Article

Differential hydroxylation efficiency of the two non-heme carotene hydroxylases: DcBCH1, rather than DcBCH2, plays a major role in carrot taproot

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Abstract

Carotene hydroxylase plays an important role in catalyzing the hydroxylation of carotene to xanthophylls, including two types: non-heme carotene hydroxylase (BCH type) and heme-containing cytochrome P450 hydroxylase (P450 type). Two BCH-encoding genes were annotated in the carrot genome. However, the role of BCHs and whether there are functional interactions between the duplicated BCHs in carrot remains unclear. In this study, two BCH encoding genes, DcBCH1 and DcBCH2, were cloned from carrot. The relative expression level of DcBCH1 was much higher than that of DcBCH2 in carrot taproots with different carotene accumulation levels. Overexpression of DcBCH1 in ‘KRD’ (high carotene accumulated) carrot changed the taproot color from orange to yellow, accompanied by substantial reductions in α-carotene and β-carotene. There was no obvious change in taproot color between transgenic ‘KRD’ carrot overexpressing DcBCH2 and control carrot. Simultaneously, the content of α-carotene in the taproot of DcBCH2-overexpressing carrot decreased, but the content of β-carotene did not change significantly in comparison with control carrot. Using the CRISPR/Cas9 system to knock out DcBCH1 in ‘KRD’ carrot lightened the taproot color from orange to pink-orange; the content of α-carotene in the taproot increased slightly, while the β-carotene content was still significantly decreased, compared with control carrot. In DcBCH1-knockout carrot, the transcript level of DcBCH2 was significantly increased. These results indicated that in carrot taproot, DcBCH1 played the main function of BCH enzyme, which could hydroxylate α-carotene and β-carotene; DcBCH1 and DcBCH2 had functional redundancy, and these two DcBCHs could partially compensate for each other.

Introduction

Carotenoids are lipid-soluble terpenoids composed of an isoprene skeleton, which exhibit bright colors including red, orange or yellow under visible light, due to the existence of multiple conjugated double bonds in their molecular structure [1–3]. As one of the important coloring substances, carotenoids endowed plants’ different organs such as flowers, fruits, seeds, roots, etc. with rich colors, and also play important roles in the process of plants resisting adversity [3–6]. In addition, carotenoids and their derivatives play a vital role in maintaining human health and delaying body aging [7, 8]. The most widely distributed carotenoids mainly include the hydrocarbon carotenes, such as lycopene, β-carotene, and α-carotene, and the xanthophylls, such as lutein, zeaxanthin, violaxanthin, neoxanthin, and capsanthin. In plants, the biosynthetic pathway of carotenoids has been extensively studied and well-characterized, and most of the enzymes and genes have been identified [3]. Carotene hydroxylases are key enzymes in the production of xanthophylls, which catalyze the hydroxylation of the β- and ε-ring at the 3’ positions of carotene (α-carotene and β-carotene) to produce lutein (α-carotene-derived xanthophyll) and zeaxanthin (β-carotene-derived xanthophyll); the latter can be further modified to produce other xanthophylls. There are two different types of carotene hydroxylases in plants: the non-heme di-iron oxygenase (BCH, also known as BHY, HYD, or HYb) and heme-containing cytochromes P450 oxygenase (CYP97A, CYP97B, and CYP97C) [9–12].

BCH mainly hydroxylates the β-ring of carotene and is widely found in organisms such as plants, bacteria, and cyanobacteria [13–15]. Phylogenetic analysis revealed that duplication events of the BCH genes occurred in higher plants after monocotylosedous and dicotylosedous splits [16]. Some studies have shown that the BCH encoded by the duplicated genes have the same function, mainly act on β-carotene, and also have a hydroxylation effect on α-carotene containing a β-ring [17, 18]. In the pulp of transgenic orange (Citrus sinensis) that silenced the expression of the β-carotene hydroxylase gene (Csβ-CHX), the β-carotene content was increased 36-fold compared to control plant [19]. In wheat (Triticum aestivum), specifically blocking the expression of the endogenous BCH in endosperm resulted in a 10.5-fold increase in endosperm β-carotene levels [20]. In Arabidopsis thaliana, the
proteins (β-hydroxylase 1 and β-hydroxylase 2) encoded by β-OHase 1 and β-OHase 2 can effectively catalyze the hydroxylation of the C3 position of carotene containing β-ring and had only weak activity on carotene containing ε-ring in vitro, while there were differences in the functional strength of the two proteins in vivo [14, 21]. Studies have also found that the functions of BCH encoded by duplicated genes in some species are not completely consistent. For example, BCH1 and BCH2 of maize (Zea mays) showed differences in enzymatic function in vitro [22]. In addition, BCH genes in some higher plants exhibited gene-specific expression patterns and contributed to different carotenoid levels in a tissue-specific manner [23]. In tomato (Solanum lycopersicum), the bch2 (CrtR-b2) mutant produced a colorless phenotype in petals, but had no effect on lutein biosynthesis in leaves [17].

Carrot (Daucus carota) is one of the top ten vegetable crops in the world; its taproot color is closely related to the composition and accumulation of carotenoids [25–27]. Orange carrot taproot accumulates large amounts of α-carotene and β-carotene [27]. There were two BCH encoding genes annotated in the carrot genome database [28]. The role of these two BCHs and what is the relationship between them, such as functional complementarity or redundancy, in carrot remains unclear. Here, we isolated the two BCH encoding genes, DcBCH1 and DcBCH2, from carrot. The relative expression level of DcBCH1 was much higher than DcBCH2 in the taproot of two carrot cultivars with different carotenoid accumulation. We overexpressed DcBCH1 and DcBCH2 in ‘KRD’ (high carotene accumulation) and investigated carotene content in transgenic carrot taproot, and further knocked out DcBCH1 in ‘KRD’ to evaluate the hydroxylation functions of carotene by these two BCH enzymes (DcBCH1 and DcBCH2) and its relationship.

