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Downregulation of the lycopene ε-cyclase gene increases carotenoid synthesis via the β-branch-specific pathway and enhances salt-stress tolerance in sweetpotato transgenic calli

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Lycopene ε-cyclase (*LCY-ε*) is involved in the first step of the α-branch synthesis pathway of carotenoids from lycopene in plants. In this study, to enhance carotenoid synthesis via the β-branch-specific pathway [which yields β-carotene and abscisic acid (ABA)] in sweetpotato, the expression of *IbLCY-ε* was downregulated by RNAi (RNA interference) technology. The RNAi-*IbLCY-ε* vector was constructed using a partial cDNA of sweetpotato *LCY-ε* isolated from the storage root and introduced into cultured sweetpotato cells by *Agrobacterium*-mediated transformation. Both semi-quantitative Reverse transcription polymerase chain reaction (RT-PCR) of carotenoid biosynthesis genes and high-performance liquid chromatography (HPLC) analysis of the metabolites in transgenic calli, in which the *LCY-ε* gene was silenced, showed the activation of β-branch carotenoids and its related genes. In the transgenic calli, the β-carotene content was approximately 21-fold higher than in control calli, whereas the lutein content of the transgenic calli was reduced to levels undetectable by HPLC. Similarly, expression of the RNAi-*IbLCY-ε* transgene resulted in a twofold increase in ABA content compared to control calli. The transgenic calli showed significant tolerance of 200 m*M* NaCl. Furthermore, both the β-branch carotenoids content and the expression levels of various branch-specific genes were higher under salt stress than in control calli. These results suggest that, in sweetpotato, downregulation of the ε-cyclization of lycopene increases carotenoid synthesis via the β-branch-specific pathway and may positively regulate cellular defenses against salt-mediated oxidative stress.

Abbreviations – ABA, abscisic acid; CaMV, cauliflower mosaic virus; CHY-β, β-carotene hydroxylases; DAB, 3,3-diaminobenzidine; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GGPS, geranylgeranyl pyrophosphate synthase; HPLC, high-performance liquid chromatography; LCY-β, lycopene β-cyclase; LCY-ε, lycopene ε-cyclase; MS, Murashige and Skoog; NCED, 9-cis-epoxycarotenoid dioxygenase; NT, non-transgenic; PDS, phytoene desaturase; PSY, phytoene synthase; RNAi, RNA interference; ROS, reactive oxygen species; RT-PCR, Reverse transcription polymerase chain reaction; RWC, relative water content; ZDS, ζ-carotene desaturase; ZEP, zeaxanthin epoxidase.

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Introduction

Carotenoids are a class of terpenoid pigments naturally found in higher plants, fungi and photosynthetic microorganisms (Cunningham and Gantt 1998, Fraser and Bramley 2004), where they are synthesized via general isoprenoid biosynthesis pathways in the chloroplasts and chromoplasts (Galpaz et al. 2006). They serve in various roles, such as accessory pigments for light harvesting and scavengers of the reactive oxygen species (ROS) produced by the photosynthetic machinery (Tao et al. 2007). These functions are involved in the interaction with chlorophyll that enables energy usage. Thus, carotenoids absorb light and pass its energy to chlorophyll for photosynthesis, in addition to transferring excess energy away from chlorophyll. The latter function protects the photosynthetic apparatus from ROS-mediated damage under stress conditions (Romer and Fraser 2005, Han et al. 2008). Recent studies, including genetic modification of carotenoid biosynthesis in *Arabidopsis* and tobacco plants, showed that carotenoids increase tolerance to high light conditions, UV irradiation, herbicides and salt stress (Davison et al. 2002, Götz et al. 2002, Han et al. 2008).

In humans, carotenoids are an important dietary component but are also used in cosmetics applications (Fraser and Bramley 2004, Taylor and Ramsay 2005, Botella-Pavía and Rodríguez-Concepción 2006). β-carotene is the precursor of vitamin A (retinol) (Lakshman and Okoh 1993), a deficiency of which causes the malfunction of light absorption by the retina (Ye et al. 2000). In addition, protection of the eyes from blue light radiation (Krinsky and Johnson 2005) and from macular degeneration (Landrum and Bone 2004) is achieved with carotenoids. The supply of carotenoids to most animals, including humans, is mainly contributed by dietary crops, albeit in concentrations that may be inadequate. Consequently, there has been significant interest in increasing carotenoid levels in crops, e.g. by using metabolic engineering. Carotenoids are enzymatic cascade metabolites of phytoene, whose synthesis is catalyzed by phytoene synthase (*PSY*) from geranylgeranyl pyrophosphate synthase (*GGPS*) that originate in the isoprenoid pathway (Cunningham et al. 1996). Two enzymes, phytoene desaturase (*PDS*) and ζ-carotene desaturase (*ZDS* or *CRTISO*), convert phytoene to lycopene via phytofluene and ζ-carotene. Lycopene is the substrate for two competing cyclases, ε-cyclase (*LCY-*ε) and β-cyclase (*LCY-*β). *LCY-*ε introduces a single ε-ring, thus converting lycopene to δ-carotene, and *LCY-*β introduces one or two β-rings at either end of lycopene to form β-carotene. Two carotenoid biosynthetic pathways proceed from lycopene, the α-branch (from α-carotene to lutein) and the β-branch (from β-carotene to neoxanthin), which play distinct and complementary roles in the mechanisms involved in photo-protection (Dall'Osto et al. 2007).

