



Article

Changes in Carotenoid Concentration and Expression of Carotenoid Biosynthesis Genes in *Daucus carota* Taproots in Response to Increased Salinity

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Abstract: Studying the changes of carotenoids in the taproot of carrots under salt treatment is helpful to probe the salt stress response mechanism of carrots. The carotenoid concentration and the expression profiles of 10 carotenoid-related genes were determined in two carrot cultivars with different taproot colors. Under salt stress, the biosynthesis of carotenoids in the taproot of both ‘KRD’ and ‘BHJS’ was activated. RT-qPCR manifested that the expression levels of *DcPSY1*, *DcPSY2*, *DcZDS1*, *DcCRT1* and *DcCRT2* increased significantly in both ‘KRD’ and ‘BHJS’ under salt stress, but *DcCHXE* transcripts decreased and *DcPDS* transcripts maintained a basal level compared to that of the control. In the taproot of ‘KRD’, the expression level of *DcLCYB*, *DcLCYE* and *DcCHXB1* climbed dramatically. However, there was no significant change in the taproot of ‘BHJS’. The study showed that salt stress can stimulate the biosynthesis of carotenoids. The accumulation of lutein in the taproots of ‘KRD’ and ‘BHJS’ may be mainly attributed to the variation in *DcLCYE* and *DcCHXB1* transcripts. The increase in β -carotene accumulation is speculated to increase salt tolerance.

Keywords: carotenoids; salt stress; *DcPSY*; *DcLCYE*; β -carotene; carrot



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1. Introduction

The carrot (*Daucus carota* L.) is the most representative root vegetable of the Apiaceae family, which is grown all over the world [1,2]. Carotenoid accumulation in carrot taproots is a complex regulatory process. The study of carotenoid metabolism and its response to stress in carrot taproots has important guiding significance for carrot production [3]. As a lipophilic molecule, carotenoids play a critical role in photosynthesis [4], photomorphogenesis [5] and plant development [6,7] in carrots. In the past decades, carotenoid biosynthesis pathways in many plants have been studied, such as tomato [8], pepper [9], citrus [10], watermelon [11], carrot [12,13] and celery [14].

Carrots are good model plants for carotenoid research since they are rich in different carotenoids thus their taproot’s color is various [15,16]. Initially, the root of a carrot was colorless ahead of domestication, and numerous breeding works brought up diverse varieties rich in carotenoids, such as anthocyanins, lycopene, α -carotene, lutein, and β -carotene [17]. Carotenoid profiles also experienced remarkable change during the growth period [18,19]. During the early stage, the root is thin and colorless. After two months, the roots begin to thicken and start accumulating carotenoids. Latterly, secondary root growth results in root enlargement and increasing production of carotenoids.

Carotenoids are mainly synthesized in plastids [20] and commenced by geranylgeranyl diphosphate (GGPP) in the methylerythritol 4-phosphate (MEP) pathway [21]. The C40 carotenoid phytoene is formed by condensation of two molecules of the C20 GGPP, produced from isopentenyl pyrophosphate (IPP) and dimethylallyl pyrophosphate (DMAPP), which is catalyzed by phytoene synthase (PSY) [22]. Phytoene is transformed into lycopene via a train of desaturation and isomerization [23]. At this time, linear carotene biosynthesis is completed, and then lycopene is cyclized by lycopene ϵ -cyclase (LCYE) and lycopene β -Cyclase (LCYB) to produce α -carotene and β -carotene [24]. α -carotene is typically modified to lutein by hydroxylation. β -carotene is converted into assorted volatiles and phytohormones (such as strigolactones and abscisic acid) catalyzed by cross-reactive carbohydrate determinants (CCDs) and 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) produces [21,25].

Salt stress can exert numerous negative impacts on plants and provoke a serious reduction in crop growth and yield. If plants suffer from inordinate amounts of salt, this will lead to various metabolic perturbations, cutback in water potential (ψ_w), disordered membrane potential, weaken photosynthesis and diminish nitrogen assimilation [26–28]. However, plants have evolved many adaptative mechanisms in response to the condition of high salinity, which involves interacting physiological traits, biochemical or metabolic pathways, and molecular mechanisms [29]. Among them, carotenoids are proved to respond to salt stress [30,31]. Carrots are among the vegetable crops that have a very low salinity threshold [32] and are rich in carotenoids, but the specific salt response mechanism is not clear so far. Hence, it is necessary to investigate the dynamic changes and mechanisms of carotenoids under salt stress in carrots.

