Plant Physiology and Biochemistry 117 (2017) 24–33

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Suppression of the *β-carotene hydroxylase* gene increases β-carotene content and tolerance to abiotic stress in transgenic sweetpotato plants

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ARTICLE INFO

Article history:
Received 21 December 2016
Received in revised form
26 May 2017
Accepted 26 May 2017
Available online 29 May 2017

Keywords:
β-carotene hydroxylase
Carotenoid
Oxidative stress
RNA interference
Salt stress
Sweetpotato

ABSTRACT

β-carotene, a carotenoid that plays a key photo-protective role in plants is converted into zeaxanthin by β-carotene hydroxylase (CHY-β). Previous work showed that down-regulation of *IbCHY-β* by RNA interference (RNAi) results in higher levels of β-carotene and total carotenoids, as well as salt stress tolerance, in cultured transgenic sweetpotato cells. In this study, we introduced the RNAi-*IbCHY-β* construct into a white-fleshed sweetpotato cultivar (cv. Yulmi) by *Agrobacterium*-mediated transformation. Among the 13 resultant transgenic sweetpotato plants (referred to as RC plants), three lines were selected for further characterization on the basis of *IbCHY-β* transcript levels. The RC plants had orange flesh, total carotenoid and β-carotene contents in storage roots were 2-fold and 16-fold higher, respectively, than those of non-transgenic (NT) plants. Unlike storage roots, total carotenoid and β-carotene levels in the leaves of RC plants were slightly increased compared to NT plants. The leaves of RC plants also exhibited tolerance to methyl viologen (MV)-mediated oxidative stress, which was associated with higher 2,2-diphenyl-1- picrylhydrazyl (DPPH) radical-scavenging activity. In addition, RC plants maintained higher levels of chlorophyll and higher photosystem II efficiency than NT plants after 250 mM NaCl stress. Yield of storage roots did not differ significantly between RC and NT plants. These observations suggest that RC plants might be useful as a nutritious and environmental stress-tolerant crop on marginal lands around the world.

1. Introduction

Sweetpotato [*Ipomoea batatas* (L.) Lam] is the seventh most important staple food crop in the world (Pradhan et al., 2015) and has the potential to be commercially utilized as a healthy food and in industrial materials such as starch and ethanol (Duvernay et al., 2013; Kasran et al., 2015). Sweetpotato is a rich source of dietary fiber, potassium, and natural antioxidants, including carotenoids, anthocyanin, vitamin A, C, and E (Krishnan et al., 2012; Islam et al., 2016; Ji et al., 2016). The dramatic increase in global population

Abbreviations: CHY-β, β-carotene hydroxylase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; HPLC, high-performance liquid chromatography; LCY-β, lycopene β-cyclase; LCY-ε, lycopene ε-cyclase; MV, methyl viologen; ROS, reactive oxygen species; qRT-PCR, quantitative reverse transcription-polymerase chain reaction; 2,4-D, 2,4-dichlorophenoxyacetic acid; RNAi, RNA interference.

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http://dx.doi.org/10.1016/j.plaphy.2017.05.017
0981-9428/

poses a grave challenge to energy and food supplies, healthcare, and environmental protection. Therefore, the development of sweetpotato plants with high nutritional value and environmental tolerance could not only help to solve the food crisis and malnutrition in many developing countries, but also attract the attention of developed countries (Mohanraj and Sivasankar, 2014; Laurie et al., 2015; Park et al., 2015a).

Carotenoids, which are antioxidants, are among the most diverse classes of natural compounds and are widely distributed in plants, algae, fungi, and bacteria (Domonkos et al., 2013; Avalos and Limón, 2015; Chang et al., 2015). In higher plants, carotenoids are mainly present in plastids, including chromoplasts and chloroplasts, where they play essential roles in protecting the photosynthetic machinery from photo-oxidative damage (Lucas et al., 2014; Nisar et al., 2015). Carotenoids also provide color to flowers and fruits and contribute to the production of volatile scents and flavors that attract insects and animals for pollination and seed dispersal (Wessinger, 2015). Furthermore, carotenoids ameliorate age-related macular degeneration of the eye and can serve as precursors for vitamin A in human body (Hill and Johnson, 2012).

In higher plants, carotenoids are synthesized from the 2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate pathway (MEP/DOXP pathway) of isoprenoid biosynthesis. The isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) are converted to geranylgeranyl pyrophosphate (GGPP) by the catalytic activities of IPP isomerase (IPI) and GGPP synthase (GGPS), and then two molecules of GGPP are converted into phytoene by phytoene synthase (PSY) (Cunningham and Gantt, 1998; Nisar et al., 2015). All-*trans* lycopene is produced from phytoene by a complex set of four reactions requiring phytoene desaturase (PDS), 15-cis-ζ-carotene isomerase (Z-ISO), ζ-carotene desaturase (ZDS), and carotenoid isomerase (CRTISO) (Isaacson et al., 2004; Han et al., 2014). All-*trans* lycopene is in turn the substrate of two competing cyclases: lycopene β-cyclase (LCY-β) and/or lycopene ε-cyclase (LCY-ε), which catalyze the branch point in the carotenoid biosynthesis pathway (Cunningham and Gantt, 1998). LCY-β and LCY-ε acting together form α-carotene, whereas LCY-β acting alone forms β-carotene (Fraser et al., 2001). β-carotene is redundantly hydroxylated by non-heme di-iron β-carotene hydroxylase (CHY-β) and cytochrome p450-type β-hydroxylase (Tian and DellaPenna, 2004; Tian et al., 2004). β-xanthophylls are epoxidated and de-epoxidated by zeaxanthin epoxidase (ZEP) and violaxanthin de-epoxidase (VDE), respectively, giving rise to the xanthophyll cycle (Gómez-García and Ochoa-Alejo, 2013). A family of carotenoid cleavage dioxygenases (CCD) such as 9-*cis*-epoxycarotenoid dioxygenase (NCEDs) cleave violaxanthin and neoxanthin to produce xanthoxin, which is oxidized to xanthoxic acid by aldehyde oxidase; xanthoxic acid is then converted to abscisic acid (ABA) by short chain dehydrogenase reductase (SDR) (Cunningham and Gantt, 1998; Shumskaya and Wurtzel, 2013; Hou et al., 2016). The strong antioxidant activity of carotenoids, which are primarily associated with their ability to quench single oxygen and scavenge free radicals, has been demonstrated *in vivo* and *in vitro* (Palozza and Krinsky, 1992).

