500 bp).
bp) and ‘Kurodagosun’ (3,000 bp).

Supplemental Table S1. List of primers used in this study.

678  Supplemental Figure S1. Overexpression of *DcMYB7* in Arabidopsis plants.

679  Arabidopsis plants transformed with the pCAMBIA 1301 vector were used as controls.

686  Supplemental Figure S2. Phylogenetic relationships among DCAR_002739 (DcbHLH3), DCAR_004632 and bHLHs involved in anthocyanin biosynthesis in other plant species. Accession numbers of proteins in GenBank are in parentheses. The numbers at nodes represent values from 1,000 bootstrap replicates, and the scale bar indicates number of substitutions per site.

681  Supplemental Figure S3. Expression of DCAR_002739 (*DcbHLH3*) and DCAR_004632 in different root tissues of 11 purple and non-purple carrot cultivars. Data are means of biological triplicate RT-qPCR reactions ± SDs. For cultivar abbreviations, see Figure 1.

682  Supplemental Figure S4. Region containing *DcMYB6* (green highlight), *DcMYB8* (red highlight) and *DcMYB7* (blue highlight) on chromosome 3 of the DH1 orange
carrot genome. The promoter region of \textit{DcMYB7} is interrupted by a non-functional
duplication of itself (gray background).

**Supplemental Figure S5.** Alignment analysis of the predicted protein sequences of
\textit{DcMYB7} and \textit{DcMYB8} from the carrot genome. Identical sequences are indicated by
the black background.

**Supplemental Figure S6.** Alignment analysis of the promoter sequences of \textit{DcMYB7}
from ‘Deep purple’ (3,000 bp), ‘Cosmic purple’ (2,775 bp), ‘Sanhongliucun’ (3,500
bp) and ‘Kurodagosun’ (3,000 bp). The poly (dA-dT) element is underlined with a
green wave pattern. The tandem repeat element is underlined with blue arrows, and
the Tc1/Mariner and hAT transposons are underlined in red.

**Supplemental Table S1.** List of primers used in this study.

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**Figure Legends**

**Figure 1.** Expression patterns of *DcMYB6* and *DcMYB7* in carrot roots. A, Four-month-old purple and non-purple carrots of the 11 cultivars used in this study. Cultivar abbreviations: DPP, ‘Deep purple’; PP68, ‘Purple 68’; TZ2H, ‘Tianzi2hao’; ZBC-S, ‘Zibacun’ with solid purple roots; ZBC-P, ‘Zibacun’ with purple peridermal roots; CPP, ‘Cosmic purple’; KRD, ‘Kurodagosun’; SHLC, ‘Sanhongliucun’; JCH, ‘Junchuanhong’; QTH, ‘Qitouhuang’; BY, ‘Baiyu’. Separate images are shown as a composite for comparison. B, Relative transcript levels of *DcMYB6* and *DcMYB7* in different root tissues of 11 purple and non-purple carrot cultivars. Data are means of biological triplicate RT-qPCR reactions ± SDs.

**Figure 2.** Functional detection of *DcMYB6* and *DcMYB7* in carrots. A and B, Overexpression of *DcMYB6* (A) and *DcMYB7* (B) in orange ‘Kurodagosun’ carrots. Separate images are shown as a composite for comparison. C, Total anthocyanin contents in roots of untransformed, 35S:*DcMYB6* and 35S:*DcMYB7* transgenic ‘Kurodagosun’ carrots. Values are means of three biological replicates with error bars representing ± SDs. D, Untransformed and *DcMYB7*-knockout ‘Deep purple’ carrots. Separate images are shown as a composite for comparison. E, Total anthocyanin contents in roots of untransformed and *DcMYB7*-knockout ‘Deep purple’ carrots. Data are means of three technical replicates ± SDs.
Figure 3. Role of *DcMYB7* in regulating *DcbHLH3* and structural genes in the anthocyanin pathway. A, Yeast two-hybrid assays validating the interaction of *DcMYB7* with *DcbHLH3*. B, Relative transcript levels of *DcbHLH3* and structural genes in roots of untransformed and 35S:*DcMYB7* transgenic ‘Kurodagosun’ carrots. Data represent means of biological triplicate RT-qPCR reactions ± SDs. C, Relative transcript levels of *DcbHLH3* and structural genes in roots of untransformed and *DcMYB7*-knockout ‘Deep purple’ carrot lines. Data are means of three technical replicates with error bars representing ± SDs.

