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Molecular Characterization of Carotenoid Cleavage Dioxygenases and the Effect of Gibberellin, Abscisic Acid, and Sodium Chloride on the Expression of Genes Involved in the Carotenoid Biosynthetic Pathway and Carotenoid Accumulation in the Callus of *Scutellaria baicalensis* Georgi

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Supporting Information

ABSTRACT: Three cDNAs encoding carotenoid cleavage dioxygenases (SbCCD1, SbCCD4, and SbNCED) were isolated from *Scutellaria baicalensis*, an important traditional herb in Asia and North America. Amino acid sequence alignments showed that they share high identity and similarity to their orthologs in other plant species. Quantitative real-time polymerase chain reaction analysis revealed that *SbCCD1* and *SbCCD4* were most strongly expressed in flowers, whereas *SbNCED* was expressed at the highest level in roots. The expression levels of phytoene synthase (*SbPSY*), phytoene desaturase (*SbPDS*), ξ-carotene desaturase (*SbZDS*), β-ring carotene hydroxylase (*SbCHXB*), zeaxanthin epoxidase (*SbZEP*), *SbCCD1*, *SbCCD4*, and *SbNCED* in the callus of *S. baicalensis* varied under different concentrations of gibberellic acid (GA_3) and abscisic acid (ABA). Under NaCl treatment, expression levels of all genes increased with increasing NaCl concentrations. Except for zeaxanthin, increasing GA_3, ABA, and NaCl concentrations caused higher losses in the total carotenoid content. The total carotenoid content substantially decreased with increasing GA_3, ABA, and NaCl concentrations, with the biggest reductions observed in the NaCl treatment. The isolation and characterization of SbCCD1, SbCCD4, and SbNCED together with the study on the effect of GA_3, ABA, and NaCl on carotenoid biosynthesis will be helpful to elucidate the carotenoid biosynthesis mechanism in *S. baicalensis* and may set new trends in metabolic engineering of carotenoids in plants.

KEYWORDS: *Callus, carotenoid cleavage dioxygenases, carotenoids, abiotic stress, Scutellaria baicalensis*

INTRODUCTION

Carotenoids represent the largest group of pigments in nature and are found in all plants.[1] They play various functions in plants, such as stabilization of lipid membranes, harvesting light for photosynthesis, and protecting the photosystem from oxidation.[2,3] Carotenoids also provide the attractive coloration, ranging from yellow to red, in fruits and flowers, acting as attractants for pollination and seed dispersal.[4] Furthermore, oxidative cleavage of carotenoids produces a number of apocarotenoids, which act as signals in plant development, serve as antifungal agents, and contribute to the flavor and aroma of flowers and fruits.[5] The best-studied apocarotenoid derivative is abscisic acid (ABA), a plant hormone, which regulates a wide range of biological processes, including seed dormancy, fruit maturation, and response to various environmental stresses in plants.[6] Dietary carotenoids are essential nutrients for humans and animals because they are precursors of vitamin A, and its deficiency leads to xerophthalmia, blindness, and premature death.[7] Owing to their strong antioxidant properties, carotenoids absorbed through dietary intake can prevent certain forms of cancer, heart disease, and macular degeneration.[8,9]

The biosynthesis of carotenoids has been extensively studied in higher plants, and most steps in the biosynthesis pathway have been identified (Figure 1).[1,10] During their synthesis, carotenoids can be cleaved at any of the conjugated double bonds by enzymes of the carotenoid cleavage dioxygenase (CCD) family to form a wide range of apocarotenoids.[5] In *Arabidopsis*, the CCD enzyme family consists of nine members: four CCDs (CCD1, CCD4, CCD7, and CCD8) and five 9-*cis*-epoxycarotenoid dioxygenases (NCEDs; NCED2, NCED3, NCED5, NCED6, and NCED9). Orthologs in other plants species are named according to their homology to CCDs in *Arabidopsis*. CCD1 and CCD4 appear to cleave the 9,10 (9′,10′) double bonds in multiple carotenoid substrates to

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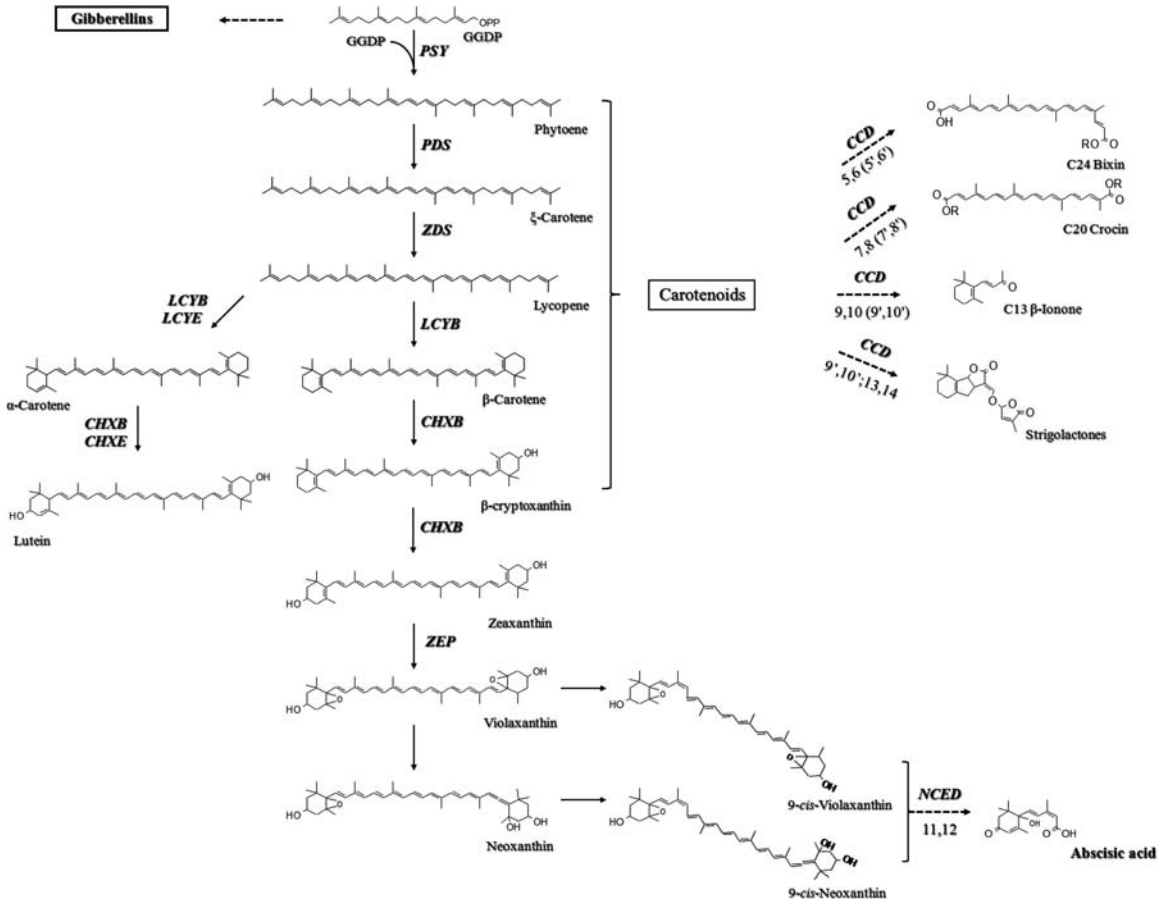