Genetic engineering of carotenoid biosynthesis in crop plants has been used to improve nutritional value and environmental stress tolerance (Zhao et al., 2014; Decourcelle et al., 2015). For example, modification (overexpression or RNAi) of genes encoding key enzymes of the carotenoid biosynthesis pathway can increase β-carotene, lutein, and violaxanthin content (Fujisawa et al., 2008; Pasare et al., 2013). Simultaneous expression of *Arabidopsis PSY* or maize *PSY* and *Erwinia uredovora phytoene desaturase* (*CrtI*) induces accumulation of β-carotene and β-xanthophylls, resulting in 'Golden Rice' (Ye et al., 2000; Paine et al., 2005). Transgenic potato tubers overexpressing the cauliflower Orange (*OR*) gene, which is responsible for chromoplast differentiation in plants, showed accumulation of carotenoids and continuously increased β-carotene content over the course of long-term cold storage (Lopez et al., 2008). Transgenic sweetpotato calli overexpressing the *IbOr* gene have high carotenoid content and tolerance to salt stress (Kim et al., 2013a). In addition, down-regulation of the *LCY-ε* or *LCY-β* gene by RNAi increases carotenoid synthesis and tolerance to abiotic stress in transgenic sweetpotato calli (Kim et al., 2013b). A recent study in orange carrots revealed that a loss of function in the carotene hydroxylase gene is responsible for high carotene content (Arango et al., 2014).

CHY-β is a key regulatory enzyme in the synthesis of β-branch carotenoids (Sun et al., 1996). Previously, we reported that down-regulation of *CHY-β* increases β-carotene and total carotenoid content and increases salt tolerance in transgenic sweetpotato calli (Kim et al., 2012). However, the effects of CHY-β on overall carotenoid levels in whole plants remain unknown. In this study, we generated the transgenic sweetpotato plants by down-regulating expression of *CHY-β* gene. These plants exhibited elevated carotenoid content, antioxidant capacity, and salt stress tolerance.

2. Materials and methods

2.1. Plant materials

White-fleshed sweetpotato (cv. Yulmi) was used in this study. Sweetpotato plants were cultivated by the cutting propagation method for 4 weeks in a growth chamber at 25 ± 1 °C with 16 h day length, and maximum irradiance was approximately 10,500 μmol m^{-2} s^{-1}. Embryogenic calli were induced from shoot meristems of sweetpotato and cultured on MS1D solid medium (MS basic salt medium supplemented with 1 mg L^{-1} 2,4-D, 3% sucrose, and 0.3% Gelrite) in the dark in a 25 °C incubator. Embryogenic calli were propagated by subculture in fresh medium at 3 week intervals.

2.2. Construction of the RNAi vector

To construct the *IbCHY-β* RNAi vector, a double-stranded RNA construct was produced that contained the cauliflower mosaic virus 35S promoter (35Sp), a sense fragment of *IbCHY-β* cDNA [*IbCHY-β*-S, a region of *IbCHY-β* not similar to other genes, which was identified using BLOCK-iT™ RNAi Designer (Invitrogen, CA, USA)], a 120-nucleotide intron of *Arabidopsis thaliana RTM1*, the *IbCHY-β* fragment in antisense orientation (*IbCHY-β*-AS), and a *Nos* terminator. All fragments were obtained using Pfu-X DNA polymerase (Solgent, Daejeon, Korea) and the indicated primer pairs (listed in Supplemental Table S1). The 35Sp-(*IbCHY-β*-S)-Intron-(*IbCHY-β*-AS)-Nos (35Sp:RNAi-*IbCHY-β*) constructs were obtained using the In-fusion HD cloning kit (Takara, Dalian, China). The plasmid was constructed in the plant expression vector pCAMBIA3300.

2.3. Agrobacterium-mediated transformation

35Sp:RNAi-*IbCHY-β* plasmids were introduced into *Agrobacterium tumefaciens* EHA105 using the freeze-thaw method (Höfgen and Willmitzer, 1988). Transformed *A. tumefaciens* were grown overnight on a shaker at 200 rpm in YEP liquid media containing 200 μM acetosyringone (AS) and 100 mg L^{-1} kanamycin at a temperature of 28 °C, and then transformed into sweetpotato embryogenic callus using the *Agrobacterium*-mediated transformation method as described by Kim et al. (2012). The calli were placed on MS1D plates containing 100 μM AS and cultivated in a dark room at 28 °C for co-culture. After co-culture at 28 °C for 2–3 days, embryogenic calli were rinsed five times with sterile H_2O and

transferred to sterile filter paper. The air-dried calli were transferred to 1DCP selection medium [MS (basic salt) medium containing 1 mg L^{-1} 2,4-D, 400 mg L^{-1} cefotaxime, 1 mg L^{-1} PPT (DL-phosphinothricin), 3% sucrose, and 0.3% Gelrite] and subcultured onto freshly prepared medium at 1–2-week intervals for putative transgenic callus selection. Cefotaxime was added to prevent contamination caused by rapid growth of *Agrobacterium*, this step can be skipped if the calli are thoroughly washed and bacteria with scarcely any more growth.

2.4. Gene expression analysis

Total RNA was extracted from the indicated plant tissues using the GeneAll Ribospin Plant™ kit (GeneAll, Seoul, Korea). All quantitative RT-PCR (qRT-PCR) analyses were performed with a CFX real-time PCR system and CFX system software (Bio-Rad, CA, USA) using Ever-Green 20 fluorescent dye (BioFact, Daejeon, Korea). For cDNA production, 2 μg of total RNA was reverse-transcribed using an RT-PCR kit (Enzynomics, Daejeon, Korea). The reaction mixture was diluted to 100 μL with sterilized water and 2 μL of each reaction subjected to real-time qRT-PCR. The gene-specific primers used in this study are listed in Supplemental Table S1. The program used for PCR was as follows: initial denaturation for 5 min at 95 °C, followed by 40 cycles of 95 °C for 20 s, 60 °C for 40 s, and 72 °C for 1 min. Three biological repeats and three technical repeats were performed for each data point.

