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Research article

Enhanced accumulation of carotenoids in sweetpotato plants overexpressing *IbOr-Ins* gene in purple-fleshed sweetpotato cultivar

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ABSTRACT

Sweetpotato [*Ipomoea batatas* (L.) Lam] is an important root crop that produces low molecular weight antioxidants such as carotenoids and anthocyanin. The sweetpotato orange (IbOr) protein is involved in the accumulation of carotenoids. To increase the levels of carotenoids in the storage roots of sweetpotato, we generated transgenic sweetpotato plants overexpressing *IbOr-Ins* under the control of the cauliflower mosaic virus (*CaMV) 35S* promoter in an anthocyanin-rich purple-fleshed cultivar (referred to as *IbOr* plants). *IbOr* plants exhibited increased carotenoid levels (up to 7-fold) in their storage roots compared to wild type (WT) plants, as revealed by HPLC analysis. The carotenoid contents of *IbOr* plants were positively correlated with *IbOr* transcript levels. The levels of zeaxanthin were ~12 times elevated in *IbOr* plants, whereas β-carotene increased ~1.75 times higher than those of WT. Quantitative RT-PCR analysis revealed that most carotenoid biosynthetic pathway genes were up-regulated in the *IbOr* plants, including *PDS*, *ZDS*, *LCY-β*, *CHY-β*, *ZEP* and *Pftf*, whereas *LCY-ε* was down-regulated. Interestingly, *CCD1*, *CCD4* and *NCED*, which are related to the degradation of carotenoids, were also up-regulated in the *IbOr* plants. Anthocyanin contents and transcription levels of associated biosynthetic genes seemed to be altered in the *IbOr* plants. The yields of storage roots and aerial parts of *IbOr* plants and WT plants were not significantly different under field cultivation. Taken together, these results indicate that overexpression of *IbOr-Ins* can increase the carotenoid contents of sweetpotato storage roots.

1. Introduction

Sweetpotato [*Ipomoea batatas* (L.) Lam] is one of the most nutritionally dense root crops worldwide because it serves as an abundant source of health-promoting low molecular weight antioxidants such as vitamins, anthocyanins and carotenoids (Teow et al., 2007). In particular, the colors of yellow- and purple-fleshed sweetpotato storage roots are derived from high quantities of carotenoids and anthocyanins, respectively. These pigmented bioactive compounds are of particular interest due to their free radical-scavenging activity, which is implicated in slowing the aging process and preventing chronic degenerative diseases such as cancer (Chen et al., 2005; Luceri et al., 2008).

Abbreviations: ABA, abscisic acid; ANS, anthocyanidin synthase; AsA, ascorbic acid; CaMV, cauliflower mosaic virus; CCDs, carotenoid cleavage dioxygenases; CHY-β, β-carotene hydroxylase; CRTI, carotenoid desaturase; DFR, dihydroflavonol 4-reductase; DW, dry weight; F3H, flavanone-3-hydroxylase; GAP, glyceraldehyde-3-phosphate; GGPP, geranylgeranyl diphosphate; HPTII, hygromycin resistant gene; LCY-β, lycopene β-cyclase; LCY-ε, lycopene ε-cyclase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; NXS, neoxanthin synthase; PDS, phytoene desaturase; Pftf, plastid fusion/translocation factor; PSY, phytoene synthase; SZM, Sinzami; UF3GT, UDP glucose flavonoid-3-O-glucosyltransferase; ZDS, ξ-carotene desaturase; ZEP, zeaxanthin epoxidase.

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All photosynthetic organisms and some non-photosynthetic bacteria and fungi produce carotenoids. Carotenoids are lipophilic secondary metabolites that are synthesized from isoprenoids (Cazzonelli et al., 2010). These compounds are not only necessary for light harvesting and preventing photo-oxidative damage to chlorophyll, but they are also a primary dietary source of provitamin A for animals and humans (Cazzonelli et al., 2010). The prevalence of vitamin A deficiency is a serious problem in developing countries. Accordingly, molecular breeding to improve the provitamin A contents of staple crops is important for relieving vitamin A deficiency (Low et al., 2007).