Figure 1. Carotenoid biosynthesis pathway in plants. GGDP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, *ξ*-carotene desaturase; LCYB, lycopene *β*-cyclase; LCYE, lycopene *ε*-cyclase; CHXB, *β*-ring carotene hydroxylase; CHXE, *ε*-ring carotene hydroxylase; ZEP, zeaxanthin epoxidase; CCD, carotenoid cleavage dioxygenase; NCED, 9-*cis*-epoxycarotenoid dioxygenase.

produce apocarotenoids that contribute to the color, flavor, and aroma of fruits and flowers.[11,12] Recently, additional cleavage activities of CCD1 at the 5,6 (5′,6′) and 7,8 (7′,8′) double bonds in specific carotenoid substrates under certain buffer conditions have been reported.[13,14] Functional analysis has revealed that the other two CCDs, CCD7 and CCD8, are involved in the biosynthesis of a novel type of plant hormone known as strigolactone, which is involved in shoot branching.[15,16] NCEDs specifically cleave the 11,12 double bonds in 9-*cis* isomers of epoxycarotenoids to yield the C_{15} product xanthoxin, which is the precursor of ABA, the final product of the carotenoid biosynthesis pathway.[17] On the other hand, ABA was proven to control the abiotic stress signaling pathway in plants.[18] Therefore, carotenoid accumulation is highly regulated by the activity of CCD genes, the biosynthesis of ABA, and abiotic stresses, such as drought and salinity.[19] In addition, carotenoid accumulation is also partially regulated by gibberellin (GA) biosynthesis, which requires geranylgeranyl diphosphate, the initial precursor of the carotenoid biosynthetic pathway (Figure 1).

Scutellaria baicalensis contains a variety of flavones, phenylethanoids, amino acids, sterols, and essential oils, and it has been used an important traditional drug in Asia and North America for centuries.[20,21] Pharmacological reports have revealed that *S. baicalensis* has powerful medicinal properties, including anti-inflammatory, antidiabetic, antiviral, antihypertension, antioxidant, and anticancer effects.[22−24] However, except for flavonoids, which are believed to be the major component of *S. baicalensis*, very few studies have described the biosynthesis of other compounds as well as the contribution of them to the considerable medicinal properties of *S. baicalensis*. Recently, the cell culture of *S. baicalensis* has been developed and studied for the production of secondary metabolites.[25,26] The plant cell culture may therefore be an alternative to investigate the biosynthetic mechanism of secondary metabolites because of a similar genetic background of plant tissues. It has been reported that the carotenoid content and composition in citrus cultured *in vitro* were similar to those in citrus fruits ripening on trees.[27]

The aim of the present study was to investigate the effects of different physiological parameters [gibberellic acid (GA_3), ABA, and NaCl] on the expression of genes involved in the carotenoid biosynthetic pathway and accumulation of carotenoids in particular and gain insight into the molecular mechanisms of carotenoid biosynthesis in *S. baicalensis* in general. Three cDNAs belonging to three distinct groups of the CCD family, namely, SbCCD1, SbCCD4, and SbNCED, were isolated and characterized in *S. baicalensis*. In addition, the effect of GA_3, ABA, and NaCl on the transcription of eight genes involved in the carotenoid biosynthetic pathway (*SbPSY*, *SbPDS*, *SbZDS*, *SbCHXB*, *SbZEP*, *SbCCD1*, *SbCCD4*, and *SbNCED*) and carotenoid accumulation was investigated.

MATERIALS AND METHODS

Plant Materials. *S. baicalensis* Georgi plants were grown under greenhouse conditions for 1 month and then transferred to the

experimental farm of Chungnam National University (Daejeon, Korea). After flowering, the plants were collected and the roots, stems, leaves, and flowers were dissected. All samples were immediately frozen in liquid nitrogen and then stored at −80 °C and/or freeze-dried for RNA isolation and/or high-performance liquid chromatography (HPLC) analysis.