Typically, the carotenoid content of plant cells is increased by either ectopic gene expression or downregulation of key pathway enzymes. For example, in *Arabidopsis*, ectopic expression of the *PSY* gene in seed resulted in the enhancement of certain carotenoids as well as chlorophyll content, together with an increase in abscisic acid (ABA) levels (Lindgren et al. 2003). The downregulation of *LCY-*ε and β-carotene hydroxylases (*CHY-*β) was shown to enhance β-carotene accumulation in potato tubers (Paine et al. 2005, Diretto et al. 2007b, Diretto et al. 2007a, Van Eck et al. 2007, Zhu et al. 2009), while silencing of the zeaxanthin epoxidase gene (*ZEP*) increased zeaxanthin levels, also in potato (Romer et al. 2002). In our recent study, the concentrations of carotenoids derived from the α- and β-branch pathways, including β-carotene and lutein, were increased in RNAi (RNA interference)-*CHY-*β transgenic calli (Kim et al. 2012), which also resulted in better tolerance of NaCl-mediated oxidative stress.

LCY-ε genes have been isolated from many plants, such as *Arabidopsis*, potato, maize, autumn olive and rape (Cunningham et al. 1996, Diretto et al. 2006, Yu et al. 2008, Bai et al. 2009, Guo et al. 2009). They are involved in regulating the mechanisms underlying carotenoid synthesis, coordinating the expression of carotenoid biosynthesis-related genes. While it is known that *LCY-*ε catalyzes ε-cyclization of lycopene and thus plays an active role in both the carotenoid pathway and ROS scavenging, other potential functions are unknown.

Sweetpotato [*Ipomoea batatas* (L.) Lam] ranks seventh in annual production among global food crops. It is an alternative source of bio-energy and rich in natural antioxidants. Its wide adaptability on marginal lands in tropical temperature zones and its high nutrient density, including various phytochemicals, anthocyanins, vitamin C, carbohydrates, potassium and dietary fiber (Yoshinaga et al. 1999, Teow et al. 2007), can be exploited to secure the food supply and to improve malnutrition in developing countries. The non-profit Center for Science in the Public Interests designated sweetpotato as 1 of 10 health-improving super foods. The USDA reported that sweetpotato yields two to three times as much carbohydrate as field corn and approaching the amount obtained from some varieties of sugarcane (Ziska et al. 2009). Despite these many healthful and useful properties, few studies have examined the biosynthesis and genetic engineering of carotenoids in sweetpotato (Kim et al. 2012).

In this work, transgenic sweetpotato calli were generated by the downregulation of *IbLCY-ε* using white-fleshed cv. Yumli. Carotenoids accumulation was found to occur more rapidly in transgenic cells than in whole plants and could be distinguished by the degree of yellow coloration. The aim of this study was to understand carotenoid synthesis via the *β*-branch-specific pathway in *IbLCY-ε* silenced sweetpotato cultured cells. Accordingly, carotenoid biosynthetic genes, the levels of altered carotenoids and the antioxidant capacity of these compounds in transgenic calli were characterized. In addition, ABA content and 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity under conditions of salt stress were investigated.

Materials and methods

Plant materials

Sweetpotato (*Ipomoea batatas* L. Lam. cv. Yulmi) plants were kindly provided by the Bioenergy Crop Research Center, National Institute of Crop Science, Rural Development Administration, South Korea. The plants were cultivated in soil for 50 days in a growth chamber maintained at 25°C with a photo-cycle of 16 h light/8 h dark. Non-embryogenic calli of sweetpotato induced from shoot apical meristems were used as experimental material, following the method reported by Kim et al. (2012).

Isolation of the *LCY-ε* gene and vector construction

To clone a partial *IbLCY-ε* cDNA from sweetpotato, primers for *LCY-ε* were synthesized based on the sequence of the *LCY-ε* gene from morning glory (*Ipomoea nill)* (accession no. TA10964_35883) deposited in the TIGR Plant Transcript Assemblies database. A partial *IbLCY-ε* fragment was amplified from the sweetpotato cDNA by RT-PCR using the Advantage 2 pfu DNA polymerase mix (Clontech, Tokyo, Japan) and a *LCY-ε* primer set, 5′-GAACAA ACTAATGTTAAGACTGGAGACA-3′ and 5′-AGGATTT CTGGGATCAACTCTATC-3′. The amplified product was cloned into pGEM-T Easy vector (Promega, Madison, WI) and then sequenced. To construct the *IbLCY-ε* RNAi vectors, *IbLCY-ε*-specific primers were designed from the sequence of *IbLCY-ε*, which contained the following *Eco*RI and *Xho*I restriction sites: 5′-*GA ATTC*GAACAAACTAATGTTAAGACTGGA-3′ and 5′-*C TCGAG*GATAGAGTTGATCCCAGAAATCCT-3′. The PCR product was digested with *Eco*RI and *Xho*I and ligated into the pENTR11 vector (Invitrogen, Carlsbad, CA). The pENTR11-*IbLCY-ε* clones were then subjected to site-specific recombination in a plant RNAi expression vector, pH7GWIWG2(I), together with the CaMV (cauliflower mosaic virus) 35S promoter (Park et al. 2011), in a reaction catalyzed by the LR Clonase enzyme mix (Invitrogen).

Transformation of sweetpotato calli

Recombinant plasmids with the pH7GWIWG2(I)-*IbLCY-ε* construct were introduced into *Agrobacterium tumefaciens* strain EHA105 and then co-cultured with sweetpotato calli. The *Agrobacterium*-mediated transformation of sweetpotato calli was carried out according to the manual, as previously described (Kim et al. 2012).