The carotenoid biosynthetic pathway and its metabolic enzymes in higher plants are well documented. Carotenoids are synthesized from pyruvate and glyceraldehyde-3-phosphate (GAP), which is converted to geranylgeranyl diphosphate (GGPP) via the non-mevalonate pathway. Phytoene, the first carotenoid in this pathway, is formed from two GGPP molecules; this reaction is catalyzed by phytoene synthase (PSY). The production of lycopene from phytoene is then catalyzed by phytoene desaturase (PDS) and ξ-carotene desaturase (ZDS) through the addition of four double bonds. Then, α- and β-carotene are produced from lycopene. Two β-rings of β-carotene are created by the action of lycopene β-cyclase (LCY-β), which creates β-ionone rings. Lycopene ε-cyclase (LCY-ε) creates one ε-ring using LCY-β to produce α-carotenoid. β-Hydroxylase (CHY-β) converts β-carotene into zeaxanthin, and zeaxanthin epoxidase (ZEP) then mediates the formation of violaxanthin through the addition of C-5, C-6-epoxy groups into the C-3-hydroxy-β-rings of antheraxanthin. Neoxanthin synthase (NXS) converts violaxanthin into neoxanthin (Cazzonelli et al., 2010; Cunningham and Gantt, 1998).

Carotenoids are a source of apocarotenoids in plants, including diverse signaling molecules such as abscisic acid (ABA) and strigolactone (Cunningham and Gantt, 1998; Giuliano, 2014). Carotenoid cleavage dioxygenases (CCDs) produce apocarotenoids from carotenoids. CCDs can affect the contents and composition of carotenoids. CCDs cleave specific bonds in the conjugated carotenoid polyene chain (Walter and Strack, 2011). In *Arabidopsis thaliana*, the plant hormone ABA is produced by five 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), and strigolactones, which regulate auxiliary branching and tillering, are synthesized by the enzymes CCD7 and CCD8 (Auldridge et al., 2006). In addition, *CCD1* and *CCD4* are involved in the production of apocarotenoid-derived pigments, flavors and aromas of various plants through the degradation of carotenoids (Auldridge et al., 2006; Gonzalez-Jorge et al., 2013).

Efforts to improve the carotenoid contents of plants through metabolic engineering have focused on modifying the expression of carotenoid pathway genes. For examples, the β-carotene rich golden rice (*Oryza sativa*) was engineered by overexpressing daffodil *PSY* and carotenoid desaturase (*crtI*) from *Erwinia uredovora* in rice (Paine et al., 2005). Silencing of *CHY-β* in orange fruit significantly increases β-carotene levels (Pons et al., 2014). Although sweetpotato is recognized as a good source of health-promoting bioactive compounds including carotenoids, the metabolic engineering of carotenoids in transgenic sweetpotato plants has not been adequately investigated to date. In our previous studies, down-regulation of *CHY-β, LCY-ε* and *LCY-β* by RNA interference technology led to higher β-carotene and total carotenoid levels in sweetpotato calli (Kim et al., 2012, in press, 2013b).

Lu et al. (2006) identified a splicing mutation of the *Orange* (*Or*) gene (which is not a carotenoid pathway gene) that increases carotenoid accumulation via induction of chromoplast differentiation in cauliflower. Or protein contains a DnaJ cysteine-rich zinc-binding domain, and its sequence is highly conserved among plant species (Lu et al., 2006). Overexpression of *Or* increased the carotenoid and β-carotene contents of transgenic potato tubers, which continuously increased under cold storage conditions (Li et al., 2012; Lopez et al., 2008). Moreover, overexpression of Arabidopsis *Or* (*AtOr*) increases the carotenoid contents of rice calli (Bai et al., 2014). Furthermore, overexpression of sweetpotato *Or* (*IbOr*) not only increases the β-carotene content in transgenic *IbOr* calli, but also significantly increases the α-carotene, lutein, β-crytoxanthin and zeaxanthin contents. The transgenic *IbOr* calli also exhibit increased antioxidant activity and tolerance to salt stress (Kim et al., 2013a).