In this study, two carrot cultivars with different taproot colors were used as models. The concentration of lycopene, lutein, α -carotene, β -carotene and the total carotenoids, besides the expression of carotenoid biosynthesis-related genes were detected and analyzed. Under salt stress, the biosynthesis of carotenoids in the taproot of both two carrot cultivars was activated and dynamic changes in the carotenoid synthetic pathway were also observed. This study provided a potential theoretical basis for the mechanism of carotenoid biosynthesis response to salt stress in carrots.

2. Materials and Methods

2.1. Plant Materials, Growth Conditions and Stress Treatments

Two carrot cultivars were selected based on their different taproot colors: 'Kurodagosun (KRD)' (orange) and 'Benhongjinshi (BHJS)' (red). Carrot seeds were preserved in the State Key Laboratory of Crop Genetics and Germplasm Enhancement of Nanjing Agricultural University. The carrot seeds were germinated on filter paper for 5 days, and then transferred into containers (plastic, 32 cm in diameter and 25 cm in height) filled with organic substrate and vermiculite (1:1; *v/v*). Carrot seedlings were incubated in the greenhouse at Nanjing Agricultural University (25 °C, 14 h light period and 18 °C, 10 h dark period). Salt stress treatment began 45 days after sowing (DAS). Plants were subjected to salt stress by watering each container with 500 mL of NaCl solution (300 mmol·L⁻¹) or distilled water (for control group). Four days after the first exposure, the treatment was repeated until nine rounds of stress treatment within the same time period. The roots of each carrot cultivar were sampled at 80 DAS and three biological repetitions which comprise a pool of different roots of the same carrot cultivar under the same treatment were arranged. The samples that were separated into fragments were frozen in liquid nitrogen immediately and ground to powder, then stored at -80 °C until further experiments.

2.2. Determination of Carotenoids

Carotenoids were purified and quantified in accordance with the method of Ma et al. [33]. A total of 50 mg of vacuum freeze-dried powder from each sample was utilized for carotenoid extraction, which was performed by using 2 mL of acetone in a 50 °C water bath each time. Then, the combined extraction supernatants (2 mL) were filtered through 0.45 μ m filters and analyzed by HPLC on a Shimadzu LC-20A HPLC System (Shimadzu,

Kyoto, Japan), and three biological repetitions were set. Twenty microliters of supernatants were injected into a Hadera ODS-2 C₁₈ analytical column (250 mm × 4.6 mm, 5 μm nominal particle size; Shimadzu) with mobile phase consisting of mixtures of methanol: acetonitrile (90:10, v/v). The flow rate was 1 mL·min⁻¹, and elution was detected with a Shimadzu diode-array detector at 450 nm. All data were quantified on the basis of their standard curves. Standards were derived from Shanghai yuanye Bio-Technology (Shanghai, China). Three biological replicates were set for each assay.

2.3. Total RNA Isolation and Reverse Transcription

Total RNA of carrot was isolated using an RNA simple Total RNA Kit (Tiangen, Beijing, China) according to the instructions. Nanodrop ND-spectrophotometer (NanoDrop Technologies, Waltham, MA, USA) was used to detect the concentration of each RNA sample. Then, the total RNA (1 μg) was reverse transcribed into cDNA using Prime Script RT reagent Kit (Takara, Dalian, China).

2.4. Quantitative Real-Time PCR

Ten carotenoid biosynthesis-related genes (*DcPSY1*, *DcPSY2*, *DcPDS*, *DcZDS1*, *Dc-CRT1*, *DcCRT2*, *DcLCYE*, *DcLCYB1*, *DcCHXE*, *DcCHXB*) and reference gene *DcActin* were selected for gene expression analysis [34,35]. Primers were designed by Premier 6.0 and gene sequences were same as those reported in our previous work [33,34]. RT-qPCR analysis was performed according to the operating instructions of SYBR premix *Ex Taq* Kit (Takara, Dalian, China) and CFX96 PCR detection system (Bio Rad, CA, USA) with three technical replicates. RT-qPCR reactions were performed in 20 μL volume: SYBR Green I mix 10 μL, ddH₂O 7.2 μL, cDNA template 2.0 μL. The forward and reverse primers were 0.4 μL (10 μmol/L) respectively. The gene expression amount is calculated according to the relative quantitative method. The gene expression was calculated by 2^{-ΔΔC_t} method [36], and the gene expression level was analyzed and mapped using Microsoft Excel. The results were expressed as the mean of three independent biological replicates.