Results
Isolation and analysis of DcBCH1 and DcBCH2
Based on the carrot genome database, a total of two BCH genes (DcBCH1 and DcBCH2) were annotated. DcBCH1 and DcBCH2 were located on chromosomes 6 and 4 in carrot, respectively, with the same structure both containing seven exons (Fig. 1a and b). The full-length open reading frames (ORFs) of DcBCH1 and DcBCH2 isolated from ‘KRD’ were 930 bp and 912 bp, encoding 309 and 303 amino acids, respectively (Figs S1 and 2, see online supplementary material). DcBCH1 and DcBCH2 from ‘KRD’ and the genome database differ by 7 and 19 sites different at the amino acid level, respectively. In addition, the predicted molecular weights and isoelectric points of DcBCH1 and DcBCH2 proteins from ‘KRD’ were 34.2 kDa and 33.89 kDa, 9.04 and 8.66, respectively.

The similarity between DcBCH1 and DcBCH2 protein sequences was 65.61% (Fig. 1c). Further sequence multiple alignment of DcBCH1 and DcBCH2 with BCHs from other plant species found that DcBCH1 and DcBCH2 contained four conserved histidine domains: ‘HX8H’ (HE/KALWH), ‘HX6H’ (HESHHH), ‘HX4H’ (HDGLHVH), and ‘HX4H’ (HQLHH) to ensure the catalytic activity of carotene hydroxylase (Fig. 1c; Fig. S3, see online supplementary material). Evolutionary analysis results showed that DcBCH1 and DcBCH2 were most closely related to BCH1 from Apium graveolens (AgBCH1) (Fig. S4, see online supplementary material).

Subcellular localization of DcBCH1 and DcBCH2
To analyse the subcellular localization of DcBCH1 and DcBCH2, the recombinant vectors DcBCH1-EGFP and DcBCH2-EGFP were introduced individually into tobacco mesophyll cells. The results showed that the green fluorescence signals of DcBCH1-EGFP and DcBCH2-EGFP were distributed in the chloroplast and overlapped with the red fluorescence signal of the chloroplast, indicating that both DcBCH1 and DcBCH2 were localized in the chloroplast (Fig. 2).

Expression analysis of DcBCH1 and DcBCH2 in carrot taproot
The contents of α-carotene, β-carotene and lutein in the taproot of two carrot cultivars with different taproot colors were determined. As shown in Fig. 3a and b, a large amount of α-carotene and β-carotene accumulated in the taproot of the orange carrot (‘KRD’), while the taproot of ‘BY’ was pale yellow and nearly white, only a small amount of lutein was detected.

RT-qPCR analysis showed that the expression level of DcBCH1 in the taproot of ‘KRD’ and ‘BY’ was significantly higher than that of DcBCH2. In addition, the expression level of DcBCH1 in ‘KRD’ was significantly higher than that in ‘BY’. DcBCH2 expression level was not significantly different between ‘KRD’ and ‘BY’ (Fig. 3c). Among other 14 carotenoid metabolism-related genes, DcGGPS1, DcPSY1, DcPSY2, DcPDS, DcZDS1, DcCRTISO, DcLCYE, DcZEP, and DcCCD1a showed a lower transcript level in ‘BY’ than that in ‘KRD’, while the transcript level of DcLYC, DcCCD4, and DcCED3 in ‘BY’ was higher than that in ‘KRD’ (Fig. 3d).

Generation of transgenic carrot plants overexpressing DcBCH1 and DcBCH2
Transgenic carrots overexpressing DcBCH1 and DcBCH2 were generated to investigate the effect of up-regulation of DcBCH1 and DcBCH2 expression on the accumulation of carotenoids in carrot taproot, and differences in function between DcBCH1 and DcBCH2. After verification by PCR amplification (Figs 4b and 5b), transgenic carrot lines overexpressing DcBCH1 (BCH1–2, BCH1–8, and BCH1–9) and DcBCH2 (BCH2–1 and BCH2–5) were selected to further analysis. Transcript level assessment determined by RT-qPCR revealed that the expression level of DcBCH1 was the highest in the BCH1–8, and the BCH2–5 had the highest transcript level of DcBCH2 (Figs 4c and 5c).

Effects of DcBCH overexpression on carotenoids content and expression profiles of metabolism-related genes in carrot taproot
The taproot phenotype of three DcBCH1-overexpressing transgenic carrot lines (BCH1–2, BCH1–8, and BCH1–9) and control carrot (KRD) were observed. As shown in Fig. 4d, there was a clear difference in the taproot color between the transgenic and control carrots, the taproot of transgenic carrot hosting DcBCH1 gene was yellow, while the control carrot was orange.