2.5. Carotenoid analysis

Carotenoids were extracted from sweetpotato storage roots and leaves, and analyzed by HPLC as described (Kim et al., 2014). Carotenoid extraction was conducted under low light to prevent loss due to degradation. Lyophilized sweetpotato storage roots (200 mg) was ground in a mortar and pestle (pre-cooled with liquid N_2) with 5 ml of acetone containing 0.01% butylated hydroxytoluene (BHT), sea sand, Na_2SO_4, and $NaHCO_3$. The resultant extract was centrifuged at 4 °C at 5000 rpm for 5 min and the supernatant concentrated *in vacuo* and re-dissolved in CH_2Cl_2:acetone (1:1, 200 μL). The solution was filtered through a 0.45-μm membrane filter (Whatman, PTFE, 13 mm) and analyzed on an Agilent 1100 HPLC system (Hewlett-Packard, Palo Alto, CA, USA). Standard or sample solution (20 μL) was injected directly onto a YMC C30 carotenoid column (3 μM, 4.6 × 250 mm, Japan) with solvent A [methanol:*tert*-butylmethyl ether (MTBE):H_2O, 81:15:4, v/v)] and solvent B (MeOH:MTBE:H_2O, 6:90:4, v/v) using a step gradient elution of 100% solvent A for the first 15 min, followed by a gradient from 100% solvent A to 100% solvent B over the next 35 min. A conditioning phase (50–60 min) was used to return the column to its initial state. The flow rate was 0.7 ml min^{-1} and the column temperature was 22 °C. The eluent was detected at 450 nm on a UV–Visible detector. ChemStation software (Hewlett–Packard, Palo Alto, CA, USA) was used to operate the HPLC-DAD system. An external calibration method was used for carotenoid quantification. One milligram of each standard was dissolved in 10 ml CH_2Cl_2/0.01% BHT, and then working calibration solutions (50, 20, 10, 5.0, 2.5, 1.0, 0.50, 0.25, 0.10, and 0.025 μg ml^{-1}) were prepared by diluting stock solutions of the external standards. Standards of α-carotene, β-carotene, β-cryptoxanthin, and zeaxanthin were purchased from CaroteNature (Lupsingen, Switzerland). Under these chromatographic conditions, standard carotenoids produced peaks at t_R (min) values of 37.3 for α-carotene, 39.2 for β-carotene, 33.5 for β-cryptoxanthin, and 26.6 for zeaxanthin. Carotenoid content was measured by HPLC analysis in five different sweetpotato storage roots. All content levels were expressed as the mean (average content per g dry weight) ± SD (standard deviation) of two independent determinations.

2.6. Ion leakage analysis

Ion leakage was analyzed using leaf discs according to the method of Kim et al. (2011), with some modifications. Round leaf discs (1.0 cm in diameter) were punched from the fifth fully expended leaves of 1-month-old plants using a hole puncher. Seven leaf discs were floated in a solution containing 0.4% (w/v) sorbitol (sterilized) and freshly made methyl viologen (MV) at various final concentrations (0, 1, 2.5, 5 and 10 μM), and placed in the dark for 12 h to facilitate diffusion of the MV into the leaf discs. Afterward, the leaf discs were moved to a 25 °C incubator under light conditions. The electrical conductance the MV solution was measured every 12 h using an ion conductivity meter (Istek, Model 455C), and total conductivity was measured after the solutions containing the leaf discs were autoclaved after the last time point. Based on the relative ion leakage, loss of cytoplasmic solutes following the MV treatment was calculated by comparing instant electrical conductance with total conductivity. Data are expressed as the mean of three replicates, with each replicate consisting of seven leaf discs.

2.7. DPPH radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity of sweetpotato plants was measured as described by Kim et al. (2012), with some modifications. Storage root samples (200 mg) of the RC plants and NT plants were ground in 1 mL methanol and the extract centrifuged at 13,000 rpm for 5 min. An aliquot of each supernatant (100 μL) was added to 0.5 mM DPPH in methanol (750 μL). After vortexing, the mixture was incubated in the dark for 15 min. Absorbance of each sample was recorded at 517 nm against a blank (methanol). L-Ascorbic acid (AsA, 0.015–0.125 mM) was used as the standard for the calibration curve. DPPH radical-scavenging activity was expressed as mol AsA equivalent per gram of tested sample. Graphs display means of three values for each sample.

2.8. NaCl treatments

Three-week-old RC and NT plants were treated with 250 mM NaCl solution every 3 days for 6 days. After 6 days of treatment, the NaCl solution was discarded and replaced with water.

2.9. Analysis of photosynthetic activity

Photosynthetic activity in leaves was estimated based on chlorophyll fluorescence determination of photochemical yield (Fv/Fm), which represents the maximal yield of the photochemical reaction in photosystem II (PSII). Fv/Fm values were detected from the fifth intact fully expanded leaves (from the shoot apical meristem) of sweetpotato plants following 30 min of dark adaption, using a portable chlorophyll fluorescence meter (Handy PEA, Hansatech, England).

2.10. Chlorophyll content

Chlorophyll content of sweetpotato plants was measured using leaf disks as previously described (Aron, 1949) with some modifications. Round leaf discs of three-week-old RC and NT plants were prepared as described in this study (as referred to in 2.6 ion leakage analysis). Sufficient leaf discs were floated in a solution containing 250 mM NaCl and placed in 25 °C incubator with 16 h day length. 0.1 g fresh leaf powder were dispensed and extracted twice using 80% acetone, after centrifuged, supernatant was measured by

spectrophotometric method and total amount of chlorophylls ($a+b$) was calculated.

2.11. Detection of ABA in leaves

The ABA detection was performed as previously described (Artsaenko et al., 1995). Fifth intact fully expanded leaves of three-week-old RC and NT plants were sampled and frozen in N2. 0.4 g fresh leaf powder was suspended with 4 ml 80% acetone-H_2O (80:20, v/v) at 4 °C overnight in the dark. After centrifuged at 4000 rpm for 10 min, the supernatant was diluted 1:10 and 1:100 with TBS buffer (provided in kit), ABA amount was determined with a Phytodetek ABA enzyme immunoassay test kit (Agdia Inc. Elkhart, USA) following manufacturer instruction, and absorbance at 405 nm was detected with Victorx multilabel readers (PerkinElmer, USA). Three biological repeats and three technical repeats were performed for each data point.

2.12. Data analysis

Statistical analyses were performed using SPSS (Statistical Package for the Social Sciences). *P < 0.05 or **P < 0.01 (determined by the by t-test) was interpreted as statistically significant.

3. Results

3.1. Characterization of RC transgenic sweetpotato plants

RNAi is an effective tool for experimentally manipulating gene expression and probing gene function on a genome-wide scale in both plants and animals (Hannon, 2002). Previously, we generated transgenic sweetpotato calli in which *CHY-β* was down-regulated by RNAi. In the current study, an RNAi construct was introduced into sweetpotato plants (referred to as RC) by *Agrobacterium*-mediated transformation to generate *CHY-β* knockdown transgenic plants. Transgenic plants were selected on medium containing BASTA (*Bar*) (Fig. 1A). Thirteen independent RC plants were confirmed by genomic PCR analysis using *bar* gene primers (Fig. 1B) and three lines (RC5, RC6 and RC7) were selected for further study based on RT-PCR analysis (Fig. 1C).