Callus Induction. Callus induction of *S. baicalensis* were performed using a previously described method.[26] Briefly, leaf explants (grown *in vitro*) were cut aseptically into approximately 7 × 7 mm^2 sections at the ends and placed on agar-solidified culture medium, which consisted of salts and vitamins of MS medium (Murashige and Skoog, 1962), supplemented with 2.0 mg L^{-1} 2,4-dichlorophenoxy acetic acid (2,4-D), and 30 g L^{-1} sucrose. After 4 weeks, calli were subcultured in the same culture medium at 25 °C in a growth chamber in dark conditions (24 h of darkness).

Stress Treatments. After 4 weeks of subculture under dark conditions, calli were transferred to the same culture medium supplemented with GA_3 (50, 100, and 200 μM), ABA (50, 100, and 200 μM), or NaCl (100, 200, and 400 mM) for 3 weeks. Calli grown on the culture medium alone for 3 weeks were used as controls, and all callus samples were maintained in a growth chamber under standard cool white fluorescent tubes with a flux rate of 35 μmol s^{-1} m^{-2} and a 16 h photoperiod. The experiments were repeated 3 times, and the mixture of three independent replicate callus was used for the further analysis of gene expression and carotenoid accumulation. All samples were frozen in liquid nitrogen immediately after harvesting and then stored at −80 °C and/or freeze-dried for RNA isolation and/or HPLC analysis.

RNA Isolation and cDNA Synthesis. Callus samples were ground to a powdered form in a mortar with liquid nitrogen, and total RNA was isolated using a Plant Total RNA Mini Kit (Geneaid, Taiwan) according to the instructions of the manufacturer. The quality and concentration of total extracted RNA was determined by both 1% agarose gel electrophoresis and spectrophotometric analysis. For quantitative real-time polymerase chain reaction (PCR), 1 μg of total RNA was used for reverse transcription using the ReverTra Ace-R kit (Toyobo, Osaka, Japan). The resulting cDNA was diluted 20-fold into 20 μL and used as a template for subsequent PCR analyses.

Isolation of cDNAs Encoding Carotenoid Cleavage Dioxygenases. For a previous study, we had obtained 39 581 different genes from *S. baicalensis* using next-generation DNA sequencing (NGS) platforms (Roche/454 GS_FLX+ and Illumina/Solexa HiSeq2000; data not reported). Three full-length cDNAs encoding CCD1, CCD4, and NCED were indentified from the *S. baicalensis* NGS database. They were then analyzed individually for homology with known sequences and designated SbCCD1, SbCCD4, and SbNCED.

Sequence Analyses. The deduced amino acid sequences of the carotenoid cleavage dioxygenases from *S. baicalensis* were analyzed for homology using the BLAST program at the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/BLAST). The predicted molecular mass of protein was calculated online at the website http://www.sciencegateway.org/tools/proteinmw.htm. Sequence alignments were carried out using BioEdit Sequence Alignment Editor, version 5.0.9 (Department of Microbiology, North Carolina State University, Raleigh, NC). A phylogenic tree was constructed at the online website (http://www.phylogeny.fr/).

Real-Time PCR. On the basis of the following amplified sequences of *S. baicalensis*: SbPSY, SbPDS, SbZDS, SbCHXB, SbZEP, SbCCD1, SbCCD4, SbNCED, and SbActin (GenBank accession numbers KC417312, KC417313, KC417314, KC417315, KC417316, KC760147, KC760148, KC760149, and HQ847728, respectively), real-time PCR primers were designed using the Primer3 website (http://frodo.wi.mit.edu/primer3/) (Table 1) and then tested for the specificity of fragment sizes and melting curves by PCR and real-time PCR, respectively. For quantification of standards, PCR products amplified from cDNA were purified and the concentration of the products was measured to calculate the number of cDNA copies. The expression of eight genes involved in the carotenoid biosynthetic pathway was calculated by the method of relative quantification with *actin* as the reference.

Table 1. Primers Used for Real-Time PCR

primer	sequence (5′ → 3′)	amplicon (base pairs)
SbPSY F	ACCACAGAGGCTGTCTATAATGCTG	126
SbPSY R	TGCCAACTCATCTTGAGGTAGGTAG	
SbPDS F	TGGAGATTGGTACGAGACTGGTTTA	183
SbPDS R	GGTGCAGGTAAGACTTCAGGAAAAT	
SbZDS F	GAACTTCTTCCTTGCTGGCTCTTAC	130
SbZDS R	CTTCCGTAATTCCACCAACTCTTCT	
SbCHXB F	GAGAAGGAAAGTGGAGAGGAGAATG	136
SbCHXB R	AAGTAATCCCGAAGCTGGACATAAC	
SbZEP F	AAAGTAATGTGGTGGACTTCGATGA	175
SbZEP R	CCAGTGTAACAGGTGTAGCCAGAGT	
SbCCD1 F	TAAAAATGGTCTGGCTTCACAGAAA	192
SbCCD1 R	ACTTCAAGCTTGTCCTTTCCACTCT	
SbCCD4 F	TCATAATCACTCTCTCACACCACCA	200
SbCCD4 R	TCTTTCTTCTTCCCTTGATTTCACC	
SbNCED F	CACTACAGCCAGACCATAAAGTGCT	149
SbNCED R	TTCTGAATGATGTTCCATTGAGGTT	
SbActin F	GACTGAAGCTCCTCTTAATCCCAAG	102
SbActin R	ACAGCCTGAATGGCAACATACATAG	

Real-time PCR reactions were carried out in a 20 μL reaction mix, containing 5 μL of template cDNA, 10 μL of 1× SYBR Green Real-Time PCR Master Mix (Toyobo, Osaka, Japan), 0.5 μL of each primer (10 μM), and DEPC-treated water. Thermal cycling conditions were as follows: 95 °C for 5 min, 40 cycles of 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 20 s. PCR products were analyzed using Bio-Rad CFX Manager 2.0 software. Triplicates of each sample were used for real-time analysis.