The accumulation of carotenoids in the taproot of the above carrot materials was further determined. Compared with control carrot (KRD), the content of α-carotene in transgenic carrot taproot was reduced by 65%–70% (BCH1–9 and BCH1–2), and the accumulation of α-carotene was not detected in BCH1–8 (Fig. 4e). In the taproot of BCH1–2 and BCH1–8, the accumulation of β-carotene was not detected, and the β-carotene content in BCH1–9 was significantly lower than that in control carrot (Fig. 4f). In addition, the lutein content in transgenic carrot taproot was slightly higher than that in the control carrot (Fig. 4g).
To further analyze the changes in carotenoid accumulation, the relative expression levels of carotenoid metabolism-related genes in the taproot of control carrot (KRD) and DcBCH1-overexpressing transgenic carrot were measured. As shown in Fig. 4h, in transgenic carrot (BCH1–8 and BCH1–9), the relative expression levels of DcGGPS1, DcPSY1, DcCRTISO, and DcCCD4 were increased to different degrees compared with control carrot. The expression levels of DcPDS and DcECH were similar between control carrot and transgenic carrot. Compared with the control carrot, the relative expression levels of DcPSY2, DcLCYB, and DcZEP were increased in the three transgenic carrot lines (BCH1–2, BCH1–8, and BCH1–9), and the expression level of DcZEP in three transgenic carrot lines was 3–5.4 times higher than that in the control carrot. On the contrary, the expression levels of DcLCYE, DcCYP97A3, and DcNCED3 in three transgenic carrot lines were reduced in comparison with control carrot. In addition, in BCH1–8 and BCH1–9, the expression level of DcBCH2 was about 1.6 times higher than that in control carrot, while the expression level of DcBCH2 in BCH1–2 decreased by 87%, compared with control carrot.

**Effects of DcBCH2 overexpression on carotenoids content and expression profiles of metabolism-related genes in carrot taproot**

There was no obvious difference in taproot color between DcBCH2-overexpressing transgenic carrot (BCH2–1 and BCH2–5) and control carrot (KRD) (Fig. 5d). The content of α-carotene in taproot of the transgenic carrot was significantly lower than that of control carrot, decreasing by 37% (BCH2–1) and 31% (BCH2–5), respectively (Fig. 5e). Compared with the control carrot, the content of β-carotene in BCH2–5 was reduced by 17%, while the content of β-carotene in BCH2–1 was not significantly changed (Fig. 5f). In addition, lutein content in taproot between transgenic and control carrot was no significant difference (Fig. 5g).

Further analysis of the relative expression levels of carotenoid metabolism-related genes in the taproot of transgenic and control carrot (KRD). As shown in Fig. 5h, among the 15 genes detected (DcGGPS1, DcPSY1, DcPSY2, DcPDS, DcZDS1, DcCRTISO, DcLCYE, DcLCYB, DcECH, DcBCH1, DcCYP97A3, DcCCD1a, DcCCD4, DcZEP, and DcNCED3), except for DcBCH1 and DcGGPS1, the other 13 genes expression levels in transgenic carrot taproot were significantly higher than those in control carrot. The relative expression levels of DcLCYB and DcECH in BCH2–1 and BCH2–5 were 4.6- and 7.6-fold, 7.8- and 5-fold higher than those in control carrot, respectively. Compared with the control carrot, the relative expression level of DcCYP97A3 was increased by 1.7-fold in BCH2–1, while the expression in BCH2–5 was not significantly different from that in control carrot. There was no significant difference in the relative expression level of DcECH in BCH2–1 and control carrot, while in BCH2–5, the expression level of DcBCH1 was 1.6-fold higher than that in control carrot.

**Generation of DcBCH1 knockout mutant carrot by CRISPR/Cas9**

It was observed that overexpression of DcBCH1 significantly reduced carotene accumulation in transgenic carrot taproot and changed the color of taproot. Therefore, we further utilized the CRISPR/Cas9-mediated genome editing system to knock out DcBCH1 in ‘KRD’. Based on the sequence of DcBCH1, four target sites (T1, T2, T3, and T4) located on the first, second, fourth, and fifth exons were selected (Fig. 6a). The four target site sgRNAs driven by the promoters AtU3b, AtU3d, AtU6–1, and AtU6–29, respectively, were assembled and inserted into the pYLCRISPR/Cas9Pubi-H vector to obtain a knockout expression vector (Fig. 6b). Genetic transformation of carrot was carried out using Agrobacterium-mediated method. The fragments containing the target site were amplified from the obtained resistant plant gDNA by PCR using specific primers, and then sequenced directly...
to detect the mutation of the target site. The direct sequencing results showed that the target site 2 (targeted by AtU3d-driven sgRNA) was mutated in the obtained transgenic resistant plant, and there were no changes in the other three target sites (target sites 1, 3, and 4). Further analysis showed that biallelic mutation occurred in target site 2 (Fig. 6c).

**Effects of DcBCH1 knockout on carotenoids content and expression profiles of metabolism-related genes in carrot taproot**

The taproot phenotypes of two plants of DcBCH1 mutant carrot (DcBCH1-Knockout-1 and DcBCH1-Knockout-2) were observed. As shown in Fig. 6d, the taproot color of DcBCH1-Knockout carrot became lighter, showing a pink-orange phenotype. Carotenoids content determination results showed that the content of \( \alpha \)-carotene in DcBCH1-Knockout carrot taproot was slightly higher than that of control carrot (Fig. 6e). In DcBCH1-Knockout carrot taproot, the content of \( \beta \)-carotene was significantly reduced, which was about 45% lower than that of control carrot (Fig. 6f). In addition, the lutein content of DcBCH1-Knockout and control carrot was not significantly different or slightly lower than that of control carrot (Fig. 6g).