Figure 4. Functional investigation of *DcMYB7* gDNA sequences from various carrot cultivars. A, PCR amplification of *DcMYB7* gDNA from 11 different carrot cultivars. B, Schematic representation of full-length *DcMYB7* from 11 different carrot cultivars having three exons (black boxes) and two introns (lines between exons). C, Overexpression of *DcMYB7* gDNA from different carrot cultivars in the orange ‘Kurodagosun’ carrot under the control of the CaMV 35S promoter. ‘Kurodagosun’ explants transformed with the pCAMBIA 1301 vector were used as control.

Figure 5. Functional investigation of the *DcMYB7* promoter from various carrot cultivars. A, Physical locations of *DcMYB6*, *DcMYB7* and *DcMYB8* on chromosome 3 of the DH1 orange carrot genome. B, PCR amplification of the sequence upstream of *DcMYB7* from 11 different carrot cultivars. C, Schematic representation of *DcMYB6*, *DcMYB7* and *DcMYB8* from 11 different carrot cultivars. D, Functional investigation
of the *DcMYB7* promoter from different carrots in ‘Kurodagosun’ and ‘Sanhongliucun’ carrots.

**Figure 6.** Anthocyanin modifications in carrots. A, Anthocyanin composition profiles from roots of ‘Deep purple’ carrots and 35S:*DcMYB7* transgenic ‘Kurodagosun’ carrots (line 1). B, Schematic of the proposed biosynthetic pathway of Cy3XSGG. UCGXT and SAT were identified in the present study.

**Figure 7.** Role of *DcMYB7* in regulating *DcUCGXT1* and *DcSAT1*. A, Relative transcript levels of *DcUCGXT1* and *DcSAT1* in roots of untransformed and 35S:*DcMYB7* transgenic ‘Kurodagosun’ (top), as well as untransformed and *DcMYB7*-knockout (bottom) ‘Deep purple’ carrot lines. Data are means of three biological (top) or technical (bottom) replicates with error bars representing ± SDs. B, Yeast one-hybrid assays showing that DcMYB7 binds to the promoter fragments of *DcUCGXT1* and *DcSAT1*. C, Transient expression assays showing that DcMYB7 promotes the expression of *DcUCGXT1* and *DcSAT1*. D, Promoter activities of *DcUCGXT1* and *DcSAT1* expressed as a ratio of firefly luciferase (LUC) to Renilla luciferase (REN) activity. Data are means of six replicate reactions with error bars representing ± SDs.

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biosynthesis in carrot (*Daucus carota* L.) using RNA-Seq. BMC Genomics 19: 811


composition of black carrot (*Daucus carota* ssp. *sativus* var. *atrorubens* Alef.)


DcMYB6, is involved in regulating anthocyanin biosynthesis in purple carrot taproots. Sci Rep 7: 45324


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Figure 7. Role of DcMYB7 in regulating DcUGX7T1 and DcSAT1. A, Relative transcript levels of DcUGX7T1 and DcSAT1 in roots of untransformed and 35S:DcMYB7 transgenic ‘Kurodagosun’ (top), as well as untransformed and DcMYB7-knockout (bottom) ‘Deep purple’ carrot lines. Data are means of three biological (top) or technical (bottom) replicates with error bars representing ± SDs. B, Yeast one-hybrid assays showing that DcMYB7 binds to the promoter fragments of DcUGX7T1 and DcSAT1. C, Transient expression assays showing that DcMYB7 promotes the expression of DcUGX7T1 and DcSAT1. D, Promoter activities of DcUGX7T1 and DcSAT1 expressed as a ratio of firefly luciferase (LUC) to Renilla luciferase (REN) activity. Data are means of six replicate reactions with error bars representing ± SDs.


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