Gene expression analysis

Total RNA was extracted from sweetpotato calli using the Easy-Spin™ total RNA extraction kit (iNtRON, Daejeon, Korea). First-strand cDNA was synthesized from total RNA (1 μg) using M-MLV reverse transcriptase (MBI-Fermentas, St. Leon-Rot, Germany) according to the manufacturer's instructions. The expression levels of *IbLCY-ε* and other carotenoid biosynthesis-related genes were analyzed by semi-quantitative RT-PCR using the gene-specific primers listed in Table 1.

Determination of carotenoid content

Carotenoids from sweetpotato calli (100 mg freeze-drying) were extracted and analyzed using the Agilent 1100 HPLC (high-performance liquid chromatography) system (Hewlett-Packard, Palo Alto, CA) according to the method of Lim et al. (2009) and Kim et al. (2012). The HPLC-DAD system was operated via CHEMSTATION software (Hewlett-Packard). Carotenoids were quantified by an external calibration method. *β*-carotene, *β*-cryptoxanthin, lutein, violaxanthin and zeaxanthin standards were obtained from CaroteNature (Lupsingen, Switzerland). Under these conditions, the peaks of standard carotenoids at t_R (min) values were as follows: violaxanthin (peak 11.5), lutein (peak 23.3), zeaxanthin (peak 26.6), *β*-cryptoxanthin (peak 33.5) and *β*-carotene (39.2).

Analysis of radical-scavenging activity

The DPPH radical-scavenging activity of sweetpotato callus was evaluated as described by (Pieroni et al. 2002), with slight modifications. The absorbance of the resulting solution at 517 nm was measured spectrophotometrically against a blank (MeOH). L-ascorbic acid (AsA, 0.015–0.125 m*M*) was used as the standard and DPPH radical-scavenging activity was expressed as mol AsA g^{-1} tested sample.

Table 1. Primers used to amplify carotenoid biosynthesis-related genes from the sweetpotato.

Target gene	Sequence	Direction	Amplicon size (bp)
GGPS	AGTAGGTGTGTTGTATCAAGTTGT	FOR	308
	AACAGGTAAGAGCATATAGTGTAGC	REV	
PSY	TATTTACCTCAAGATGAATTAGCTC	FOR	399
	TCAGCTTCTTCAGTACAGTATTACA	REV	
ZDS	GGTGTTATACAAAACAGGATTACAT	FOR	302
	AAAGGAAAAGAGAAGAGAAGAACTA	REV	
LCY-ε	GAAAATTGTACGTATATATCGACTTC	FOR	365
	TAGTTATTTGTGAAAGGAAGATCAG	REV	
LCY-β	TAGATATGAAGGATATTCAGGAAAG	FOR	358
	AGTAGAATATCCATACCAAAACAGA	REV	
CHY-β	GTTTACTGTTTAGTCCTTTAAGTCG	FOR	334
	AACATCTCAGTATATGGAACTTCTC	REV	
ZEP	GTAGTAAACATGGTACTTGGATCAC	FOR	355
	CATTTCTAGTGATTTCTTGTTCTG	REV	
NCED	GGGAAGATCCCGGAGTGTAT	FOR	381
	GTGGTACGGCAAATCGTCTT	REV	
α-Tubulin	CAACTACCAGCCACCAACTGT	FOR	220
	CAAGATCCTCACGAGCTTCAC	REV	

Determination of ABA content

The ABA content of sweetpotato callus was measured using a Phytodetek ABA enzyme immunoassay test kit (Agdia, Inc., Elkhart, IN), as described elsewhere (Artsaenko et al. 1995), with slight modifications. Color was detected with the Bio-Rad i-Mark Microplate Reader at a wavelength of 405 nm (Bio-Rad, Hercules, CA, USA). Two independent experiments were carried out.

Salt stress

To examine salt-stress tolerance, 2-week-old calli were incubated for 24 h in Murashige and Skoog (MS) liquid medium containing 3% sucrose, 5% Phyto agar and 1 mg 2,4-D l^{-1} and supplemented with 150 or 200 m*M* NaCl.

Qualitative and quantitative analysis of H_2O_2

Sweetpotato calli were incubated in a solution containing 1 mg of 3,3-diaminobenzidine (DAB)-HCl (pH 3.8) ml^{-1} for 5 h at 25°C under continuous light, as described by Chadwick et al.(1995). Oxidized DAB was visualized as a dark-polymerization product resulting from the reaction between DAB and H_2O_2. To quantify H_2O_2 production, the absorbance of the DAB solution from each callus was measured at 460 nm. The oxidized DAB content was determined from a calibrated DAB standard curve.

Analysis of RWC

Tissue dehydration was analyzed according to the relative water content (RWC) of the transgenic calli after treatment with 150 or 200 m*M* NaCl. The RWC was measured as follows: RWC (%) = (fresh weight − dry weight)/fresh weight (Zhao et al. 2004).

Statistical analysis

Experimental data were determined by one-way ANOVA. Subsequent multiple comparisons were examined based on the least significant difference (LSD) test. All statistical analyses were carried out following the method described by Kim et al. (2012).