2.5. Data Analysis

SPSS version 25.0 was utilized to statistically analyze data based on one-way ANOVA. Duncan's multiple range test at *p* < 0.05 was selected for significance test.

3. Results

3.1. Growth Analysis of Two Carrot Cultivars under Different Treatments

At 80 DAS, the phenotypes of 'KRD' and 'BHJS' under different treatments are shown in Figure 1, the taproots of 'BHJS' are red and the taproots of 'KRD' are orange. The color difference between 'KRD' and 'BHJS' is caused by the different carotenoid accumulation although the color difference is not very obvious due to the light. Previous studies have shown that high osmotic stress and high ionic stress caused by salt injury led to plant growth inhibition and root biomass reduction in carrots [37]. Consistently, the volume of taproots and the length of petioles of carrots decreased significantly, and there was no significant difference in the number of fibrous roots, petioles, taproots color and leaf color. The taproot length, maximum taproot diameter and leaf length of carrot were decreased due to salt stress. Under salt stress, the average taproot length of 'KRD' and 'BHJS' decreased by 15.79% and 14.00%, and the maximum diameter of the average taproot decreased by 38.71% and 30.77%, and the average leaf length decreased by 16.22% and 12.5%, respectively (Figure 2).



Figure 1. Phenotype of carrot under salt stress. (A) ‘BHJS’ CK; (B) ‘BHJS’ under salt stress; (C) ‘KRD’ CK; (D) ‘KRD’ under salt stress. Bar = 5 cm. Salt Stress: NaCl solution (300 mmol·L⁻¹). CK: Distilled water.

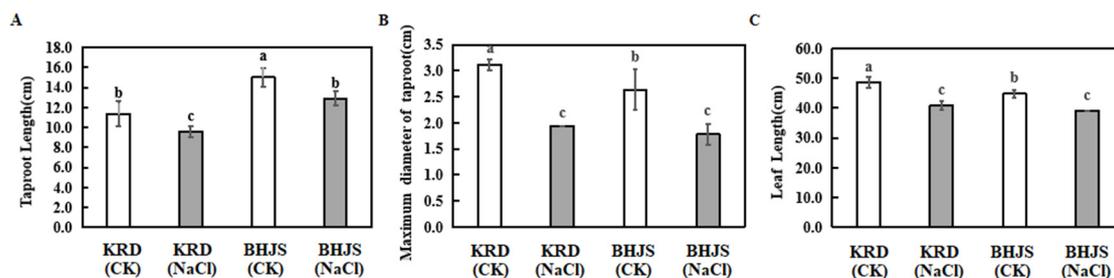


Figure 2. Morphological parameters of carrot under salt stress. (A) Taproot length; (B) Maximum diameter of taproot; (C) Leaf length. Different lowercase letters indicate significant differences among different carrot cultivars and treatment at the $p < 0.05$ level.

3.2. Effects of Salt Stress on Carotenoids Accumulation in Taproots of Carrot

The accumulation of various carotenoids in the taproots of the ‘KRD’ and ‘BHJS’ under 0 (control) and 300 mmol L⁻¹ salt treatment are shown in Figures 3 and 4. The total carotenoid concentration of ‘KRD’ and ‘BHJS’ under salt treatment was higher than the control. The changes in carotenoid concentration in the taproots under salt stress varied among different cultivars.

The concentration of lutein, α -carotene, β -carotene and total carotenoids in the taproots of ‘KRD’ under salt treatment increased significantly by 4.81%, 33.75%, 48.54% and 38.01%, respectively. The concentration of lycopene, α -carotene, β -carotene and total carotenoids in the taproots of ‘BHJS’ increased significantly by 19.13%, 20.91%, 10.47% and 11.52%, respectively, but the concentration of lutein decreased significantly by 3.29% compared to the control.