The expression level of DcBCH2 in DcBCH1-Knockout carrot was 5.5-fold (DcBCH1-Knockout-1) and 6.6-fold (DcBCH1-Knockout-2) than that in control carrot (KRD), respectively. Compared with the control carrot, the expression levels of DcPSY1, DcPSY2, DcPDS, DcZDS1, DcCRTISO, DcLCYE, DcLCYB, DcECH, DcZEP, and DcCCD1a in DcBCH1-Knockout carrot were all up-regulated to varying degrees. DcNCED3 transcript level in DcBCH1-Knockout carrot was 4 ∼ 13.5-fold higher than that in control carrot. In addition, the expression of DcCYP97A3 in DcBCH1-Knockout carrot was reduced by 51%–70% in comparison with the control carrot (Fig. 6h).

**Discussion**

Most of higher plants contain two or more BCH (BHY) with high sequence similarity [17, 29]. In *A. thaliana*, two BCH encoding genes, \( \beta \)-OHase 1 and \( \beta \)-OHase 2, were identified with 70% sequence similarity, and located on chromosomes 4 and 5, respectively [21]. In the maize genome, two functional BCH genes, Zmbch1 and Zmbch2 (located on chromosomes 2 and 10, respectively, with protein similarity of 76.6%), two pseudogenes of BCH, and more than two BCH homologous genes with unknown functions were annotated [22, 30]. Carrot, a biennial herb of the genus *Daucus* in the Apiaceae family, is an important root vegetable crop [31]. With the development of high-throughput sequencing technology, more and more genome sequences of some important plants belonging to the Apiaceae family have been completed [28, 32–34]. A total of two BCH encoding genes were annotated in the carrot genome, located on chromosomes.
4 and 6, respectively [28]. We cloned these two BCH encoding genes from carrot (‘KRD’) and named them as DcBCH1 and DcBCH2. The protein sequence similarity between DcBCH1 and DcBCH2 was 65.61%. Studies have confirmed that BCH proteins from different species contained the characteristic motifs, four conserved histidine domains (‘HX4H’, ‘HX3HH’, ‘HX4H’, and ‘HX2HH’) to ensure the normal activity of BCH [35, 36]. Sequence alignment results showed that both DcBCH1 and DcBCH2 contained four complete conserved histidine domains in the amino acid sequence.

In plants, the presence of isozymes often reflects the need for the same catalytic action in different subcellular compartments [37]. Plastids are the main site for carotenoid biosynthesis and storage in plants. Subcellular localization analysis confirmed that BCH from other species was localized in plastid (chloroplast) [38]. Our study showed that DcBCH1 and DcBCH2 were also located in the plastid (chloroplast). These results indicated that DcBCH1 and DcBCH2 had normal hydroxylase activity and played catalytic roles in the same subcellular location. We further detected the expression of DcBCH1 and DcBCH2 to preliminarily analyse whether there was a functional difference between DcBCH1 and DcBCH2. This is similar to previous research that the orange carrot taproots mainly accumulated a lot of \( \alpha \)-carotene and \( \beta \)-carotene, and the nearly white carrot only accumulated little lutein or almost no carotenoids [25, 26, 39]. Gene expression is affected by many factors, such as materials, planting management, growth and development, environmental factors, etc. [18, 38, 40]. RT-qPCR analysis showed that many carotenoid metabolism-related genes expression levels in the orange carrot taproot were higher than those in the nearly white carrot taproot; both DcBCH1 and DcBCH2 were expressed in the orange and nearly white carrot taproots, and the relative expression level of DcBCH1 was much higher than that of DcBCH2. These results indicated that DcBCH1 may play a major role in carrot taproot.

Overexpression of DcBCH1 in carrot with high carotene (\( \alpha \)-carotene and \( \beta \)-carotene) accumulation changed the color of the transgenic carrot taproot from orange (the phenotype of the control carrot) to yellow. Corresponding to the phenotypic results, the contents of \( \alpha \)-carotene and \( \beta \)-carotene in transgenic carrot taproot were greatly reduced compared with those of the control carrot, especially the content of \( \beta \)-carotene. In transgenic tomato fruits hosting kiwifruit AcBCH1, the \( \beta \)-carotene content significantly decreased, in AcBCH1 overexpressing transgenic kiwifruit leaves, the \( \beta \)-carotene content decreased and zeaxanthin content increased [41]. Overexpression of chyB in A. thaliana increased the contents of total carotenoids and xanthophyll such
as zeaxanthin and violaxanthin, and decreased β-carotene content [42]. In DcBCH1-overexpressing transgenic carrot taproot, the lutein (α-carotene-derived xanthophyll) content was increased. These findings implied that the β-carotene-derived xanthophylls content may also be increased, and the decrease of α-carotene and β-carotene content was mainly due to the production of DcBCH1 hydroxylation products (α- and β-carotene-derived xanthophylls). There was no significant difference in taproot color between DcBCH2-overexpressing transgenic and control carrot. In two DcBCH2-overexpressing transgenic carrot lines taproot, the α-carotene content was significantly lower than that of control carrot; the content of β-carotene in BCH2–1 taproot did not change significantly, while in BCH2–5 taproot was decreased, compared to control carrot. In A. thaliana, β-OHase 1 and β-OHase 2 were expressed in all experiment tissues, and the β-OHase 1 transcript level was 10–50 times higher than that of β-OHase 2. Both β-hydroxylase 1 (β-OHase 1) and β-hydroxylase 2 (β-OHase 2) were confirmed to effectively hydroxylate the β-ring, and had
poor hydroxylation ability to substrates containing the ε-ring in vitro [21]. MpBHY can hydroxylate the β-ring of β-carotene and α-carotene, and promote the biosynthesis of zeaxanthin and lutein [43]. In Escherichia coli BL21 (DE3), CitHYb from sweet orange could catalyze the hydroxylation of the β-ring of β-carotene and α-carotene [18]. These results indicated that both DcBCH1 and DcBCH2 have the activity of hydroxylating β-ring in vivo.