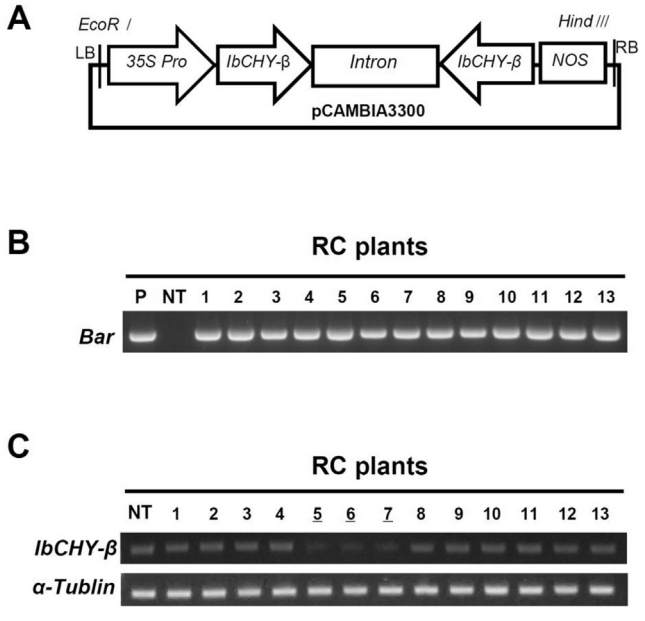

Fig. 1. Generation of transgenic sweetpotato plants by down-regulation of *IbCHY-β* (RC plants). (A) Schematic diagram of the constructs. (B) Genomic DNA PCR analysis of the RC plants with *bar* gene. P, positive control; NT, non-transgenic plant; Numbers (1–13) represent independent transgenic lines. (C) Transcript levels of *IbCHY-β* gene in NT and 13 independent RC plants, as determined by RT-PCR. The sweetpotato *tubulin* gene was used as an internal control.

To determine whether down-regulation of *CHY-β* resulted in increased carotenoid synthesis in RC transgenic sweetpotato plants, we first evaluated the morphological phenotypes of NT and RC plants. Sweetpotato stem cuttings (NT, RC5, RC6 and RC7) were grown in a living modified organisms (LMO) field for 4 months. One-month-old RC plants exhibited no significant phenotypic alterations in aerial plant parts (Fig. 2A). In addition, the yields of aerial parts and tuberous roots were not significantly different between NT and RC plants under field conditions (Supplemental Fig. 1). However, storage roots of RC plants harvested from LMO fields exhibited somewhat deeper color densities (faint orange color) than those of the NT plants (faint yellow color) (Fig. 2A).

3.2. Carotenoid content and RT-PCR analysis in RC transgenic sweetpotato plants

To determine whether the pigmentation in storage roots of RC plants was the result of carotenoid accumulation, we analyzed the carotenoid content in storage roots and leaves of NT and RC plants by HPLC. RC transgenic plants exhibited variations in composition and levels of carotenoids in storage roots (Table 1) and leaves (Table 2). The total carotenoid levels in storage roots of RC5 (20.71 μg g^{-1} DW), RC6 (23.14 μg g^{-1} DW) and RC7 (24.18 μg g^{-1} DW) were 2.26–2.63 fold higher than those of NT (9.18 μg g^{-1} DW) (Table 1). RC plants contained 16.22–18.53 times more β-carotene in their storage roots than NT plants, whereas α-carotene was statically not changed. Isomer of β-carotene such as 13Z-β-carotene and 9Z-β-carotene was increased up to 2.67 and 1.53 fold compared to NT, respectively. Interestingly, β-cryptoxanthin content (μg g^{-1} DW) was significantly increased ranged from 1.73 (RC5) to 2.51 (RC7), while that of NT plants were 0.30. Furthermore, zeaxanthin contents were slightly increased ranged from 2.68 to 3.65 compared to NT (2.64) (Table 1). In the leaves, the total carotenoid levels of RC5 (3302.0 μg g^{-1} DW), RC6 (3338.2 μg g^{-1} DW) and RC7 (3205.4 μg g^{-1} DW) were 1.1–1.2 fold higher than those of NT (2857.7 μg g^{-1} DW) (Table 2). Unlike storage roots, β-carotene, 13Z-β-carotene, and 9Z-β-carotene were slightly increased compared to NT plants. Interestingly, violaxanthin content in leaves of RC plants were 1.7–1.8 fold higher than those of NT. Taken together, these results suggested that down-regulation of *CHY-β* in RC plants lead to elevated carotenoid content, including a marked increase in the β-carotene levels.

Next, we investigated transcript levels of genes related to carotenoid biosynthesis in storage roots of NT and RC plants. As expected, *CHY-β* transcript levels were reduced in RC plants. In addition, the transcript levels of upstream genes involved in carotenoid biosynthesis pathways were significantly lower in RC than in NT plants (Fig. 2B). By contrast, the transcript levels of *LCY-β* and *LCY-ε*, which encode the enzymes that catalyze the branch point in the carotenoid biosynthesis pathway, did not significantly differ between NT and RC plants. Interestingly, transcript levels of downstream genes such as *ZEP* and *NCED* were also significantly decreased relative to those in NT plants (Fig. 2B).

3.3. RC plants exhibit increased oxidative stress tolerance

Carotenoids from various sources have received increasing attention due to the desirable physiological effects associated with their antioxidant properties. To evaluate the oxidative stress tolerance of RC plants, we incubated detached leaves in 5 μM MV, a