In the current study, we introduced *IbOr-Ins* into purple-fleshed sweetpotato plants to produce both anthocyanin and carotenoids in their storage roots. We measured the carotenoid contents and transcription levels of carotenoid biosynthetic pathway genes in the transgenic plants and characterized the molecular physiological properties of the plants. In addition, we compared the yields of storage roots and aerial parts of transgenic versus wild-type (WT) plants under field cultivation conditions.

2. Materials and methods

2.1. Plant materials and expression vectors

The WT sweetpotato cultivar Sinzami (SZM) was obtained from the Bioenergy Crop Research Center, National Institute of Crop Sciences, Rural Development Administration (RDA), Korea. SZM produces high amounts of anthocyanin in its storage roots (~3.7 mg g^{-1} dry weight [DW]; data not shown). To increase the levels of carotenoids in these anthocyanin-rich sweetpotato plants, the pGWB11 binary vector harboring *IbOr* insertion type (*IbOr-Ins*, which encodes seven additional amino acids between 131 and 142 of WT IbOr protein) under the control of the cauliflower mosaic virus (*CaMV) 35S* promoter was constructed as previously described (Kim et al., 2013a) (Fig. 1A).

2.2. Transformation and regeneration of sweetpotato

Embryogenic calli were isolated from SZM plants and used for *Agrobacterium*-mediated transformation as described previously (Lim et al., 2004). The transformed calli were selected on MS medium containing 400 mg L^{-1} cefotaxime and 25 mg L^{-1} hygromycin. Transformed calli were sub-cultured to fresh medium at 3-week intervals. Regenerated plantlets were confirmed by genomic PCR of the hygromycin resistance gene (*hygromycin phosphotransferease*, *hpt*) and transferred to pots for further experiments.

2.3. Gene expression analysis

Total RNA was extracted from storage roots and leaves using the cetyl trimethylammonium bromide method (Kim and Hamada, 2005). Extracted RNA was treated with DNase I according to the manufacturer's instructions (Takara, Japan) to remove any traces of genomic DNA. Total RNA (2 μg) was reverse-transcribed using oligo-dT primers and an M-MLV cDNA Synthesis Kit (Clontech, USA) according to the manufacturer's instructions. Gene-specific primers were designed using a stringent set of criteria to enable application of universal reaction conditions. The sequence and accession numbers for each primer pair are indicated in Table 2. RT-qPCR analysis was carried out in 96-well plates with a CFX Real-time PCR System and CFX system software (Bio-Rad, USA) using the EvaGreen-based PCR assay. Each reaction (final volume of 20 μl) contained 1 μl of diluted cDNA, 10 μl of EvaGreen PCR Master Mix (Solgent, Korea) and 1 μl of each primer. The PCR conditions were as follows: 95 °C for 15 min, followed by 40 cycles of 95 °C for 20 s, 60 °C for 40 s and 72 °C for 20 s. The melting curves were analyzed at 65–95 °C after 40 cycles. Each RT-qPCR was performed in duplicate. *ADP-ribosylation factor (ARF*) was used as the reference