The accumulations of α -carotene, β -carotene and total carotenoids in the taproots of ‘KRD’ and ‘BHJS’ were activated under the salt stress treatment. Lycopene was barely detected in the taproot of ‘KRD’ in both the control and the salt treatment group, but a high concentration of lycopene was detected in the control group and salt treatment group of ‘BHJS’, 205.37 μ g/g and 244.66 μ g/g, respectively, reflecting the difference in lycopene concentration in the taproots of ‘KRD’ and ‘BHJS’. The lutein concentration in the taproots of ‘KRD’ and ‘BHJS’ increased and decreased significantly, respectively, under salt treatment (300 mmol·L⁻¹ NaCl), reflecting the difference in the mechanism of lutein response to salt stress between ‘KRD’ and ‘BHJS’.

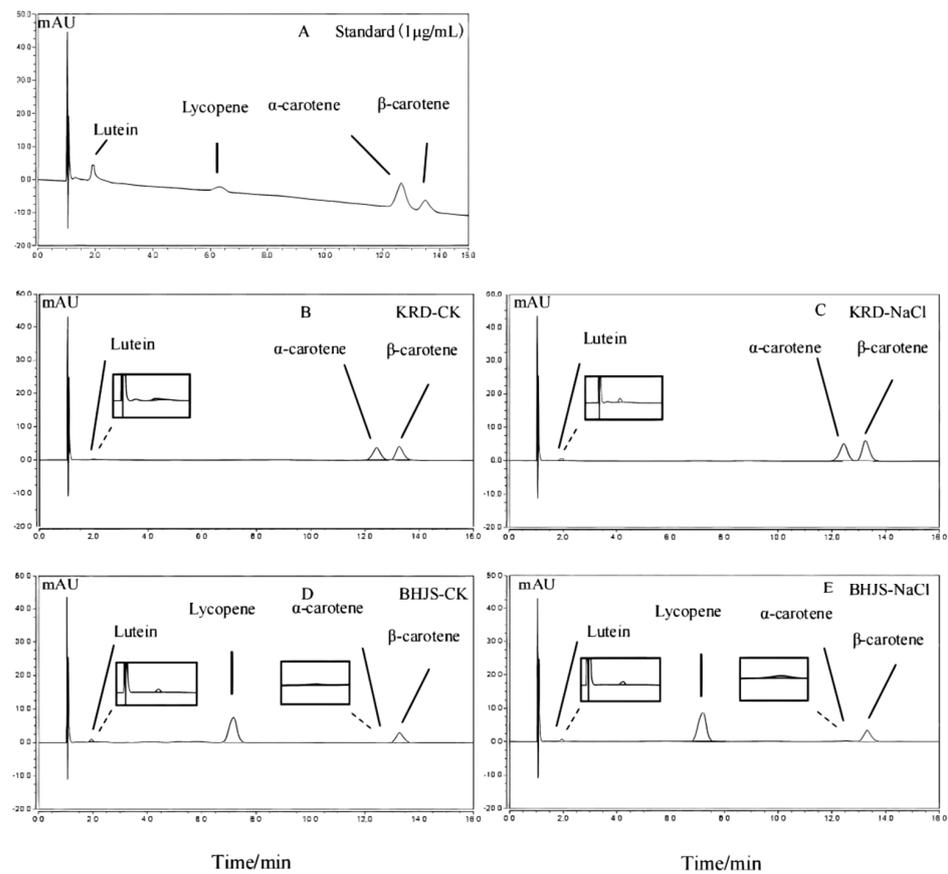


Figure 3. UPLC chromatograms of carotenoids in different carrot cultivars and treatments. (A) Standard UPLC chromatograms of carotenoids; (B) UPLC chromatograms of carotenoids in ‘KRD’ of control group; (C) UPLC chromatograms of carotenoids in ‘KRD’ of salt treatment group; (D) UPLC chromatograms of carotenoids in ‘BHJS’ of control group; (E) UPLC chromatograms of carotenoids in ‘BHJS’ of salt treatment group. Cultivar abbreviations: BHJS, Benhongjinshi; KRD, Kurodagosun.