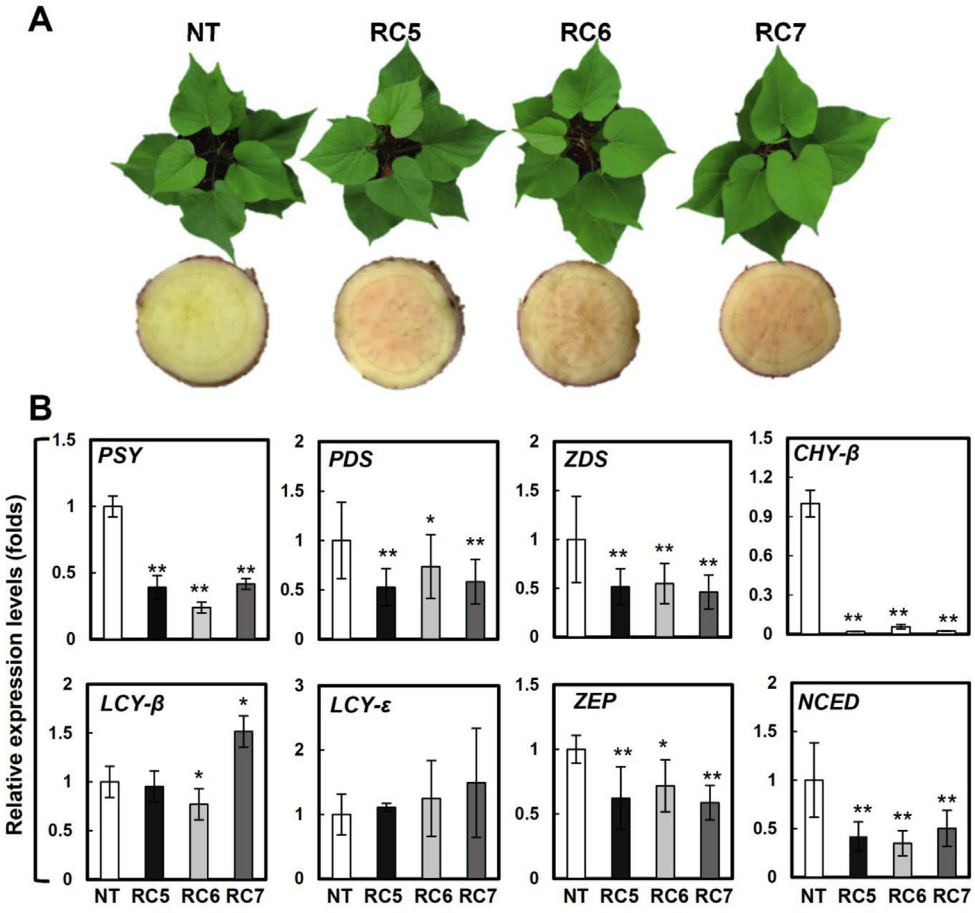

Fig. 2. Expression patterns of genes related to carotenoid biosynthesis in storage roots of RC plants. (A) Phenotypes of young plants and storage roots of NT and RC plants. (B) Transcript levels of genes related to carotenoid biosynthesis were normalized against the corresponding transcript level of the sweetpotato *tubulin* gene, which was used as an internal control. The storage roots harvested from LMO field were used for gene expression analysis. Data are expressed as the mean ± SD of three biological repeats. Asterisks indicate significant difference relative to NT at * $P < 0.05$ or **$P < 0.01$ (t-test).

Table 1
Carotenoid content in the storage roots of non-transgenic and transgenic sweetpotato plants (μg g^{-1} dry wt).

Line	α-carotene	13Z-β-carotene	9Z-β-carotene	β-carotene	β-crypto-xanthin	zeaxanthin	others	Total carotenoid
NT	0.65 ± 0.08	0.33 ± 0.03	0.32 ± 0.03	0.47 ± 0.07	0.30 ± 0.11	2.64 ± 0.63	4.44 ± 1.23	9.18 ± 2.18
RC5	0.61 ± 0.05	0.79 ± 0.05*	0.49 ± 0.04*	7.63 ± 1.16**	1.73 ± 0.20**	2.68 ± 0.32	6.77 ± 1.23*	20.71 ± 3.05**
RC6	0.62 ± 0.07	0.81 ± 0.08*	0.46 ± 0.13*	8.10 ± 0.75**	2.06 ± 0.31**	3.65 ± 0.59*	7.44 ± 1.12*	23.14 ± 3.06**
RC7	0.62 ± 0.07	0.89 ± 0.02*	0.49 ± 0.04*	8.71 ± 0.17**	2.51 ± 0.23**	3.31 ± 0.46	7.65 ± 0.70*	24.18 ± 1.70**

Table 2
Carotenoids content in the leaves of non-transgenic and transgenic sweetpotato plants (μg g^{-1} dry wt).

Line	α-carotene	13Z-β-carotene	9Z-β-carotene	β-carotene	violaxanthin	lutein	others	Total carotenoid
NT	302.4 ± 8.6	35.0 ± 2.6	99.1 ± 7.4	757.2 ± 33.4	294.9 ± 52.7	1136.0 ± 32.5	233.1 ± 12.8	2857.7 ± 130.1
RC5	252.1 ± 14.4*	44.1 ± 5.9*	112.5 ± 8.3*	970.8 ± 76.3*	487.6 ± 70.5*	1130.5 ± 45.3	304.5 ± 22.4*	3302.0 ± 149.7*
RC6	258.5 ± 11.8*	46.2 ± 2.4*	114.9 ± 3.5*	963.5 ± 39.1*	533.5 ± 28.2*	1130.8 ± 91.7	290.7 ± 4.9*	3338.2 ± 159.7*
RC7	222.1 ± 6.1**	43.0 ± 1.8*	119.3 ± 4.1*	941.3 ± 52.1*	506.3 ± 10.0*	1066 ± 70.4	307.2 ± 61.3*	3205.4 ± 118.7*

The amount of each carotenoid was measured by HPLC analysis with a minimum of five different plants. All levels are expressed as the mean (mg g^{-1} dry wt) ± SD of two independent determinations. NT: non-transgenic plant; RC5, RC6 and RC7: transgenic plant lines. Asterisks indicate significant difference relative to NT at * $P < 0.05$ or **$P < 0.01$ (t-test).

typical reactive oxygen species (ROS)-generating chemical, and quantified the loss of cytoplasmic solutes based on electrical conductance. After 24 h of MV treatment, detached leaves of NT plants exhibited more cellular damage (approximately 3.5-fold normal levels), whereas the RC plants exhibited less membrane damage (approximately 1.6-fold normal levels) (Fig. 3A and B). Furthermore, to investigate antioxidant activity in transgenic sweetpotato plants, we also analyzed 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity in storage roots of NT and RC plants. RC plants had 1.75-fold higher DPPH activity

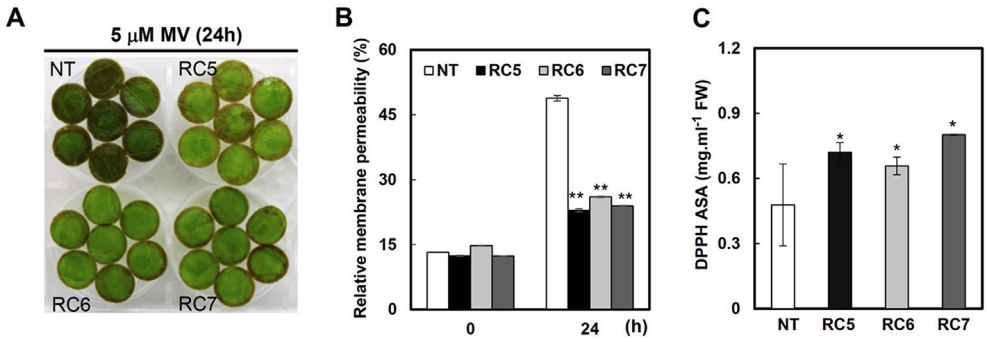

Fig. 3. Antioxidant activity of RC plants. (A) Differential visible damage of leaf discs. (B) Relative membrane permeability of fifth leaves of 1-month-old NT and RC plants over 3 days of MV-mediated oxidative stress. Percentages of relative membrane permeability were calculated using 100% to represent values obtained after autoclaving. (C) DPPH radical scavenging activity in the storage roots. Data are expressed as the mean ± SD of three biological repeats. Asterisks indicate significant differences relative to NT at * $P < 0.05$ or ** $P < 0.01$ (t-test).

than NT plants, with the highest level observed in RC7 lines (Fig. 3C). These results suggest that increased carotenoid content in RC plants confer tolerance to oxidative stress.