During maize endosperm development, the expression profiles of Zmbch1 and Zmbch2 were consistent, but in in vitro enzyme activity experiments, ZmBCH1 could hydroxylate β-carotene into β-cryptoxanthin and zeaxanthin, while ZmBCH2 could only hydroxylate β-carotene to β-cryptoxanthin alone, and the overall activity of ZmBCH2 was lower than that of ZmBCH1 [22]. In three DcBCH1-overexpressing transgenic carrot lines, the DcBCH2 transcript level in BCH1–2 was significantly lower, while in the other two transgenic lines (BCH1–8 and BCH1–9) was significantly higher in comparison with the control carrot. The expression level of DcBCH2 in BCH1–8 (with the highest DcBCH1 transcript level)
Figure 6. Effects of DcBCH1 knockout on carotenoid accumulation in carrot taproot. (a) The positions of the four target sites on the DcBCH1 gene structure. (b) Structure diagram of the assembly of four sgRNA expression cassettes. (c) Sequence peak map of successful DcBCH1 editing sites in DcBCH1-Knockout carrot. (d) Taproot phenotype of control carrot (KRD) and DcBCH1-Knockout mutant carrot, long scale bar = 2 cm, short scale bar = 1 cm. (e-g) α-carotene (e), β-carotene (f) and lutein (g) content in taproot of control carrot (KRD) and DcBCH1-Knockout mutant carrot. (h) Relative expression levels of carotenoid metabolism related genes in the taproot of control carrot (KRD) and DcBCH1-Knockout mutant carrot. Bars represent mean standard deviation (SD). The statistical significance of the measurements between DcBCH1-Knockout carrot and the control carrot (KRD) was determined using Student’s t test (*P < 0.05; **P < 0.01; ***P < 0.001).

was the highest. Correspondingly, only lutein accumulation was detected in BCH1–8 taproot, while α-carotene and β-carotene were not detected. In BCH1–9 (with the lowest DcBCH1 transcript level), the DcBCH2 expression level was slightly lower than BCH1–8; the accumulation of α-carotene and β-carotene was still detected in its taproot. In BCH1–2 (the DcBCH1 transcript level was slightly lower than BCH1–8) taproot, the DcBCH2 expression level was only 13% of control carrot, the accumulation of β-carotene was not detected; while the content of α-carotene was slightly lower than BCH1–9. Lycopene ε-cyclase (LCYE) and
lycopene β-cyclase (LCYB) work together on all-trans lycopene to produce α-carotene and β-carotene, respectively. Silencing the expression of LCYB in sweet potato (*Ipomoea batatas*) by RNAi reduced the content of α-carotene and increased the content of β-carotene in storage roots [6]. The β-carotene content in the mature fruits of transgenic tomato hosting the tobacco LCYB was increased by five times in comparison with control plants, and exhibited an orange pigmentation phenotype [44]. Compared with control carrot, the expression level of DCLECY was increased, while the expression level of DCLECY was decreased to varying degrees, in the taproot of DCBCH1-overexpressing transgenic carrot, suggesting that overexpression of DCBCH1 partially biased the carotenoid flux towards the β-carotene biosynthesis branch. In the taproot of DCBCH2-overexpressing transgenic carrot, the transcription levels of DCLECY and DCLECY were higher than those in control carrot. Among them, the transcription level of DCLECY was higher in BCH2-5, which had lower DBCH2 transcription level and higher DCBCH1 transcription level. Based on the above results, we speculated that when DCBCH1 and DBCH2 coexisted, DCBCH1 played a role in hydroxylation of both α-carotene and β-carotene, and was more inclined to the hydroxylation of β-carotene, while DBCH2 may play a complementary role to the function of DCBCH1, possibly more inclined to the hydroxylation of α-carotene.