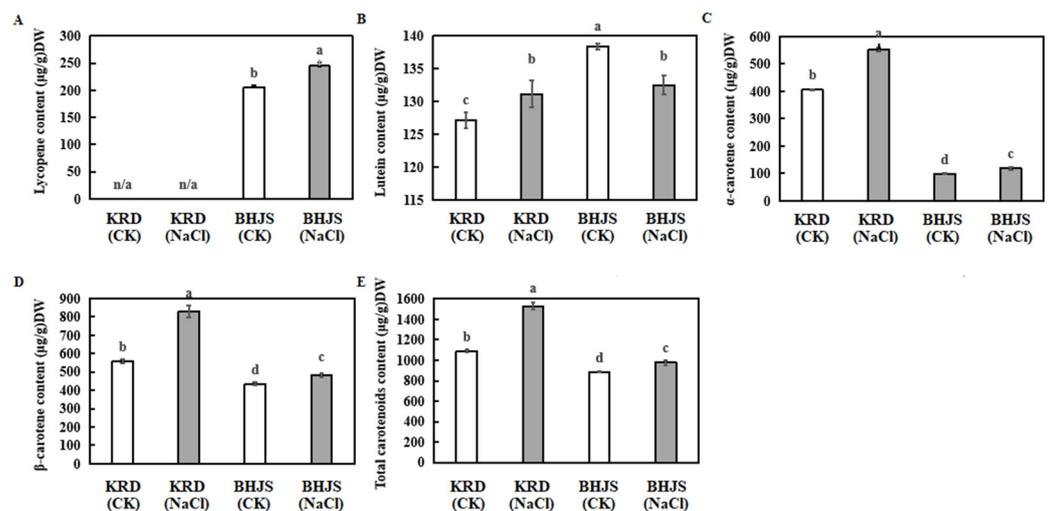


Figure 4. Carotenoids concentration in different carrot cultivars and treatments. (A) Lycopene concentration in the roots of ‘BHJS’ and ‘KRD’ under different treatment; (B) Lutein concentration; (C) α-carotene concentration; (D) β-carotene concentration; (E) Total carotenoids concentration. Cultivar abbreviations: BHJS, Benhongjinshi; KRD, Kurodagosun. “n/a” represents no concentration was detected. Different lowercase letters indicate significant differences among different carrot cultivars and treatment at the $p < 0.05$ level.

3.3. Expression Profiles of Carotenoid Biosynthesis-Related Genes

RT-qPCR results indicated that most of the 10 carotenoid biosynthesis-related genes were involved in the salt response, and the expression levels were different in 'KRD' and 'BHJS' (Figure 5). The biosynthesis of carotenoid-related genes in taproots was activated by salt stress. The changes in those selected genes showed consistency. There were also some discrepancies between the two carrot cultivars.