Carotenoid Extraction and Analyses. Extraction and measurement of carotenoids by HPLC were performed according to the method described in our previous study.[28] Briefly, carotenoids were released from the *S. baicalensis* samples (0.02 g) by adding 3 mL of ethanol containing 0.1% ascorbic acid (w/v), vortexing for 20 s, and placing in a water bath at 85 °C for 5 min. The carotenoid extract was saponified with potassium hydroxide (120 μL, 80%, w/v) in the 85 °C water bath for 10 min. After saponification, the samples were immediately placed on ice, and cold deionized water (1.5 mL) was added. β-Apo-8′-carotenal (0.2 mL, 25 g/mL) was added as an internal standard. Carotenoids were extracted twice with hexane (1.5 mL) and centrifuged at 1200*g* to separate the layers. Aliquots of the extracts were dried under a stream of nitrogen and redissolved in a 1:1 (v/v) mixture of dichloromethane/methanol before HPLC analysis. The carotenoids were separated using a C30 YMC column (250 × 4.6 mm, 3 μm; Waters Corporation, Milford, MA) with an Agilent 1100 HPLC system (Massy, France) equipped with a photodiode array detector. The chromatograms were generated at 450 nm. Solvents used for elution were as follows: solvent A, methanol/water (92:8, v/v) with 10 mM ammonium acetate, and solvent B, 100% methyl *tert*-butyl ether. Gradient elution was performed at 1 mL/min under the following conditions: 0 min, 90% A/10% B; 20 min, 83% A/17% B; 29 min, 75% A/25% B; 35 min, 30% A/70% B; 40 min, 30% A/70% B; 42 min, 25% A/75% B; 45 min, 90% A/10% B; and 55 min, 90% A/10% B. Carotenoid standards were purchased from CaroteNature (Lupsingen, Switzerland). For quantification of carotenoids in each sample, calibration curves were drawn by plotting four different concentrations of the carotenoid standards according to the peak area ratios with β-apo-8′-carotenal.

Statistical Analysis. The data for gene expression and carotenoid contents were analyzed using the computer software Statistical Analysis System (SAS, version 9.2). Treatment means were compared by Duncan's multiple range test.

RESULTS AND DISCUSSION

Sequence Analyses of CCDs from *S. baicalensis*. *SbCCD1* was 2107 base pairs (bp) long and had an open reading frame (ORF) of 1635 bp, encoding a protein of 544 amino acids with a predicted molecular mass of 61.72 kDa. *SbCCD4* was 2186 bp long, with a 1794 bp ORF encoding a protein of 597 amino acids (predicted molecular mass of 65.38 kDa). *SbNCED* was 2212 bp long with a 1797 bp ORF, encoding a protein of 598 amino acids (predicted molecular mass of 66.49 kDa).

Alignments of the deduced amino acid sequences showed that SbCCD1, SbCCD4, and SbNCED share high sequence identity and homology with their orthologs (see Supplementary Figures 1, 2, and 3 of the Supporting Information, respectively). On the basis of the phylogenic tree generated using the CCD gene family data from various plants, the three CCDs from *S. baicalensis* were classified into three distinct groups, namely, CCD1, CCD4, and NCED (Figure 2). In addition, SbNCED was closer to AtNCED3 compared to all other NCEDs from *Arabidopsis thaliana*.

Expression Levels of CCDs in Different Organs of *S. baicalensis*. Expression patterns of *SbCCD1*, *SbCCD4*, and *SbNCED* were analyzed in the roots, stems, leaves, and flowers of *S. baicalensis* by real-time PCR (Figure 3). The mRNA level of *SbCCD1* was the highest in flowers, lower in stems, and very poor in roots and leaves. *SbCCD4* mRNA was also expressed at the highest level in flowers, a relatively high level in leaves, and low levels in roots and stems. Unlike *SbCCD1* and *SbCCD4*, the mRNA level of *SbNCED* was the highest in roots, moderate in flowers, and lowest in leaves and stems.

Transcriptional Regulation of Genes Involved in Carotenoid Biosynthetic Pathway in the Callus of *S. baicalensis* under GA_3-, ABA-, and NaCl-Induced Stress. We investigated the effect of different concentrations of GA_3, ABA, and NaCl on the expression levels of genes involved in the carotenoid biosynthetic pathway in the callus of *S. baicalensis*. GA_3 treatment reduced the expression levels of *SbPSY* and *SbPDS* mRNA, with the lowest expression observed at 50 μM GA_3 (Figure 4A). Transcript levels of *SbZDS* were not affected by GA_3, whereas 50 and 200 μM GA_3 considerably increased the transcript levels of *SbCHXB* and *SbZEP*. Expression of *SbCCD1* mRNA was dramatically decreased, whereas *SbCCD4* and *SbNCED* mRNA expressions were slightly increased at all three tested concentrations of GA_3. ABA treatment did not substantially change the expression level of *SbPSY* compared to the control, while the level of *SbPDS* mRNA was decreased at all three tested concentrations of ABA (Figure 5A). Expressions of *SbZDS* and *SbCHXB* were increased with ABA treatment, and the highest expression levels were observed with 100 μM ABA. Expression of *SbZEP* varied in response to the three concentrations of ABA: 100 μM ABA did not change the expression level of *SbZEP*, whereas 50 and 200 μM ABA up- and downregulated the *SbZEP* expression, respectively. The effect of ABA treatment on the expressions of *SbCCD1* and *SbCCD4* was different. Specifically, *SbCCD1* expression was reduced at all three concentrations of ABA, whereas *SbCCD4* expression was upregulated with the highest expression observed at 50 μM ABA. The *SbNCED* mRNA level in callus treated with ABA was significantly higher than that of the control callus sample. Under NaCl treatment, all genes involved in the carotenoid biosynthetic pathway showed similar trends; in that, expression levels consistently increased with increasing NaCl concentrations (Figure 6A). There were considerable increases in expression levels of *SbPSY*, *SbZDS*, *SbCHXB*, *SbCCD1*, *SbCCD4*, and *SbNCED*, particularly at the highest concentration of NaCl (200 mM).