It was observed that overexpression of DBCH1 had a greater effect on the accumulation of carotenoids in carrot taproot than overexpression of DBCH2. We further obtained DBCH1 knockout mutant carrot, and found that the α-carotene content in the DBCH1-knockout carrot taproot was slight higher, while the β-carotene content was significantly lower in comparison with the control carrot. In potato (*Solanum tuberosum*) tuber with both CHY1 and CHY2 silenced, the contents of β-carotene and total carotenoids increased, zeaxanthin content decreased; the expression of *LusT* that encodes a β-ring hydroxylase was suppressed [45]. The contents of β-carotene-dried xanthophylls, β-cryptoxanthin and zeaxanthin, were increased in *ib*CHY-β silenced sweet potato calli, storage roots, and leaves, possibly due to the up-regulation of the *IhP450* expression level, which is also known to have β-carotene hydroxylase activity, compensated for low level of *ib*CHY-β [46, 47]. In *A. thaliana*, the b1 (β-OHase 1) mutant had a greater effect on carotenoid composition than b2 (β-OHase 2), compared with the wild type plants; b2 could compensate for the loss of b1 enzymatic activity in b1 mutant to a significant extent, and could partially compensate for the loss of b1 enzymatic activity in lut1b1 double mutant [9]. The DBCH2 expression level in DBCH1-knockout carrot was increased to varying degrees. CYP97A3, a hydroxylase belonged to cytochrome P450 class, hydroxylated both α-carotene and β-carotene, and had the main activity to α-carotene β-ring in *A. thaliana* [9]. A previous study found that the accumulation of α-carotene in orange carrot was caused by the loss of normal activity of CYP97A3 due to a frame shift insertion of CYP97A3 [48]. In DBCH1 overexpression and knockout carrots, the expression level of *DcCYP97A3* was significantly decreased compared with control carrots; whereas the *DcCYP97A3* in DBCH2-overexpressing carrot was increased. Zeaxanthin epoxidase (ZEP) catalyzes zeaxanthin to antheraxanthin, which in turn generates violaxanthin [49]. Carotenoid cleavage dioxygenases (CCDs), including CCD and NCED, are the main enzymes in the enzymatic degradation of carotenoids, and carotenoid-derived cleavage products play important roles in plant developmental processes and response to abiotic stress [3, 50]. Compared with control carrots, DCZEP expression levels were significantly increased in both DBCH1 overexpression and knockout carrots, while the *DcNCED3* expression level was lower in DBCH1 overexpression carrot, higher in DBCH1 knockout carrot. Based on these results, it can be speculated that in DBCH1 knockout carrot taproot, DBCH2 (increased expression level of DBCH2) partially supplements the loss of the enzymatic function of DBCH1, so that β-carotene was processed by hydroxylation and epoxidation reactions produce β-carotene-derived xanthophylls, which was cleaved under the action of CCDs, leading to the β-carotene content decreasing. *DcCCD1* (*DcCCD1*α in this study) has been shown to cleave δ-carotene and β-carotene in vitro [51]. *DcCCD1*α transcript level was higher in the taproot of the DBCH1 knockout carrot than in the control carrot. The cleavage of β-carotene by *DcCCD1*α may also contribute to the lower β-carotene content in the DBCH1 knockout carrot taproot.

The activity of enzymes related to carotenoid metabolism is one of the important factors affecting carotenoid accumulation in plants. Expression of endogenous carotenogenic genes is often altered by changing the levels of biosynthetic intermediates in this pathway [6, 41]. In CHY1 and CHY2 simultaneously silenced potato tuber that contained increased β-carotene and total carotenoids contents, and decreased zeaxanthin content, the expression levels of carotenoid metabolism-related genes (including *PSY*, *ZDS*, *CRTISO*, *LCYe*, *LUT1*, *LCy-b*, and ZEP) were up-regulated [45]. In *ib*CHY-β-silenced sweet potato storage root, total carotenoids and β-carotene contents were increased by 2-fold and 16-fold, respectively; the expression levels of *PSY*, *FDS*, *ZDS*, *ZEP*, and *NCED* were lower than those in non-transgenic plant [46]. In transgenic tomato fruits hosting kiwifruit acbCh, the β-carotene and lycopene contents were reduced, lutein content was increased; most genes of carotenoid metabolic pathway (*PSY*, *FDS*, *ZDS*, *CRTISO*, *LCYb*, *ECH*, *BCH*, *ZEP*, *CCD*, and *NCED*) were expressed at lower levels than control plants [41]. Compared with the respective control carrot, the expression levels of carotenoid metabolic pathway earlier enzymes encoding genes (*DcGGPS1*, *DcPSY1*, *DcPDS*, *DcZDS1*, and *DcCRTISO*) were slightly increased or not significantly changed in the taproot of DBCH1-overexpressing carrot, while the increase was greater in DBCH2-overexpressing and DBCH1-knockout carrots. The increase in one carotenoid came at the expense of others [19, 52]. PSY is considered to be the key rate-limiting enzyme in the carotenoid metabolic pathway [53]. Studies have confirmed that induced changes in the abundance of carotenoid metabolites or their downstream products negatively feed back and regulate the transcriptional or protein levels of *PSY* [48, 54]. Based on these results, it can be speculated that overexpression of DBCH1 may tend to change the proportion of different types of carotenoids in carrot taproot, so it has little effect on the transcription levels of early enzyme-encoding genes in the carotenoid metabolic pathway. In DBCH1 knockout carrot taproot, the downstream metabolites of β-carotene were degraded by CCDs, thereby negatively regulating the rate-limiting step of the carotenoid metabolic pathway. When the upstream biosynthesis rate increases, it triggers the increase of expression levels of genes encoding other related enzymes in the metabolic pathway. Alteration of carotenoid accumulation by genetic engineering is also influenced by the level of carotenoid accumulation in the species itself [52, 55]. This may be one of the reasons why the changes of carotenogenic genes in different types of transgenic carrot taproots obtained in this study were different from those in transgenic materials of other species. Even though zeaxanthin content was increased in transgenic tobacco hosting *A. thaliana chyB*, this increase was not unlimited [56]. Carotene ε-cyclase (ECH/CYP97C1) interacts with BCH on α-carotene...
and catalyzes the biosynthesis of lutein [16]. The expression level of DcBCH in DcBCH1-overexpressing carrot taproot was not significantly changed, whereas in DcBCH2-overexpressing carrot taproot was significantly higher, compared with control carrots. In DcBCH1/DcBCH2-overexpressing carrots, although α-carotene was reduced, the direct hydroxylated product lutein was not greatly increased (DcBCH1-overexpressing) or did not change significantly (DcBCH2-overexpressing), accompanied by the expression levels of CCDS (DcCCD4, DcCCD1a, and or DcNEDD3) being significantly elevated in these two types of transgenic carrot plants. These results implied that the increased expression levels of these genes may maintain the stability of carotenoid metabolism by enhancing the cleavage of carotenoids. Previous studies have suggested that DcCCD1 may compete with β-carotene hydroxylase for β-carotene substrate in vitro experiment [51]. Our previous results showed that DcCCD4 could cleave α-carotene and β-carotene [57]. It suggested there may be partly a degree of substrate competition between DcBCH and DcCCD, which may also be one of the factors affecting the changes of DcCCD transcript levels in different types of transgenic carrot taproots. In DcBCH2-overexpressing carrot, the transcription levels of DcCCD1a and DcCCD4 were significantly increased, especially DcCCD4, speculating that the deepening of the competition relationship may cause changes in the levels of intermediate metabolites and thus lead to changes in the transcription levels of other carotenogenic genes. Due to the complexity of interactions and regulation between enzymes in the carotenoid metabolic pathway, further studies are needed to elucidate the regulation of carotenoid metabolic pathway by intermediate metabolites.