3.4. RC plants exhibit increased tolerance to salt stress

To determine whether down-regulation of *CHY-β* in sweetpotato confers salt stress tolerance, we irrigated 1-month-old of NT and RC plants with 250 mM NaCl solution every 3 days for 6 days. Before treatment, the health status of NT and RC plants was indistinguishable (Fig. 4A). However, NT plants exhibited severe wilting and chlorosis, much more rapidly than RC plants, under salt stress. This difference in wilting symptoms became even more pronounced 9 days after recovery (Fig. 4A). RC plants maintained higher photosystem II efficiency at 6 days after treatment than those of NT plants (Fig. 4B). Furthermore, salinity has an inhibitory effect on chlorophyll synthesis resulting in a reduction in chlorophyll contents. Thus, we measured the chlorophyll contents in the fifth leaf (from the top) of each plant. Before salt stress treatment, all plants showed similar levels of chlorophyll content. However, the chlorophyll contents of NT plants were severely reduced by salt stress, whereas those of RC plants were slightly reduced, consistent with their salt-resistant phenotype (Fig. 4C). In addition, we investigated transcript levels of eight carotenoid biosynthesis-related genes after salinity stress. As shown in Fig. 5, after 6 days of salt stress treatment, mRNA levels of all nine genes were significantly up-regulated in RC plants, whereas transcript levels of *PSY* and *LCY-ε* were down-regulated in NT plants compared with non-treatment. Interestingly, NCED is remarkably increased in RC plants after salt stress treatment. These results indicated that down-regulation of *CHY-β* in sweetpotato plants increased carotenoid content, thereby conferring salt stress tolerance.

3.5. RC plants exhibit increased ABA biosynthesis

AtNCED3 plays a key role in ABA biosynthesis under abiotic stress conditions in *Arabidopsis* (Iuchi et al., 2001; Seo and Koshiba, 2002). The sweetpotato *NCED* gene used in this study is highly homologous to *AtNCED3* from *Arabidopsis*. Under normal condition, NCED expression is increased in RC plants compared with NT plants

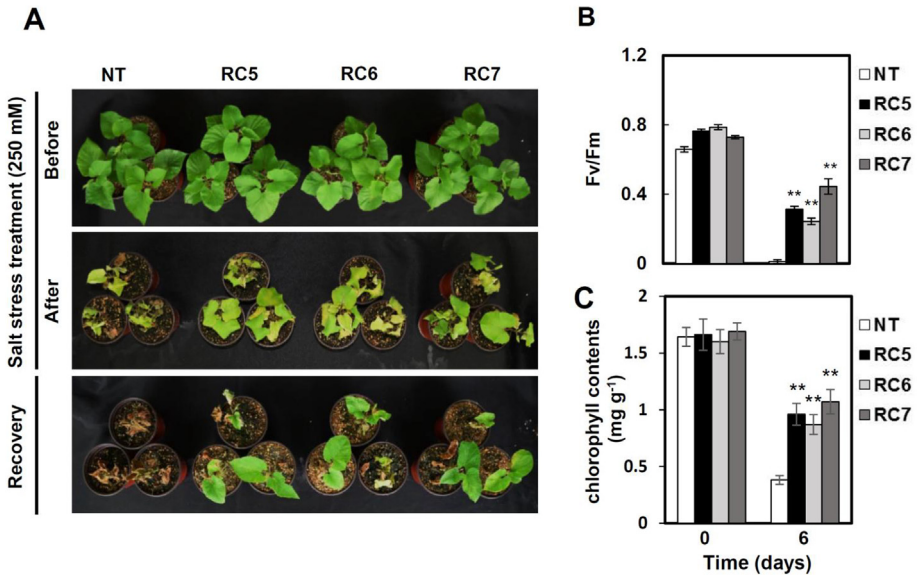

Fig. 4. Salt stress analysis of RC plants. (A) Phenotypes of 1-month-old NT and RC plants before and after 250 mM NaCl treatment for 6 days and recovery for 9 days. (B) Fluorescence-based maximum quantum yield for PS II (Fv/Fm) and (C) total chlorophyll content (Chl) in the fifth leaves of sweetpotato plants were determined 6 d after treatment. Data are expressed as the means ± SD of three biological repeats. Asterisks indicate significant differences relative to NT at * $P < 0.05$ or ** $P < 0.01$ (t-test).