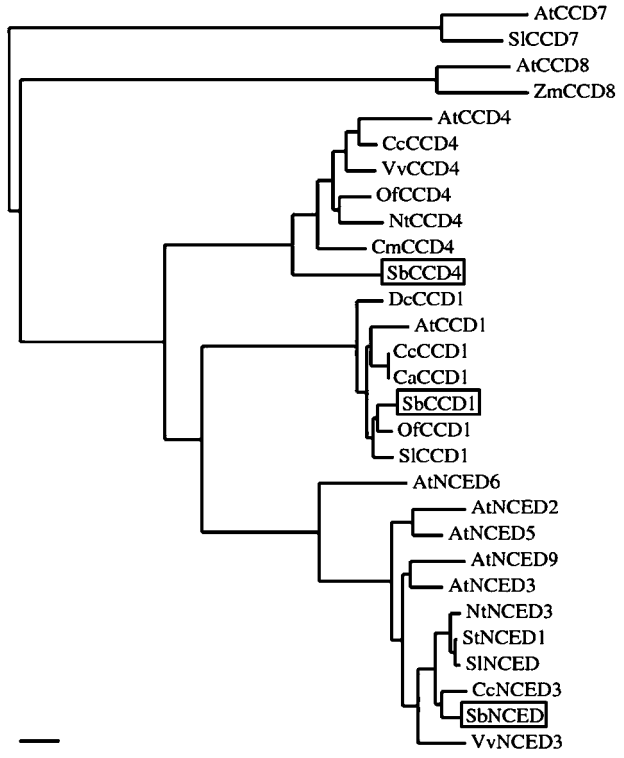

Figure 2. Phylogenic tree of SbCCD1, SbCCD4, SbNCED, and their homologues. The black boxes are the CCDs identified in this study. The bar indicates a 10% difference in amino acid sequence. *A. thaliana*, AtCCD1 (NM_116217), AtCCD4 (AY136353), AtCCD7 (NM_130064), AtCCD8 (NM_119434), AtNCED2 (NM_117945), AtNCED3 (NM_112304), AtNCED5 (NM_102749), AtNCED6 (NM_113327), and AtNCED9 (NM_106486); *Osmanthus fragrans*, OfCCD1 (AB526197) and OfCCD4 (EU334434); *Solanum lycopersicum*, SlCCD1 (NM_001247613), SlCCD7 (NM_001247504), and SlNCED (NM_001247526); *Daucus carota*, DcCCD1 (DQ192203); *Coffea canephora*, CcCCD1 (DQ157166) and CcNCED3 (DQ157167); *Coffea arabica*, CaCCD1 (DQ157170); *Chrysanthemum x morifolium*, CmCCD4 (AB247160); *Nicotiana tabacum*, NtCCD4 (JF947192) and NtNCED3 (JX101472); *Vitis vinifera*, VvCCD4 (JQ712827) and VvNCED3 (JQ319644); *Citrus clementina*, CcCCD4 (DQ309330); *Solanum tuberosum*, StNCED1 (AY662342); and *Zea mays*, ZmCCD8 (NM_001197000).

Changes in Carotenoid Accumulation in the Callus of *S. baicalensis* under GA_3, ABA, and NaCl Stress. Figures 4B, 5B, and 6B show changes in the carotenoid composition and content in the callus of *S. baicalensis* exposed to different concentrations of GA_3, ABA, and NaCl. Major carotenoids detected in the callus of *S. baicalensis* were lutein and β-carotene. With the exception of zeaxanthin, increasing GA_3, ABA, and NaCl concentrations decreased the content of all carotenoids in the *S. baicalensis* callus. In contrast, the zeaxanthin content increased with an increase in the GA_3 and ABA concentrations but decreased when 200 and 400 mM NaCl were used. The total carotenoid content substantially decreased with increasing concentrations of GA_3, ABA, and NaCl, with the biggest reduction observed in the NaCl treatment. The total carotenoid content of callus treated with 200 and 400 mM NaCl was 3.09 and 1.74 μg/g of dry weight,

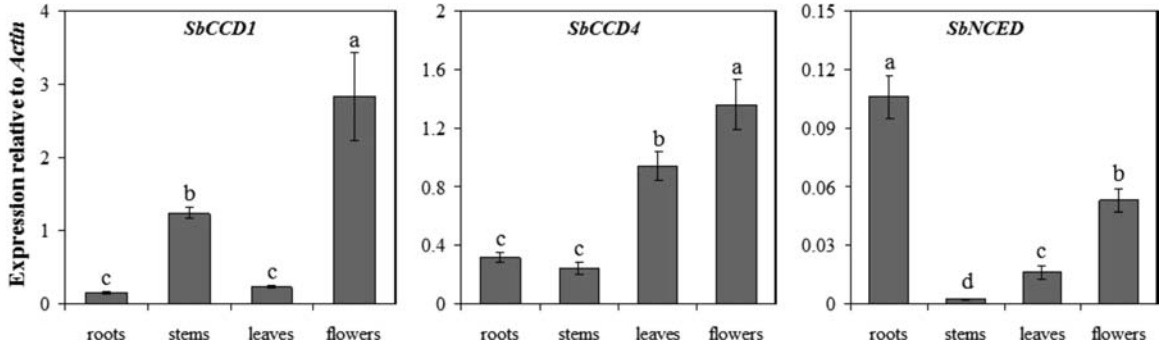

Figure 3. Expression levels of *SbCCD1*, *SbCCD4*, and *SbNCED* in different organs of *S. baicalensis*. The height of each bar and the error bars show the mean and standard error, respectively, from three independent measurements. The letters a, b, c, and d indicate significant differences at the 5% level by Duncan's multiple range test.