Results

Characterization of *IbLCY-ε* RNAi transgenic sweetpotato calli

To regulate β-branch pathway carotenoid synthesis in sweetpotato, including that of β-carotene, by suppressing the *LCY-ε* gene, a 450 bp partial fragment of *LCY-ε* (accession no. HQ828093) was isolated from sweetpotato cDNA by RT-PCR (based on the sequence of the *LCY-ε* gene from morning glory; data not shown). Transgenic sweetpotato calli expressing RNAi-suppressed *IbLCY-ε* under the control of the CaMV 35S promoter were prepared by *Agrobacterium*-mediated transformation (Fig. 1A). Twenty-nine transgenic cell lines were obtained and subsequently confirmed by genomic PCR analysis (data not shown). Three independent transgenic lines with low levels of *IbLCY-ε* gene expression and high β-carotene content were selected for further characterization. The expression of *IbLCY-ε* transcripts was reduced in all transgenic lines

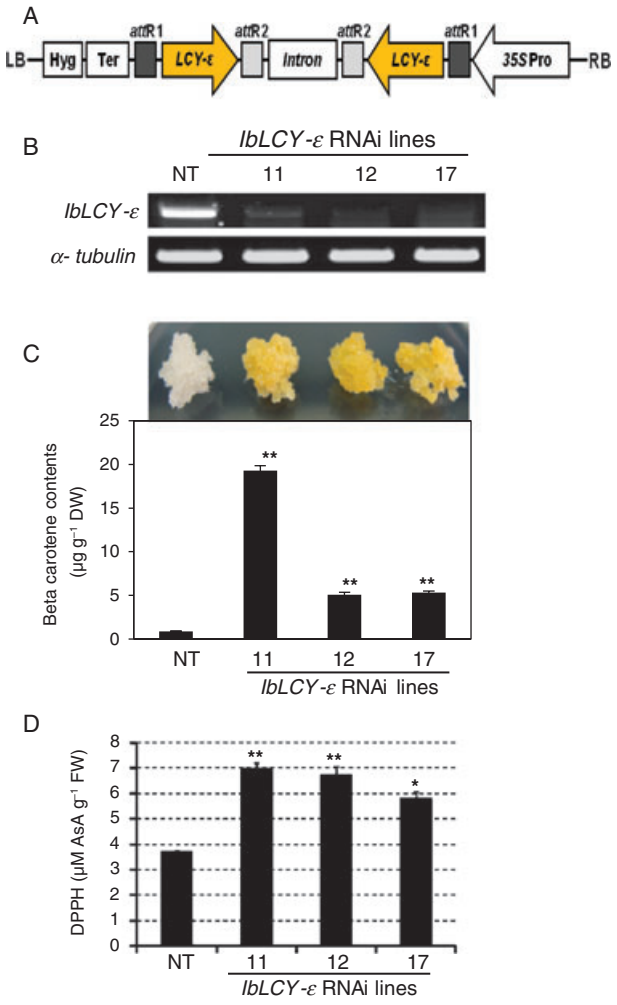

Fig. 1. Characterization of *IbLCY-ε* RNAi transgenic sweetpotato calli. (A) Construction of the CaMV 35S promoter: *IbLCY-ε* RNAi. (B) Expression of *IbLCY-ε* transgenic sweetpotato calli. Data indicate the products of 30 PCR cycles. (C) Color and total β-carotene content and (D) DPPH radical-scavenging activity in *IbLCY-ε* transgenic sweetpotato calli.

but remained high in non-transgenic (NT) lines (Fig. 1B). The *IbLCY-ε* RNAi transgenic calli were orange, whereas NT calli were white (Fig. 1C). To further ascertain whether *IbLCY-ε* expression levels in the transgenic cell lines correlated with β-carotene content, the latter was measured (Fig. 1C). In line 11, for example, the β-carotene content was 21.4-fold higher than that of NT calli. All transgenic lines in this study also showed enhanced DPPH radical-scavenging activity (Fig. 1D).

The mechanisms underlying carotenoid content regulation in sweetpotato were investigated by examining the changes in *IbLCY-ε* transgenic calli (Fig. 2A, B). Total carotenoid content in the transgenic lines was increased 7–11.2-fold. Silencing of the sweetpotato *LCY-ε* gene inhibited the α-branch of the carotenoid pathway, which produces lutein, as confirmed in the transgenic lines by HPLC analysis. In contrast, increased expression of β-branch pathway compounds was determined. Specifically, the levels of β-cryptoxanthin, zeaxanthin, violaxanthin and ABA were, respectively, 57.8–163.3, 11.7–12.3, 6.2–16 and 1.8–2 times higher than in the NT lines.

The altered carotenoid and ABA content in *IbLCY-ε* transgenic calli was further examined by semi-quantitative RT-PCR analyses of the transgenic cell lines in order to characterize the transcription pattern of several of the genes involved in carotenoid biosynthesis (Fig. 2A, C). Increased expression of upstream and downstream lycopene genes was determined in *IbLCY-ε* transgenic calli, including *PDS* (accession no. HQ828091), ζ-carotene desaturase (*ZDS*, accession no. HQ828088), lycopene β-cyclase (*LCY-β*, accession no. HQ828094), zeaxanthin epoxidase (*ZEP*, accession no. HQ828089) and 9-cis-epoxycarotenoid dioxygenase (*NCED*). In contrast, β-carotene hydroxylase (*CHY-β*, accession no. HQ828095) expression was only slightly reduced.

Enhanced tolerance of *IbLCY-ε* transgenic calli to salt stress

To evaluate the effects of altered *IbLCY-ε* expression on oxidative stress tolerance, 2-week-old transgenic calli were treated with 150 or 200 m*M* NaCl for 24 h. *IbLCY-ε* transgenic calli were the most tolerant of salt-mediated oxidative stress, as determined by qualitative and quantitative analyses of H_2O_2 (Fig. 3A, B). In addition, exposure to 200 m*M* NaCl resulted in a higher RWC in the transgenic lines than in the NT lines (Fig. 3C). Thus, it appears that *IbLCY-ε* silencing is associated with an increased tolerance of salt-mediated oxidative stress conditions.