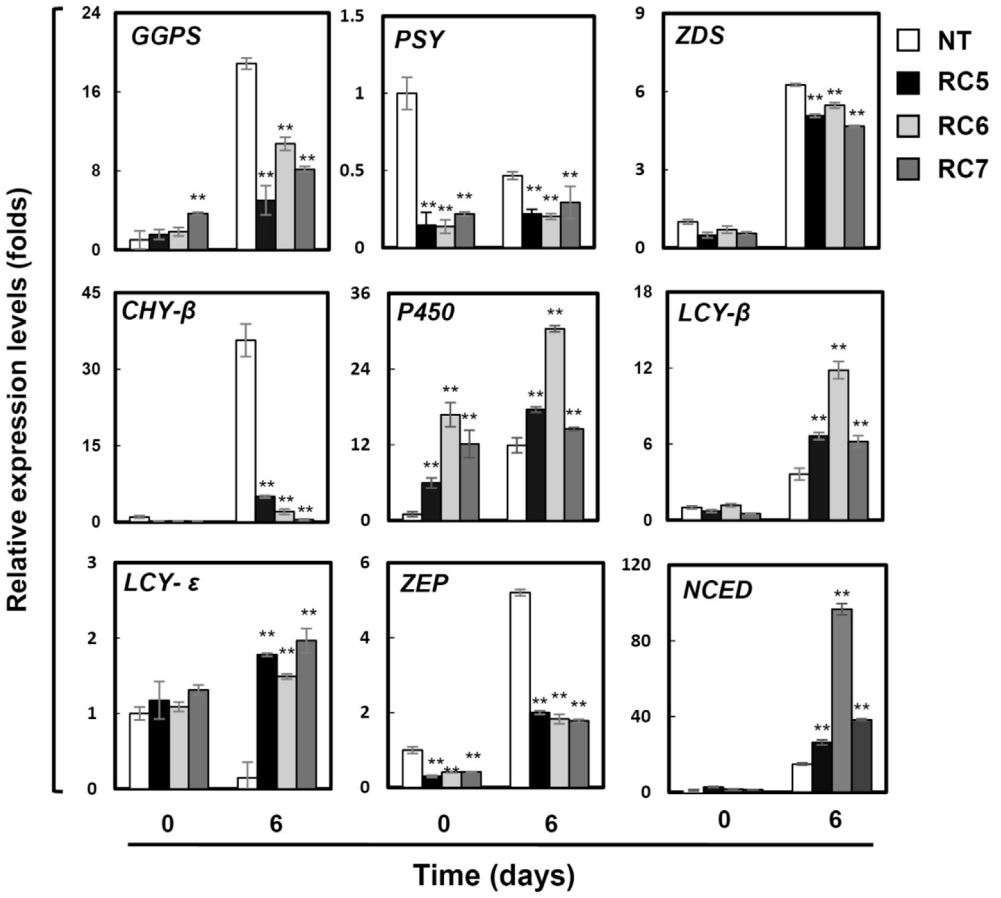

Fig. 5. Expression patterns of genes related to carotenoid biosynthesis in leaves of RC plants under salt stress. Transcript levels of genes related to carotenoid biosynthesis following treatment with 250 mM NaCl. The fifth leaves from the shoot apical meristem of plants were used for gene expression analysis. Data are expressed as the mean ± SD of three biological repeats. Asterisks indicate significant differences relative to NT at * $P < 0.05$ or **$P < 0.01$ (t-test).

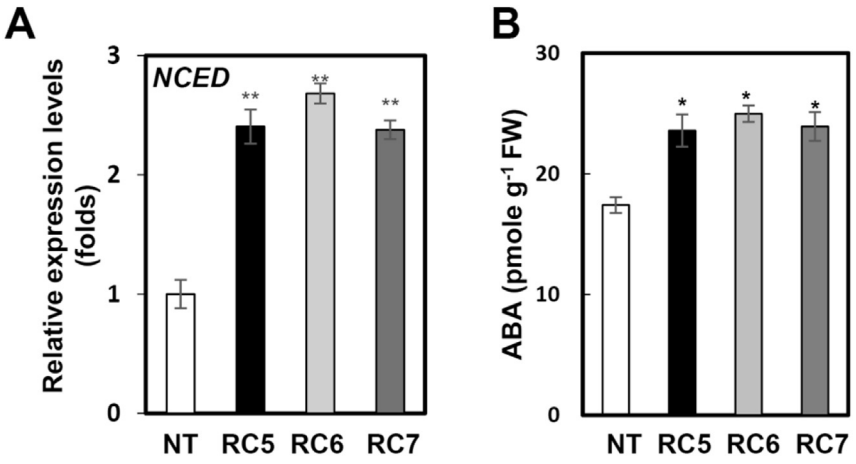

Fig. 6. Relative expression levels of NCED gene and ABA contents in RC plants. (A) Relative expression levels of NCED gene under normal condition. (B) ABA contents. One-month-old sweetpotato plants leaves (NT and RC plants) were used for RNA and ABA extraction. Data are expressed as the mean ± SD of three biological repeats. Asterisks indicate significant differences relative to NT at * $P < 0.05$ (t-test).

(Fig. 6A). Based on the result showing that the expression level of NCED was higher in RC plants and that RC plants showed the greatest tolerance to salt stress, we compared the ABA contents in leaves of both RC and NT plants. As shown in Fig. 6B, ABA contents were increased in RC plants compared to NT plants. This is consistent with our previous study which was carried out with RNAi-*IbCHY-β* culture cells (Kim et al., 2012). In addition, these results suggested that elevated ABA contents in leaves of RC plants lead to salt stress tolerance.

4. Discussion

Previous studies show that carotenoid levels can be increased by overexpressing or suppressing genes involved in carotenoid

biosynthesis pathways (Kim et al., 2012, 2013b, 2014; Park et al., 2015b). For instance, 'Golden Rice' was developed by over-expressing the maize *PSY* gene and *Erwinia uredovora* carotene desaturase (*crtI*) gene to produce high levels of β-carotene (pro-vitamin A) (Ye et al., 2000; Paine et al., 2005). A transgenic tomato with a carotenoid-enriched fruit was also developed by fruit-specific expression of *Erwinia uredovora* phytoene synthase (*crtB*); this product is potentially useful for prevention of coronary heart disease and certain cancers (Fraser et al., 2001). Overexpression of bacterial phytoene synthase (*crtB*) gene in *Brassica napus* (canola) leads to a dramatically increased carotenoid contents (Shewmaker et al., 1999). In addition, potato tuber with tuber-specific silencing of *lycopene epsilon cyclase* showed 14-fold increased β-carotene (Diretto et al., 2006). Previously, when we developed transgenic sweetpotato calli by down-regulating *IbLCY-ε*, the β-carotene content of transgenic calli was approximately 21-fold higher than of non-transgenic calli (Kim et al., 2013b). We also generated transgenic sweetpotato calli by suppressing *IbLCY-β* gene expression, resulting in significant increases in the levels of β-carotene and total carotenoids (Kim et al., 2014). These transgenic calli exhibited elevated antioxidant capacity and salt tolerance (Kim et al., 2012).