In summary, our results indicated that both DcBCH1 and DcBCH2 have hydroxylation effects on β-carotene (α-carotene and β-carotene). DcBCH1 and DcBCH2 have functional redundancy. The enzymatic activity of BCH in carrot taproot mainly depended on the function of enzyme encoded by DcBCH1 rather than DcBCH2, and these two DcBCHs can partially compensate for each other. The mutual compensation degree between DcBCH1 and DcBCH2 needs further analysis. Our results will provide a certain reference for further analysis of the metabolic mechanism of carotenoids and generation of materials with different types of carotenoids through genetic engineering.

### Materials and methods

#### Plant materials and growth conditions

The carrot ‘Baiyu’ (‘BY’, nearly white) and ‘Kurodagosun’ (‘KRD’, orange) with different taproot color were used as experimental materials. Seeds of carrot were placed on moist filter paper to germinate. Then, the germinated seeds were transferred to flower pots and grown in a glass intelligent greenhouse of the State Key Laboratory of Crop Genetics and Germplasm Enhancement of Nanjing Agricultural University. The growth conditions of the glass intelligent greenhouse were set as 12 h light/12 h dark at 28°C. Carrot taproot was sampled at 85 d after sowing, and the samples were frozen and stored at −80°C immediately.

#### Total RNA, genomic DNA (gDNA) extraction and gene cloning

We extracted total RNA and synthesized cDNA according to the previously described method [58]. gDNA was extracted from the samples using a DNAsecure plant kit (Tiangen, Beijing, China). The annotated BCH encoding DNA sequences (DcBCH1 DCAR_020269, DcBCH2 DCAR_014519) were searched in the published carrot genome [28, 59] and specific primers were designed (Table S1, see online supplementary material) to specifically amplify DcBCH1 and DcBCH2 from the cDNA of ‘KRD’. The full-length cDNA of DcBCH1 cloned form ‘KRD’ was described in our previous study [58]. The full-length cDNA of DcBCH2 was amplified by RT-PCR from carrot ‘KRD’ with the specific primers (Table S1, see online supplementary material). RT-PCR program parameters were 98°C for 3 min, 34 cycles (98°C for 10 s, 55°C for 30 s, 72°C for 15 s) and 72°C for 10 min. The amplification product was analysed by gel agarose electrophoresis and subsequently sequenced in Genscript (Nanjing, China).

#### Bioinformatic analysis

The conserved domains were analysed using the NCBI database (http://www.ncbi.nlm.nih.gov) and the amino acid sequences of BCHs from other species were also downloaded from the NCBI database. DNAMAN 6.0 software was used to perform multiple sequence alignment of the BCHs amino acid sequences. The phylogenetic tree was generated from the amino acid sequences of BCH proteins by MEGA 5.0 using the neighbor-joining method [60, 61]. The chromosomal loci and the locations of exons and introns were defined using MapChart software and Gene structure display server tool (GSDS, http://gsds.cbi.pku.edu.cn), respectively, based on the carrot genomic database [62, 63].

#### Subcellular localization analysis

The ORF of DcBCH1 and DcBCH2 without stop codon was PCR-amplified using specific primers (Table S1, see online supplementary material) from ‘KRD’ and fused with the enhanced green fluorescent protein (EGFP) gene in pSPYE vector to construct plasmid DcBCH1-EGFP and DcBCH2-EGFP, respectively. The DcBCH1-EGFP and DcBCH2-EGFP construct plasmids were introduced into Agrobacterium tumefaciens (strain GV3101) by electrotransformation, and transformed into tobacco (Nicotiana benthamiana) leaf cells using Agrobacterium infection method described previously [64]. After 5 d of Agrobacterium infection, the fluorescence signal of EGFP fusion proteins and red chloroplast autofluorescence were observed and imaged using an LSM780 confocal microscopy (Zeiss LSM 780, Oberkochen, Germany).

#### Overexpression vector construction and plant transformation

For the generation of DcBCH1 and DcBCH2 overexpression carrots, the full length of the DcBCH2 was PCR amplified from ‘KRD’ using a pair of specific primers (Table S1, see online supplementary material) and cloned into the pCAMBIA1301 vector to yield the recombinant plasmid 35S:DcBCH2. The 35S:DcBCH1 recombinant plasmid used was the same as in our previously study [58]. After the recombinant plasmids were verified by sequencing, they were introduced into A. tumefaciens (strain GV3101) by electroporation. The 35S:DcBCH1 and 35S:DcBCH2 constructs were transformed into orange carrot (‘KRD’) to generate stable transgenic carrots using Agrobacterium-mediated method, respectively [65]. Transgenic carrot was detected by PCR amplification and RT-qPCR analysis.