A potential correlation between *IbLCY-ε* silencing, stress tolerance and altered carotenoid biosynthesis was investigated by analyzing the changes in both carotenoid content and the transcription level of several carotenoid biosynthesis genes in transgenic calli subjected to 150 m*M* NaCl. Indeed, under salt-stress conditions, several β-branch pathway carotenoids (β-carotene, β-cryptoxanthin, zeaxanthin and violaxanthin), as well as total carotenoids, were higher in transgenic calli than in NT calli (Fig. 4A). Silencing of the *LCY-ε* gene reduced the lutein content of the transgenic lines under both normal culture and salt-stress conditions. Total carotenoids and β-carotene levels in the NT lines exposed to salt stress decreased by only 0.2- and 0.8-fold, respectively, whereas in transgenic lines 12 and 17, total carotenoid levels were 2.0-fold lower

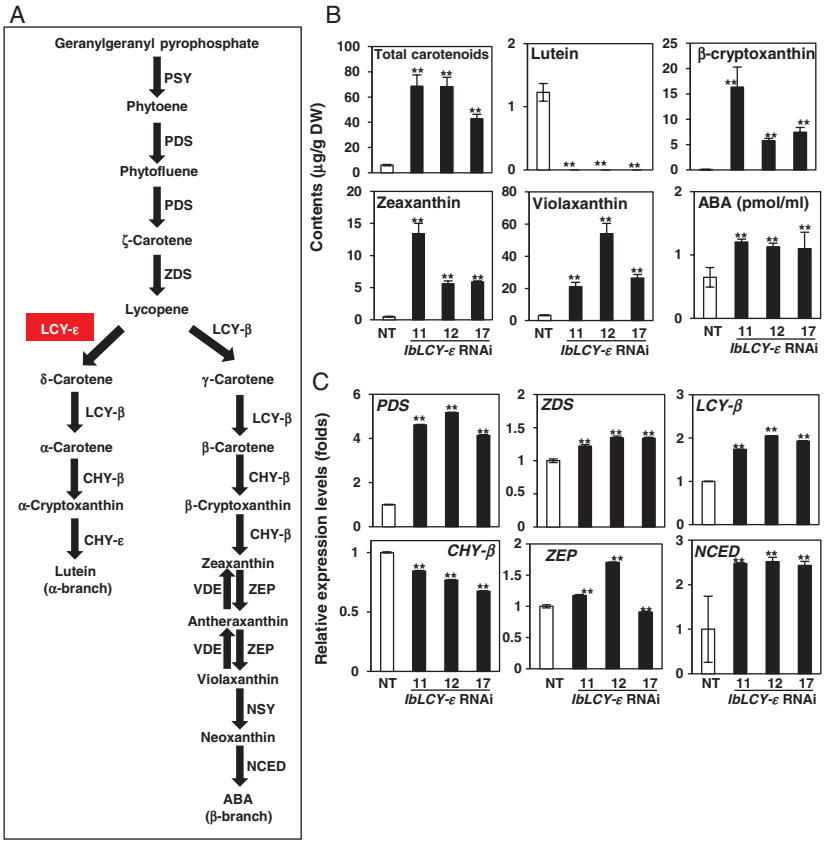

Fig. 2. Carotenoid content and expression of carotenoid-biosynthesis-related genes in *IbLCY-ε* transgenic calli. (A) The carotenoid biosynthesis pathway in plants. Box represents the carotenoids and related genes analyzed in this study. *PSY*, phytoene synthase; *PDS*, phytoene desaturase; *ZDS*, ζ-carotene desaturase; *CRTISO*, carotenoid isomerase; *LCY-β*, lycopene β-cyclase; *LCY-ε*, lycopene ε-cyclase; *CHY-β*, β-carotene hydroxylase; *CHY-ε*, ε-ring hydroxylases; *VDE*, violaxanthin de-epoxidase; *ZEP*, zeaxanthin epoxidase; *NSY*, neoxanthin synthase; *NCED*, 9-cis-epoxycarotenoid dioxygenase; ABA, abscisic acid. (B) Total and individual carotenoid contents in sweetpotato calli. The content of each carotenoid was analyzed by HPLC using five calli. The data are expressed as the mean ± SD of two replicates. (C) Expression pattern of various carotenoid-biosynthesis-related genes in sweetpotato calli.

than in the controls. In addition, salt stress reduced the expression of *IbLCY-ε* in transgenic lines compared to NT lines (Fig. 4B). Interestingly, while under salt-mediated oxidative stress the expression of *PDS*, *ZDS*, *LCY-β* and *CHY-β* was higher in the transgenic lines than in the NT lines, there was no difference in *ZEP* expression.

ABA content and *NCED* expression also increased in the transgenic and NT calli after salt treatment (Fig. 5). ABA content increased by 1.5-fold in the NT lines and by 1.8–2.6-fold in the transgenic lines after treatment with 150 m*M* NaCl.