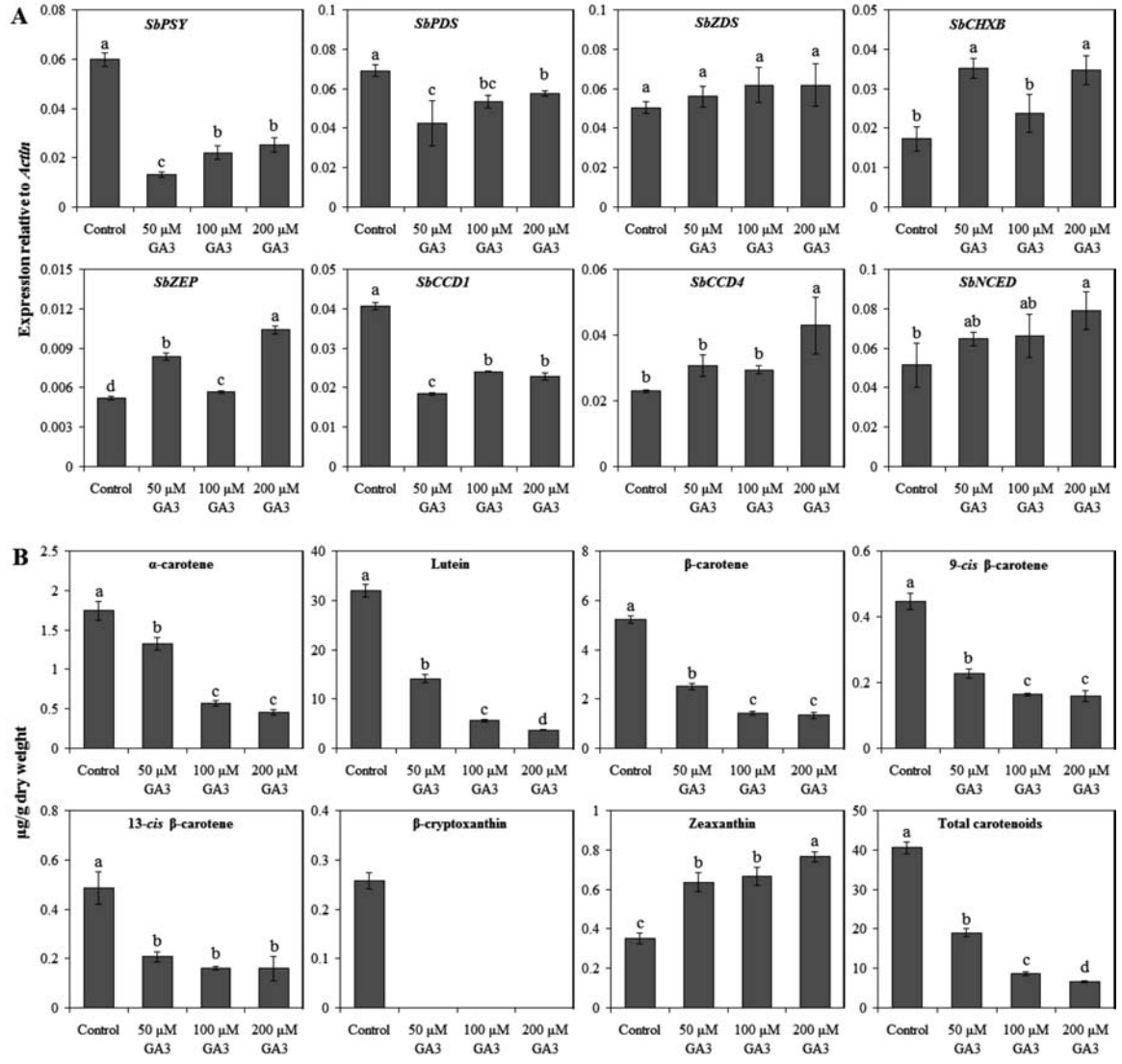

Figure 4. Expression levels of genes involved in (A) carotenoid biosynthetic pathway and (B) carotenoid accumulation in the callus of *S. baicalensis* treated with different concentrations of GA_3. The height of each bar and the error bars show the mean and standard error, respectively, from three independent measurements. The letters a, b, c, and d indicate significant differences at the 5% level by Duncan's multiple range test.

respectively, i.e., approximately 13- and 23-fold lower than that of the carotenoid content of the control sample (40.54 μg/g of dry weight).

In the present study, three cDNAs encoding CCDs (SbCCD1, SbCCD4, and SbNCED) were isolated and characterized in *S. baicalensis*. *SbCCD1* and *SbCCD4* showed the strongest expression in the flowers, whereas *SbNCED* was expressed at the highest level in the roots. In our previous study (unpublished data), small amounts of carotenoids were detected in the flowers, where *SbPSY*, *SbPDS*, and *SbZDS*