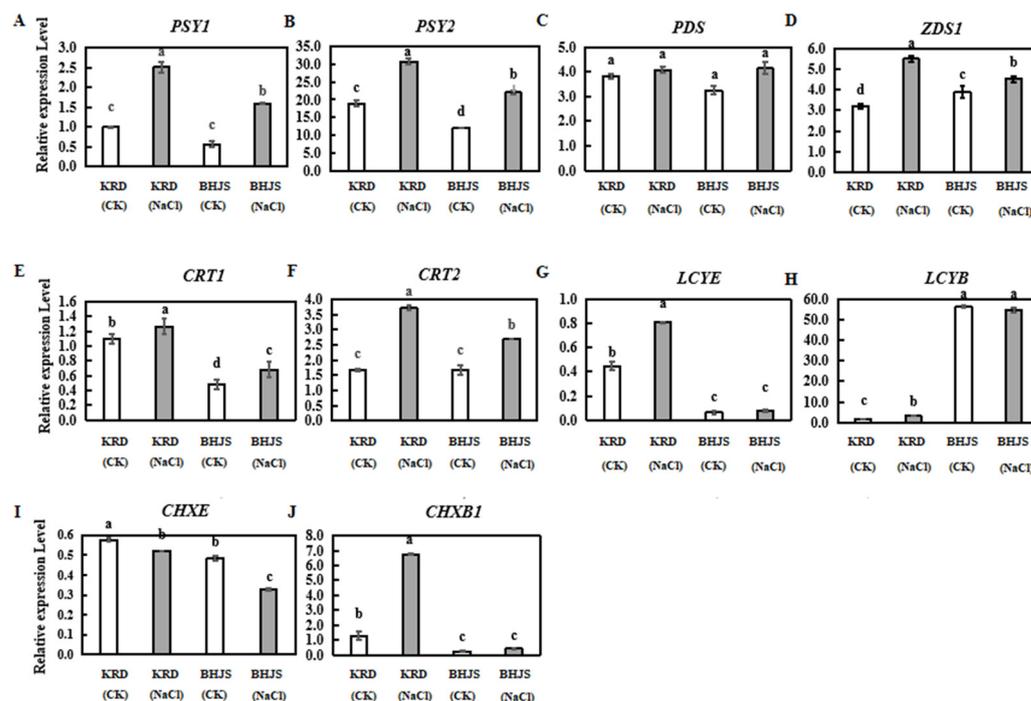


Figure 5. Relative expression levels of carotenoid biosynthesis-related genes under salt stress. (A) Relative expression level of *PSY1* in the roots of 'BHJS' and 'KRD' under different treatment; (B) *PSY2*; (C) *PDS*; (D) *ZDS1*; (E) *CRT1*; (F) *CRT2*; (G) *LCYE*; (H) *LCYB*; (I) *CHXE*; (J) *CHXB1*. Different lowercase letters indicate significant differences among different carrot cultivars and treatment at the $p < 0.05$ level.

In KRD, in contrast with the control, the expression levels of *DcPSY1*, *DcPSY2*, *DcZDS1*, *DcCRT1*, *DcCRT2*, *DcLCYE*, *DcLCYB* and *DcCHXB1* were significantly increased by 151%, 62%, 71%, 16%, 121%, 84%, 122% and 434%, respectively. *DcPDS* is basically similar to that of the control, and a 10% down-regulation of *DcCHXE* was observed.

In BHJS, the expression levels of *DcPSY1*, *DcPSY2*, *DcZDS1*, *DcCRT1* and *DcCRT2* in the taproots in the salt treatment group significantly increased by 179%, 82%, 11%, 42% and 62%, respectively, in comparison with that of the control group. The expression levels of *DcPDS*, *DcLCYE*, *DcLCYB* and *DcCHXB1* sustained steady expression levels similar to that of the control, and the *DcCHXE* transcript was significantly down-regulated by 31%.

The transcripts of *DcPSY1*, *DcPSY2*, *DcZDS1*, *DcCRT1* and *DcCRT2* in the taproots of 'KRD' and 'BHJS' were both promoted by salt stress, and the *DcPSY1* transcript was raised by more than 150%. Significantly increased levels of *DcLCYE*, *DcLCYB* and *DcCHXB1* were observed in the taproots of 'KRD' but there was no significant difference in the taproots of 'BHJS'. The changes in the expression level of *DcPDS* in the taproots of both 'KRD' and 'BHJS' are slight. The *DcCHXE* transcript declined in the taproots of both 'KRD' and 'BHJS'.

4. Discussion

Numerous reports have shown that the increase in carotenoid concentration can enhance the resilience of plants to salt stress [38–42]. Reduced expression of *CHXB* in sweet potatoes led to an increasing concentration of β -carotene and the total carotene in the cells

of transgenic plants, enhancing the antioxidant ability of plants [38]. Shi et al. [43] and Li et al. [41] overexpressed *ZDS* in sweet potato and tobacco, respectively, which improved the expression level of carotenoids, such as β -carotene and lutein, which confer enhanced salt tolerance to the plant. Salt stress could facilitate the accumulation of carotenoids in the taproots of carrots, since a concurrent increase in *DcPSY1*, *DcPSY2*, *DcZDS1*, *DcCRT1* and *DcCRT2* transcript levels with carotenoid biosynthesis was observed. A previous study confirmed that *DbPSY* plays a rate-limiting role in controlling carbon flux into the carotenogenesis pathway [23]. In this study, the expression level of *DcPSY1* and *DcPSY2* both showed a significant increase in the taproots of carrots under salt stress treatment, which authenticates the important role of *DcPSY* in prompting carotenogenesis to increase salt resistance. In maize and foxtail millet (*Setaria italica*), salt stress does not affect *PDS* [44]. In our study, *DcPDS* appears to be salt insensitive, which is in keeping with earlier findings. In a previous study, *ZDS* is characterized to be salt inducible [45–47], and Lao et al. [48] demonstrated that *DbZDS* is hypo-osmotically regulated by its promoter, and HRE (hypo-osmolarity-responsive element) is responsible for the hypo-osmotic response. In this study, the expression level of *DcZDS1* increased significantly, which is assumed to facilitate salt resistance. To fulfill the geometrical requirements of the desaturases, carotene isomerase (*CRTISO*) is employed to transform 9,15,9'-tricis- ζ -carotene into 9,9'-dicis- ζ -carotene, 7,9,9'-tricis-neurosporene into 9-*cis*-neurosporene and 7,9-*dicis*-lycopene into all-*trans*-lycopene [49], and *LcCRTISO* is able to increase carotenoid content and enhance salt tolerance in higher plants [50]. In this study, *DcCRT1* and *DcCRT2* were up-regulated under salt stress, which is speculated to improve salt tolerance. In conclusion, *DcPSY1*, *DcPSY2*, *DcZDS1*, *DcCRT1* and *DcCRT2* collaborated to activate carotenoid biosynthesis to enhance salt tolerance.