Discussion

During the life cycle of plants, carotenoids play important physiological roles in growth and in defense responses to various environmental stresses (Cunningham et al. 1996, Cunningham and Gantt 1998). Despite considerable efforts to investigate carotenoid biosynthesis, metabolic engineering has not been applied to elucidate the underlying regulatory mechanisms in several useful crop plants. Among these, sweetpotato is of considerable industrial interest as the source of materials such as starch, bio-ethanol and antioxidants, as well as a healthy food and feed crop that can be grown on marginal land (Ziska et al. 2009). The interest of our laboratory is in developing industrial transgenic sweetpotato plants with high concentrations of nutrients, including carotenoids, and an increased stress tolerance that will allow their sustainable culture.

In a previous study, we applied metabolic engineering to obtain significant increases in the concentrations of β-carotene and total carotenoids in *IbCHY-β* RNAi calli of sweetpotato (Kim et al. 2012). The results were followed up in this study, in which overall

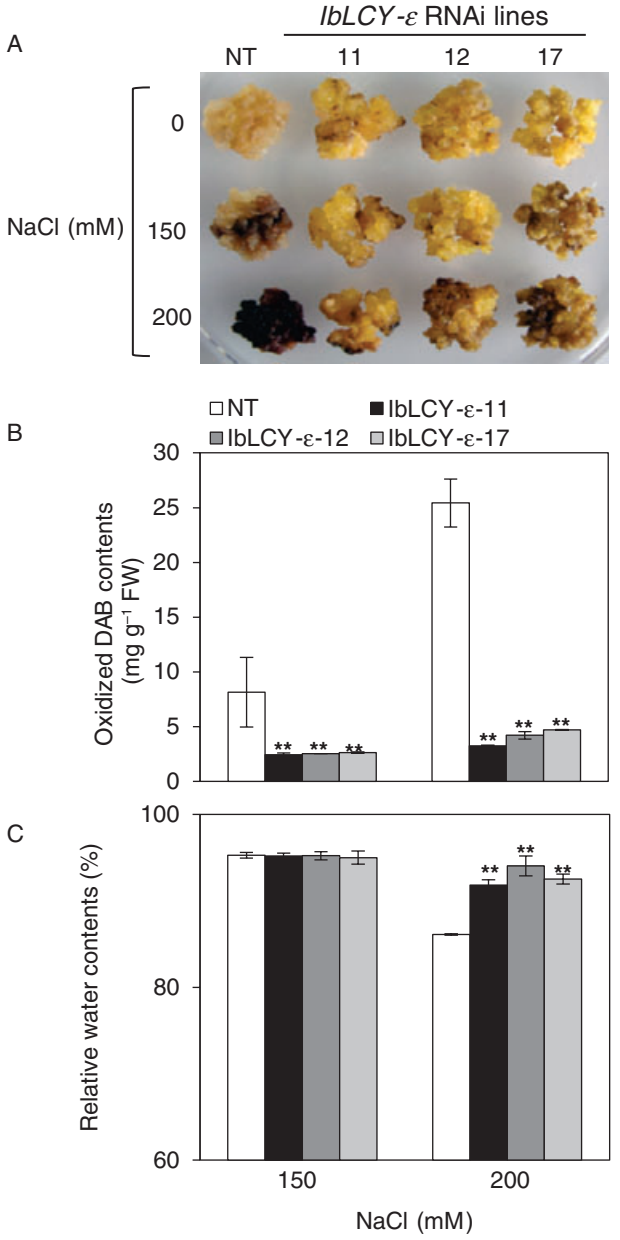

Fig. 3. Effect of salt-mediated oxidative stress on transgenic *IbLCY-ε* calli after NaCl treatment for 24 h. (A) Phenotypical analysis of H_2O_2 content and analyses, (B) H_2O_2 content and (C) relative water content (RWC) of sweetpotato calli.

carotenoid synthesis via the β-branch-specific pathway (which yields β-carotene and ABA) in sweetpotato was investigated. We found that the expression of *IbLCY-ε* could be downregulated by RNAi technology applied to the non-embryogenic calli of sweetpotato. Metabolic engineering of *IbLCY-ε* gene function using non-embryogenic sweetpotato calli was chosen because this system is similar to sweetpotato plants and transgenic calli can be easily prepared. Thus, *IbLCY-ε*-silenced transgenic sweetpotato calli under the control of the CaMV 35S promoter were successfully generated. Altered expression of the *IbLCY-ε* gene resulted in the increased production of carotenoids via the β-branch-specific pathway, while conferring increased tolerance to salt-mediated oxidative stress. The NT white cultivar of sweetpotato used in this study also expressed significant levels of endogenous carotenoid transcripts without the simultaneous accumulation of carotenoid metabolites, even though the total carotenoid content was higher in *IbLCY-ε* transgenic calli than in NT calli (Fig. 2B, C). Similar results were reported for the expression of major carotenoid biosynthesis genes in white carrot (Clotault et al. 2008).

All plant species, regardless of their color, contain carotenoid pathway in their various organs, since these compounds together with the plant hormone ABA are essential for plant growth and development as well as adaptation to unfavorable environmental stresses. Many studies have engineered a higher β-carotene content in crop plants using strategies such as the enhancement of precursor-phytoene content (Shewmaker et al. 1999, Ducreux et al. 2005) and the upregulation of *LCY-β* expression (Rosati et al. 2000, Ravanello et al. 2003). Both *LCY-ε* and *LCY-β* are required for the synthesis of α-carotene, while *CHY-β* and *CHY-ε* are required downstream for lutein synthesis via the α-branch of the carotenoid biosynthesis pathway (Pogson et al. 1996). Our previous study showed that the downregulation of *IbCHY-β* in transgenic sweet potato calli increased several carotenoids, such as α-carotene, β-carotene, β-cryptoxanthin and zeaxanthin, in addition to enhancing cellular antioxidant capacity (Kim et al. 2012). Downregulation of *IbLCY-ε* in the α-branch pathway leads to the production of β-branch pathway carotenoids, as evidenced in this study, in which calli transgenic for *IbLCY-ε* RNAi showed increased contents of the carotenoids β-carotene, β-cryptoxanthin, zeaxanthin and violaxanthin and the phyto-hormone ABA, all of which are derived from the β-branch-specific pathway (Figs 1 and 2). As expected, in the transgenic lines, these increases were accompanied by a dramatic reduction in the lutein content.