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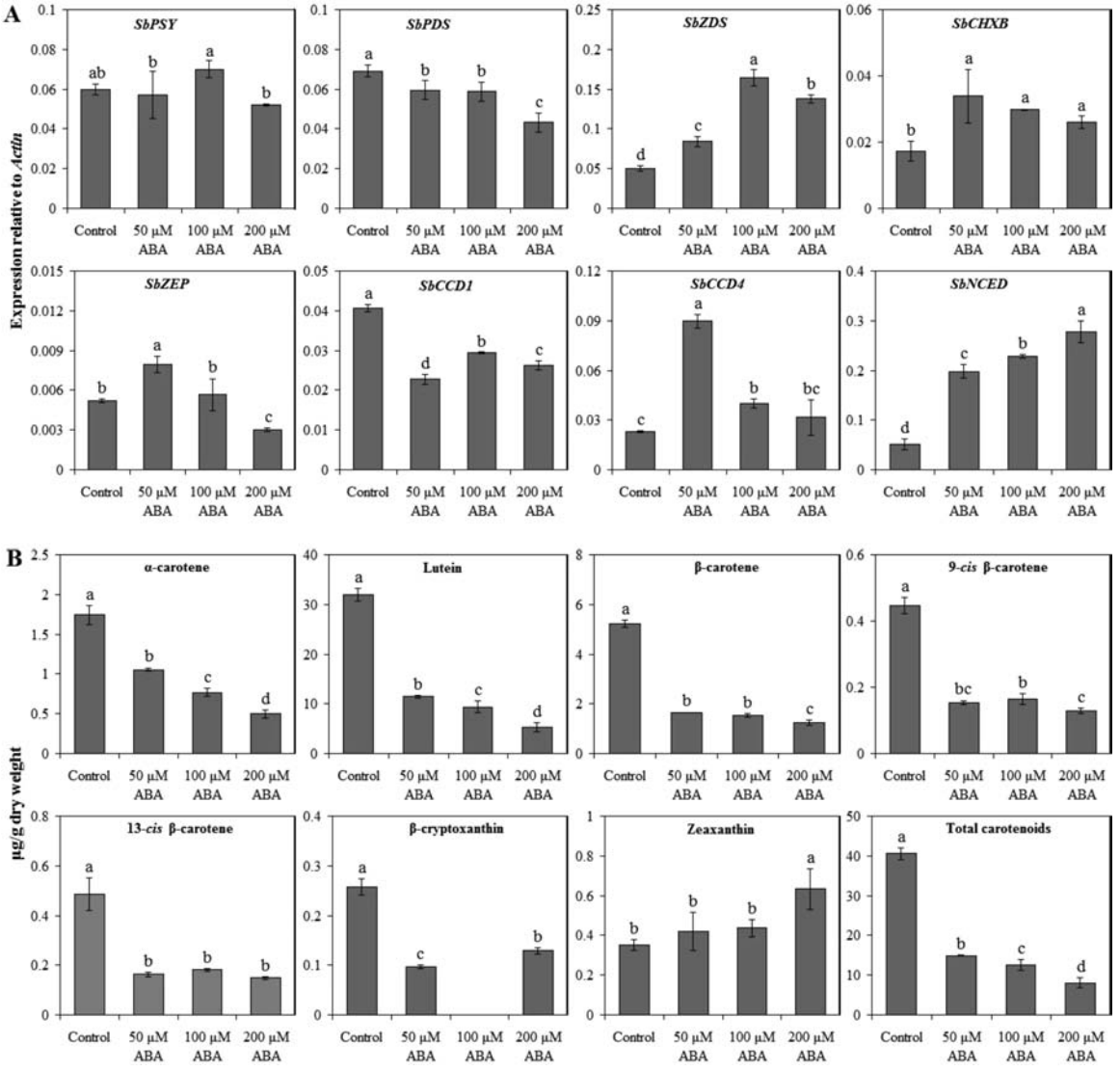

Figure 5. Expression levels of genes involved in (A) carotenoid biosynthetic pathway and (B) carotenoid accumulation in the callus of *S. baicalensis* treated with different concentrations of ABA. The height of each bar and the error bars show the mean and standard error, respectively, from three independent measurements. The letters a, b, c, and d indicate significant differences at the 5% level by Duncan's multiple range test.

were highly expressed. Activity analyses of CCD1 and CCD4 suggested that the enzymes cleaved multiple carotenoid substrates, including ζ-carotene, lycopene, β-carotene, zeaxanthin, δ-carotene, and lutein, at various positions, such as 5,6 (5′,6′), 7,8 (7′,8′), and 9,10 (9′,10′).[11,13] Therefore, it seems likely that the low content of carotenoids in the flowers of *S. baicalensis* was because of their cleavage into apocarotenoid products, which is catalyzed by the high activity of *SbCCD1* and *SbCCD4* found in this organ. The high transcription levels of CCD genes were also suggested to be responsible for the low accumulation of carotenoids in *Arabidopsis*[29] and bitter melon.[30]

GA treatment has been demonstrated to affect carotenoid biosynthesis by interacting with the early steps in the pathway.[31,32] In this study, we observed that GA_3 down-regulated *SbPSY* and *SbPDS* expression levels in the callus of *S. baicalensis*; these enzymes catalyze the first and second steps of the carotenoid biosynthetic pathway. PSY, is the key regulator of carotenoid accumulation in plants;[33,34] therefore, down-regulation of *SbPSY* expression results in a decrease in the carotenoid content. GA_3 biosynthesis is also probably induced, leading to the metabolic flux in GA biosynthesis rather than carotenoid biosynthesis.

Because ABA production uses carotenoids as precursors, the ABA level is closely related to the carotenoid content.[27] Under ABA treatment, the expression levels of the most important gene involved in the production of carotenoids (*SbPSY*) did not show big differences, whereas the expression levels of *SbNCED*, the main regulator of ABA biosynthesis, increased by several fold. The carotenoid content of the untreated callus was significantly higher than that of the ABA-treated callus. We hypothesized that exogenously added ABA stimulates endogenous ABA biosynthesis, thus decreasing the level of its precursor, i.e., carotenoid. Furthermore, ABA is well-known as a stress hormone that is active in the adaptation of higher plants to various forms of environmental stress, such as drought, high temperature, and salinity stress.[18] Therefore, under NaCl treatment, the biosynthesis of ABA could be upgraded through substantial increases in the expression levels of its biosynthetic genes, including *SbPSY* and *SbNCED*. Hence, to produce ABA