The taproot of carrots is colorful and mainly consists of white, yellow, orange, red, and purple, which is decided by accumulated pigment and content [1,51–55]. The white taproot barely has carotenoids, the yellow taproot mainly accumulates lutein, the orange taproot such as 'KRD' and 'Hongxinqicun (HXQC)' is rich in lutein, α -carotene and β -carotene and the red taproot such as 'BHJS' mainly have lutein, α -carotene, β -carotene and lycopene. In this study, lycopene was barely observed in the orange taproots of 'KRD', and both α -carotene and β -carotene are relatively higher than 'BHJS'. Hence, it is speculated that most of the lycopene in 'KRD' is used for the biosynthesis of α -carotene and β -carotene downstream. Higher content of lycopene was detected in the taproots of 'BHJS' in the salt treatment group in contrast with the control. Li et al. [50] overexpressed *PDS*, *ZDS* and *CRT* in tobacco, leading to the increased concentration of lycopene. Kong et al. [56] found that gourd and wild watermelon rootstocks boost lycopene accumulation in grafted watermelon fruit by up-regulating *PSY* and *ZDS* transcripts. Pola et al. [57] found that *PSY* in green pepper (*Capsicum annuum* L.) was significantly up-regulated at 30 °C, promoting the accumulation of lycopene. These studies show that the expression of lycopene upstream genes may exert a great impact on the changes in lycopene concentration. In this work, the lycopene concentration in the taproots of 'BHJS' under salt stress increased significantly compared to that of the control, which is assumed to be attributed to the up-regulation of carotenoid biosynthesis-related genes upstream. The taproots of 'KRD' did not accumulate lycopene, and the mechanism of lycopene accumulation in the taproots of carrots with different colors needs to be further studied.

Lycopene can be taken as a substrate to yield α -carotene and β -carotene. In the ϵ -branches, lycopene is catalyzed by *DcLCYE* to produce δ -carotene, and then formed under the catalysis of *DcLCYB* to produce α -carotene, and finally, lutein is synthesized with the catalysis of *DcCHXB* and *DcCHXE* [25]. A significant increase in lutein concentration in the taproots of 'KRD' was observed and it is speculated that the accumulation of lutein concentration was attributed to the increasing transcripts of *DcLCYE* and *DcCHXB* in this branch. However, the lutein concentration in the taproots of 'BHJS' decreased significantly, which may be caused by the dramatic decrease in *DcCHXE* transcript.

The increase in β -carotene concentration can enhance the salt tolerance of plants [41,43]. Kang et al. [40] inhibited the expression of *CHXB* in sweet potatoes, thus increasing the concentration of β -carotene in the root, and enhancing the salt tolerance of sweet potatoes. In this study, the concentration of β -carotene in the taproots of 'KRD' and 'BHJS' increased significantly under salt stress, which can partly explain the carrot responsive mechanism to salt stress.

ABA is a hormone-reinforcing plant abiotic stress response [58] and can be produced in β -branches of carotenoids biosynthesis [59]. A previous study showed that abiotic stress can induce carotenoid synthesis to produce ABA in *Arabidopsis* to increase resistance [58,60,61]. This indicates that there is a potential relationship between carotenoids and ABA, and it can be reasonably inferred that the activation of carotenoid biosynthesis is related to ABA to boost plant salt tolerance and is worth further exploration.

5. Conclusions

In conclusion, salt stress can facilitate carotenoid biosynthesis and increase carotenoid concentration in the taproots of carrots. The carotenoid compositions in taproots are various due to different carrot cultivars with different colors of the taproot. In the ϵ -branch, the difference in lutein accumulation in the taproots of carrots with different colors maybe relates to the discrepancy in the expression levels of *DcLCYE* and *DcCHXB*. In the β -branch, the significant increase in β -carotene concentration is one of the salt resistance mechanisms of carrots. This study is conducive to the investigation of dynamic changes of carotenoids in carrot taproots under salt stress.

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