ε-Cyclization of lycopene is one of the key regulatory steps in carotenoid biosynthesis via the β-branch-specific pathway in sweetpotato. However, recent studies have shown that the suppressed expression of *LCY-ε* increases carotenoid levels via both the α- and β-branch pathways. Yu et al. (2008) reported that the downregulation of *LCY-ε* in transgenic *Brassica napus* resulted in increased levels of β-carotene, β-cryptoxanthin, zeaxanthin and violaxanthin via the

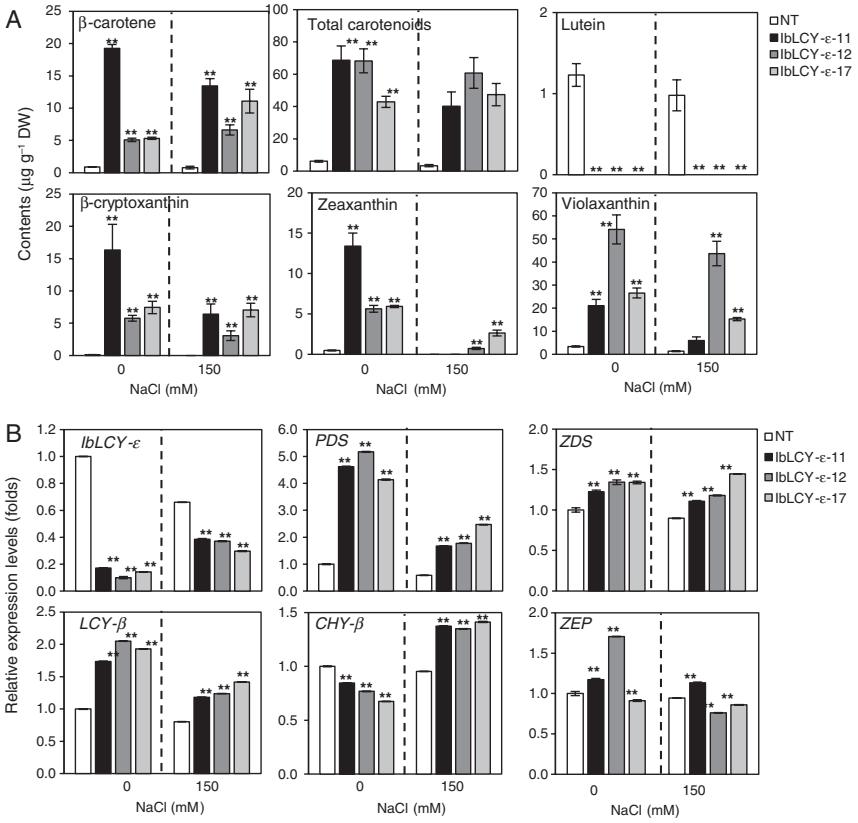

Fig. 4. Carotenoid content and expression of carotenoid-biosynthesis-related genes in *IbLCY-ε* sweetpotato calli under salt stress. (A) Quantitative analysis of total and individual carotenoid contents and (B) expression patterns of individual carotenoid biosynthesis-related genes in transgenic sweetpotato calli after treatment with 0 or 150 m*M* NaCl.

β-branch pathway, and that lutein content increased as well. In potato plants, tuber-specific silencing of the *LCY-ε* gene was associated with increased levels of carotenoids via the *β*-branch pathway and increased lutein content (Diretto et al. 2006). To our knowledge, this study is the first in which carotenoid synthesis via the *β*-branch pathway was specifically increased by the engineered silencing of *LCY-ε* in transgenic sweetpotato. We also found that NT calli (cv. Yulmi) produce violaxanthin ($3.37 \pm 0.29\,\mu g\,g^{-1}$ DW) as the main carotenoid, with comparatively lower amounts of *β*-carotene (0.9 ± 0.02) and *β*-cryptoxanthin (0.1 ± 0.06) (Figs 1 and 2). Violaxanthin usually indicates low provitamin A activity, whereas *β*-carotene and *β*-cryptoxanthin, synthesized via the *β*-branch pathway, are two of the most important provitamin-A-containing carotenoids in plants (de Pee and West 1996). Therefore, based on our analysis, silencing of the *LCY-ε* gene can be used to enhance provitamin A activity in sweetpotato calli by increasing *β*-carotene and *β*-cryptoxanthin levels (Figs 1 and 2). In addition, all transgenic lines exhibited increased ROS-scavenging activity (Fig. 1C).