CHY-β is a non-heme di-iron hydroxylase that functions in the conversion of β-carotene and β-cryptoxanthin to zeaxanthin. Overexpression of *AtCHY-β* in *Arabidopsis* showed increased total carotenoids including zeaxanthin and violaxanthin, while β-carotene was decreased compared to wild type plants (Davison et al., 2002). Therefore, we expected that silencing of *IbCHY-β* might increase β-carotene contents in sweetpotato. Here, we generated transgenic sweetpotato plants (RC plants) by down-regulating expression of *IbCHY-β* (Figs. 1 and 2). Storage roots of RC plants exhibited elevated levels of total carotenoids and changed composition of carotenoids. The main proportion of total carotenoids composition changed from 28.8% of zeaxanthin in NT to 36.8% of β-carotene in RC plants (Table 1). Interestingly, when we inhibited *CHY-β*, the content of β-cryptoxanthin was increased and zeaxanthin was not reduced in storage root of RC plants. However, the proportion of zeaxanthin in total carotenoids content was decreased from 28.8% (NT) to 8.4% (RC5). Potato and *Arabidopsis* have 3 kinds of β-carotene hydroxylase such as CHY1, CHY2 and LUT5 (P450) (Diretto et al., 2007; Fiore et al., 2006). Diretto et al. (2007) reported that silencing of *CHY-β* in potato exhibited decrease zeaxanthin contents but the downstream xanthophylls such as violaxanthin and neoxanthin were not decreased. Transgenic potatoes which decreased contents of zeaxanthin exhibited down regulated all of β-carotene hydroxylase transcripts such as *CHY1*, *CHY2* and *LUT5* (Diretto et al., 2007). Our previous study showed that down regulation of *CHY-β* increase not only β-carotene but also xanthophylls including β-cryptoxanthin and zeaxanthin in sweetpotato calli (Kim et al., 2012). Kim et al. (2012) reported that the increased expression level of *P450* gene, which is known to have function of β-carotene hydroxylase, compensates for the low levels of *IbCHY-β*. As a result, the β-cryptoxanthin and zeaxanthin were increased in sweetpotato calli. In this study, expression of *IbP450* is higher in the RC plants than in NT plants (Fig. 5). This result suggested that significantly enhanced expression of *IbP450* might up regulate contents of β-cryptoxanthin and zeaxanthin in storage roots and violaxanthin in leaves of RC plants. We speculate that down regulation of both *IbP450* and *IbCHY-β* might completely decrease β-cryptoxanthin, zeaxanthin, and violaxanthin. To evaluate this hypothesis, the suppression of both *IbP450* and *IbCHY-β* genes remains to be studied.

ROS such as superoxide radical ($O_2^{-}\bullet$), hydroxyl radical (OH•), singlet oxygen ($^1O_2$), and hydrogen peroxide (H_2O_2) are constantly produced by aerobic processes in chloroplasts, mitochondria, and peroxisomes (Ke et al., 2015). Various environmental stresses, including salt, drought, and high-intensity light, induce over-production of ROS, which can cause damage to and accumulate in DNA, protein, or lipids (Bose et al., 2014; Baxter et al., 2014). Carotenoids act as antioxidants, a function that depends on their structure, chemical properties, and location/form in biological tissues (Fiedor and Burda, 2014). Carotenoids possess a conjugated carbon double-bond system that is thought to be involved in energy transfer reactions such as those in the photosynthesis chain, and can quench singlet oxygen (Telfer, 2014; Perlík et al., 2015). Carotenoids can also scavenge superoxide anion radicals ($O_2\bullet^{-}$) and hydrogen peroxide (H_2O_2) (Fiedor and Burda, 2014). Here, RC plants exhibited lower membrane permeability than NT plants (Fig. 3A and B). In addition, DPPH is widely used to test the ability of compounds to act as free radical scavengers or hydrogen donors and to increase antioxidant activity (Aksoy et al., 2013). The storage roots of RC plants with high levels of carotenoids exhibited higher DPPH activity (Fig. 3C). These results are consistent with our previous finding that RNAi-*IbCHY-β* transgenic sweetpotato calli with elevated carotenoid content are more tolerant to oxidative stress (Kim et al., 2012). Among abiotic stresses, soil salinity represents a huge global agricultural and environmental challenge, particularly in irrigated lands (Wichelns and Qadir, 2015). Transgenic *Arabidopsis* expressing the *Salicornia europaea PSY* gene also shows elevated carotenoid content, as well as salt and oxidative stress tolerance (Han et al., 2008).

In this study, RC plants exhibited increased tolerance to salt stress (Fig. 4), which might be related to activation of ROS scavenging or a decrease in ROS production caused by increased antioxidant capacity. Previous our study indicated that silencing of *IbCHY-β* enhancing salt stress tolerance in transgenic calli with enhanced *NCED* expression and ABA contents (Kim et al., 2012). In this study, RC plants showed significantly higher salt tolerance with enhanced *NCED* transcript levels compared to NT plants (Fig. 4). Wu et al. (2015) reported that overexpression of *CHY-β* showed increased salt and drought stress in transgenic tobacco by increasing xanthophylls cycle pool. The NCED protein specifically cleaves 9-*cis* isomers of epoxy-xanthophylls such as 9-*cis*-violaxanthin and 9′-*cis*-neoxanthin to synthesize ABA (Seo and Koshiba, 2002). However, ABA and neoxanthin were not increased in transgenic tobacco, whereas violaxanthin was increased more than two times under salt stress (Wu et al., 2015). Moreover, over-expression of *AtCHY-β* also showed almost same amount of neoxanthin (Davison et al., 2002). Interestingly, down regulation of *CHY-β* in potato showed accumulation of violaxanthin and neoxanthin (Diretto et al., 2007). These results suggested that expression of *CHY-β* might not directly relate to neoxanthin and violaxanthin contents. In conclusion, enhanced transcript level of *NCED* could increase ABA contents in RC plants. These results suggest that down-regulation of *IbCHY-β* in sweetpotato increases not only total carotenoid content and β-carotene but also enhance salt stress tolerance via increased ABA level. We speculated that genetic engineering of carotenoid biosynthesis-related genes could be used to produce valuable nutrients in sweetpotato plant.

In summary, we successfully generated transgenic sweetpotato plants by down-regulating the *CHY-β* gene using RNAi technology. β-carotene content was obviously increased in transgenic sweetpotato storage roots and leaves, accompanied by a visible color change in storage roots. In addition, RC plants exhibited increased tolerance to MV-mediated oxidative stress and resistance to abiotic stresses such as salt stress, demonstrating that β-carotene plays essential roles in ROS scavenging systems and in protecting the photosynthetic machinery under conditions of oxidative and/or salt stress. Furthermore, increased carotenoid biosynthesis in sweetpotato via down-regulation of genes involved in carotenoid synthesis represents a useful proof of principle for more sophisticated

strategies for engineering β-carotenoid synthesis and composition in staple crops. Therefore, we hope that this work will be of particular value to developing countries, which face the most pressing problems related to food supply and nutritional deficiencies.

Author contributions

L. Kang, X. Deng and S.S. Kwak conceived and designed the experiments. L. Kang, S.C. Park, H.S. Kim, C.Y. Ji, S.H. Kim, Q.B. Ke, H.U. Lee, J.S. Lee, and M.J. Ahn performed the experiments. L. Kang and Q.B. Ke analyzed the data. H.S. Lee and S.S. Kwak contributed reagents/materials/analysis tools. L. Kang and S.S. Kwak wrote the paper.

Acknowledgments

This work was supported by grants from the Systems & Synthetic Agrobiotech Center (PJ01106401), the BioGreen 21 Project for Next Generation, Rural Development Administration, Korea, Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT, and Future Planning (2015053321), Korea and the 111 Project of Ministry of Education of China (no.B12007) and the KRIBB initiative program.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2017.05.017.

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