#### Generation of CRISPR/Cas9-mediated DcBCH1 knockout mutant carrot

Based on the published carrot genome database, the gDNA sequence of DcBCH1 was obtained. Four target sequences in the DcBCH1 were selected using the online tool CRISPR-GE (http://skl.scau.edu.cn/targetdesign/) for designing the sgRNA sequences. The four sgRNA expression cassettes containing four target sites driven by AtU3b, AtU3d, AtU6–1, and AtU6–29
promoter, respectively, were assembled and inserted into the corresponding site of the pYLCLRISPR/Cas9Pubi-H binary vector to obtain the expression vector DcBCH1-4xsgRNA/Cas9 according to the previously described method [65, 66]. The vector (DcBCH1-4xsgRNA/Cas9) was transferred into *A. tumefaciens* (strain GV3101) by electroporation. DcBCH1 knockout mutant carrot was generated by the *Agrobacterium*-mediated method as previously described [65]. The gDNA was extracted from obtained transgenic resistant carrot plants and amplified the target fragment containing the target site using specific primers (Table S1, see online supplementary material) by RT-PCR, and then sequenced in Genscript (Nanjing, China) to detect mutations. Mutations that produced superimposed sequencing chromatograms were decoded using the online tool DSDecode [67].

**Carotenoid contents measurements**

The samples were grated with liquid nitrogen and lyophilized in a vacuum dryer (Christ, Osterode, Germany). Approximately 50 mg of freeze-dried samples were extracted with 2 mL acetone, repeating this step until the samples were colorless and combined the extracts. The carotenoids extracts were detected using a Thermo UltiMate UHPLC system as in the previously described method [58]. Carotenoid species (α-carotene, β-carotene, and lutein) were identified in the extracts by comparison of retention times with standard samples. All data are quantified according to their respective standard curves. The specific carotenoid (α-carotene, β-carotene, and lutein) content was expressed as μg/g of dry weight (μg/g DW).

**Reverse transcription quantitative real-time PCR (RT-qPCR) analysis**

RT-qPCR analysis was performed using SYBR Premix Ex Taq kit (TaKaRa, Dalian, China) as described before [64]. The primers used for gene expression analysis were referenced from previous study or designed using the Primer Premier 6.0 software (Table S1, see online supplementary material) [26]. The data were normalized to the expression of the *DcActin* and calculated according to the 2^−ΔΔCT method [68, 69].

**Statistical analysis**

The data were analysed using SPSS 20.0 software. The significant differences in gene expression and physiological parameter data between control carrot and transgenic carrot were analysed using the one-way analysis of variance (ANOVA) with Student’s *t*-test at the significance levels of *P* < 0.05 (*), *P* < 0.01 (**), and *P* < 0.001 (***) The significant differences in DcBCH1 and DcBCH2 expression between two carrot cultivars were compared using Tukey’s multiple range test (*P* < 0.05).

**Accession numbers**

Sequence data from this article can be found in the NCBI database (http://www.ncbi.nlm.nih.gov) under the following accession numbers: *Fistacia vera* PxBCH2 (XP_031249306.1), *Petunia hybrid* PhBCH1 (Q836226.1), *Ricinus communis* RxBCH2 (XP_002513654.1), *Prunus dulcis* PdBCH1 (BBG98323.1), *Vigna angularis* VaBCH2 (XP_017413495.1), *Benincasa hispida* BxBCH1 (XP_039807152.1), *Carica papaya* CpBCH2 (XP_021892139.1), *Herrania umbritica* HuBCH1 (XP_021285832.1), *Apium graveolens* AgBCH1 (Q0CB33551.1), *Juglans regia* JrBCH2 (XP_018809669.1), *Populus euphratica* PeBCH2 (XP_010161521.1), Gentiana lutea GIBCH1 (BAE92729.1), *Adonis aestivalis* AaBCH1 (ABJ93208.1), *Rosa chinensis* RcBCH2 (XP_024191328.1), *Zea mays* ZmBCH1 (NP_001105386.2), *Z.m. atBCH2* (NP_001105907.1), *Punica granatum* PgBCH2 (XP_031383909.1), *Arabidopsis thaliana* AtBCH1 (NP_001320065.1), AtBCH2 (NP_001119420.1), *Malus domestica* MdBCH2 (XP_008343769.2), *Nicotiana tabacum* NbBCH2 (XP_016467042.1), *Solanum lycopersicum* SlBCH1 (NP_001234348.1), SlBCH2 (NP_001265981.1), *Vitis riparia* VrBCH2 (XP_034710058.1).

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**Author contributions**

A-S.X. and T.L. initiated and designed the research. T.L., J-X.L., Y-J.D., A-Q.D., and H.L. performed the experiments. T.L., J-X.L., and F-Y.Z. analysed the data. A-S.X. contributed reagents/materials/analysis tools. T.L. wrote the paper. A-S.X. and T.L. revised the paper. All authors read and approved the final manuscript.

**Data availability**

All data supporting the findings of this study are available within the paper and within its supplementary materials published online.

**Conflict of interest**

The authors declare that there are no competing interests.

**Supplementary data**

Supplementary data is available at Horticulture Research online.

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