High salinity is a major environmental stress that limits crop productivity in arid and semi-arid regions of the world (Munns et al. 2006). Recent studies noted an improved tolerance to abiotic stress induced by carotenoid metabolic engineering in plants, and that oxidative stress tolerance improves resistance to environmental stresses, including salt stress (Luo et al. 2009). Thus, in accordance with our results, it should be possible to generate transgenic plants with increased tolerance to environmental stresses by manipulating the genes involved in carotenoid biosynthesis. Han et al. (2008) reported that transgenic *Arabidopsis* expressing the *PSY* gene isolated from the halophyte plant *Salicornia europaea* showed enhanced tolerance to salt and oxidative stress. Chen et al. (2011) recently reported that the overexpression of the *LCY-β* gene from *S. europaea* and *Arabidopsis* conferred salt tolerance upon transgenic *Arabidopsis* and tobacco plants. These results are consistent with our previous finding that transgenic (RNAi-*IbCHY-β*) sweetpotato calli with enhanced carotenoids better tolerate salt-mediated oxidative stress (Kim et al. 2012).

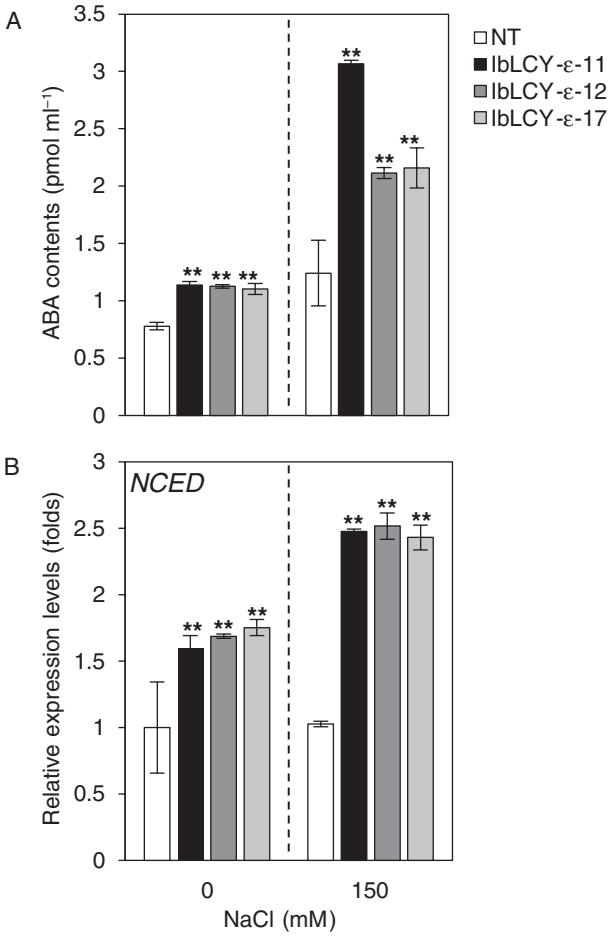

Fig. 5. Analysis of ABA content and expression of the ABA biosynthesis-related gene *NCED* in *IbLCY-ε* transgenic calli under salt-stress conditions. (A) Quantitative analysis of total ABA content and (B) expression pattern of the ABA biosynthesis-related gene *NCED* in transgenic sweetpotato calli after treatment with 0 or 150 m*M* NaCl.

However, there were no reports of enhanced salt-stress tolerance in *LCY-ε*-engineered plants. The results described herein show that, upon exposure to 150 m*M* NaCl, the transgenic lines maintained their higher levels of *β*-branch carotenoids, although as in the NT lines, carotenoid levels were generally reduced (Fig. 4); nonetheless, transgene-expressing calli exhibited both increased tolerance to salt-mediated oxidative stress and reduced ROS levels (Fig. 3). Many studies have suggested that various stresses, including salt stress, lead to oxidative stress. The increased levels of carotenoids and the higher antioxidant activity in the *IbLCY-ε* RNAi lines may contribute to overcoming salt-mediated oxidative stress in plants. High levels of vitamin C, lycopene and *β*-carotene were previously shown to enhance the antioxidant capacity of tomato plants exposed to salt stress (Krauss et al. 2005, Kim et al. 2012).

The phyto-hormone ABA is involved in abiotic stress responses, including high salinity, and is associated with stress-tolerance mechanisms induced by signaling molecules (Schachtman and Goodger 2008). Carotenoids are the precursors of ABA biosynthesis in a pathway involving NCED (Lindgren et al. 2003). As shown in this study, *IbLCY-ε* silencing in transgenic sweetpotato calli increases the ABA content by enhancing carotenoid synthesis, both under normal culture conditions and following salt treatment (Figs 2 and 5). The increased ABA levels in the transgenic lines suggested that the altered ABA content was important in the tolerance of salt-mediated oxidative or osmotic stress conditions. Indeed, consistent with the increased ABA levels, *IbCHY-β* transgenic sweetpotato calli showed enhanced *NCED* gene expression, supporting the greater tolerance of salt-mediated oxidative stress conditions by *IbCHY-β* transgenic calli than by their NT counterparts (Kim et al. 2012). Thus, the downregulation of *IbLCY-ε* seems to induce an increase in carotenoid synthesis via the *β*-branch-specific pathway, which, together with the higher ABA levels, protects against salt-mediated oxidative stress. Whether these compounds also confer tolerance to other stresses, such as drought and extreme temperature, remains to be determined.

Further studies are needed to understand the exact roles of ABA and the *IbLCY-ε* gene in the regulation of carotenoids produced via the *β*-branch-specific pathway in the whole plant. Our results suggest that the downregulation of the *IbLCY-ε* gene provides a biotechnological approach to the generation of high value-added transgenic plants with enhanced tolerance to environmental stresses and the ability to grow on marginal lands. Our current efforts are aimed at developing transgenic sweetpotato plants that produce high levels of *β*-branch carotenoids and ABA, using cultivars of different colored storage roots.

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