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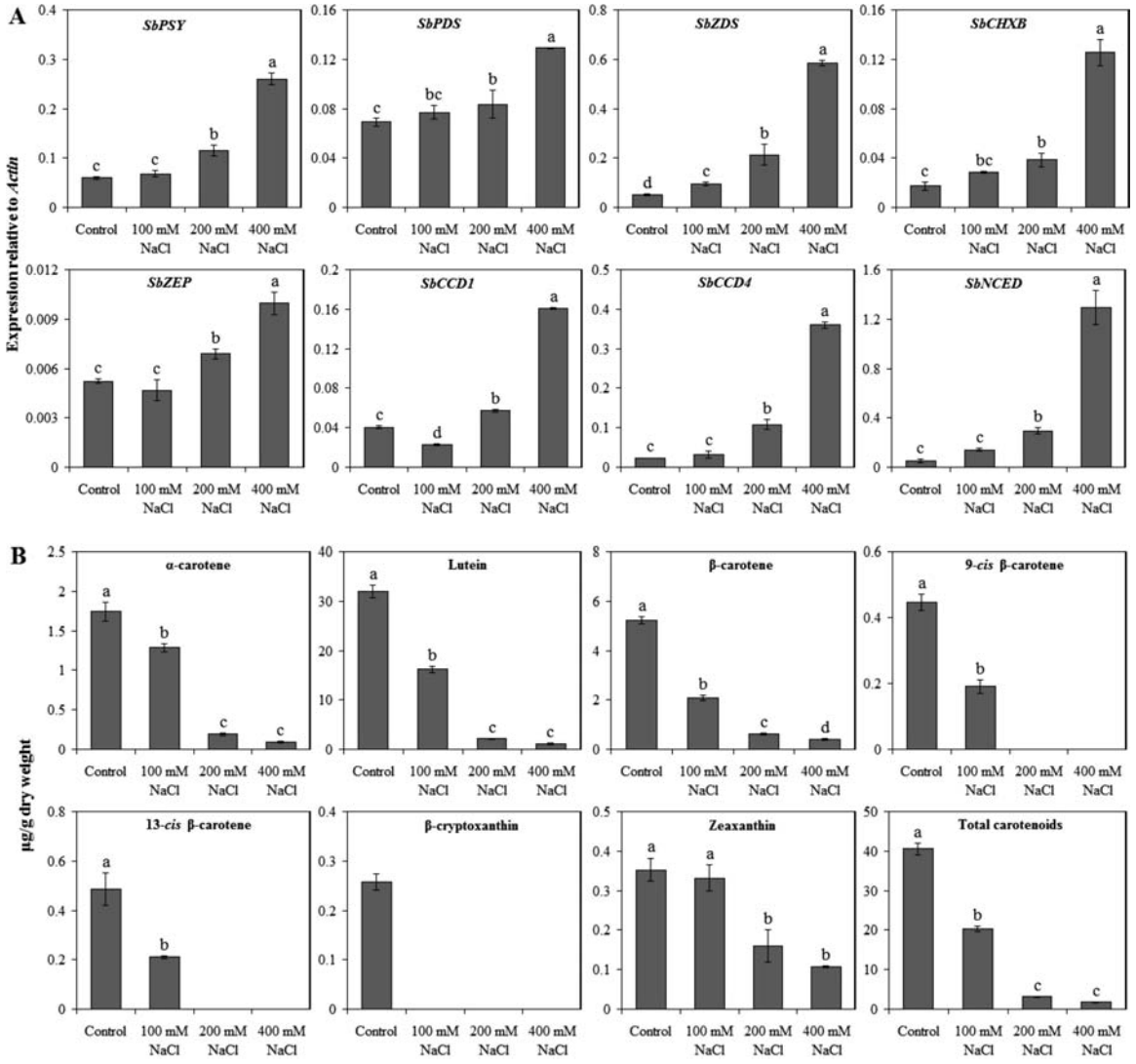

Figure 6. Expression levels of genes involved in (A) carotenoid biosynthetic pathway and (B) carotenoid accumulation in the callus of *S. baicalensis* treated with different concentrations of NaCl. The height of each bar and the error bars show the mean and standard error, respectively, from three independent measurements. The letters a, b, c, and d indicate significant differences at the 5% level by Duncan's multiple range test.

in response to salinity stress, carotenoids were degraded; consequently, the cells had a low carotenoid content. Further studies on ABA accumulation are needed to clarify the negative effect of ABA and NaCl on carotenoid accumulation in the callus of *S. baicalensis*.

Hence, the isolation and characterization of SbCCD1, SbCCD4, and SbNCED together with an understanding of the effects of GA_3, ABA, and NaCl on carotenoid biosynthesis will be helpful to elucidate the carotenoid biosynthesis mechanism in *S. baicalensis*. The negative effects of GA_3, ABA, and NaCl on carotenoid accumulation could be exploited in metabolic engineering of plants by inhibiting GA and ABA biosynthesis and/or activities of CCD genes. Our study on the induction of *SbNCED* transcription in *S. baicalensis* under high salinity stress conditions might be helpful in improving stress tolerance of other crops using transgenic plant techniques by employing NCED genes.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Multiple alignment of amino acid sequences of SbCCD1 with other CCD1s (Supplementary Figure 1), SbCCD4 with other CCD4s (Supplementary Figure 2), and SbNCED with other NCEDs (Supplementary Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

DEPC, diethylpyrocarbonate; HPLC, high-performance liquid chromatography; GGDP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ξ-carotene desaturase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; CHXB, β-ring carotene hydroxylase; CHXE, ε-ring carotene hydroxylase; ZEP, zeaxanthin epoxidase; CCD, carotenoid cleavage dioxygenase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; ABA, abscisic acid; GA, gibberellin

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