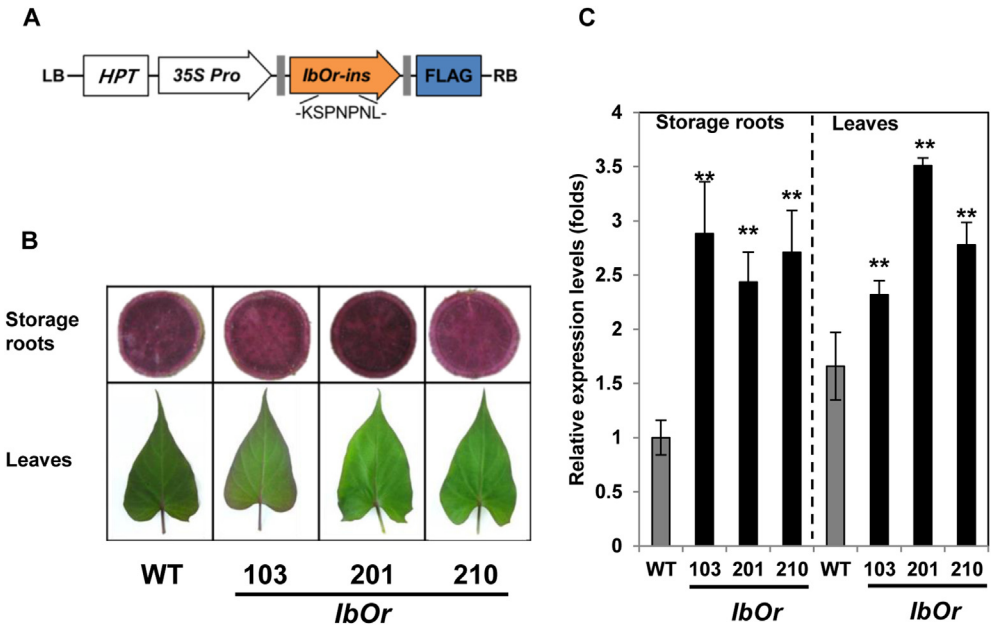

Fig. 1. Phenotypes and *IbOr* expression levels of transgenic sweetpotato lines overexpressing *IbOr-Ins*. (A) Schematic diagram of the T-DNA region of the *IbOr* constructs used for plant transformation. LB and RB, left and right T-DNA borders, respectively; *35S* Pro, *CaMV 35S* promoter; gray boxes, attB cloning site of gateway vector systems; FLAG, FLAG octapeptide. (B) Phenotypes of storage roots and leaves of three *IbOr* transgenic and wild-type (WT) lines. (C) Expression of *IbOr* in leaves and storage roots. Data are the means ± SD of three technical qRT-PCR replicates from three individual storage roots.

gene for quantification because its transcript levels are constant throughout root and leaf tissues (Park et al., 2012).

2.4. Analysis of carotenoids

Storage root samples were obtained from field-grown plants in 2012. On harvest day, ~200 g of storage roots (five roots per line) were washed, towel dried, sliced and immediately frozen at −80 °C before lyophilization. Lyophilized sweetpotato samples were stored at −80 °C until analysis of anthocyanin and carotenoid contents. Carotenoids were extracted from 0.1 g of lyophilized sweetpotato storage roots and analyzed using the Agilent 1100 HPLC system (Hewlett–Packard, Palo Alto, CA) as previously described (Kim et al., 2012, 2013a). The HPLC-DAD system was operated using Chemstation software (Hewlett–Packard). Carotenoids were quantified using an external calibration method. The β-carotene, β-cryptoxanthin, lutein, α-carotene, 13Z-β-carotene, 9Z-β-carotene and zeaxanthin standards were obtained from CaroteNature (Lupsingen, Switzerland). Under these conditions, the peaks of standard carotenoids at retention time (min) values were as follows: lutein (peak: 23.3), zeaxanthin (peak: 26.6), β-cryptoxanthin (peak: 33.5), α-carotene (peak: 37.5), 13Z-β-carotene (peak: 37.9), β-carotene (peak: 39.2) and 9Z-β-carotene (peak: 40.5). The carotenoid levels were measured in a minimum of three different storage roots. The levels were expressed as the mean (average content in μg g^{-1} DW) ± SD (standard deviation) of two independent determinations.

2.5. Analysis of anthocyanins

Anthocyanins were extracted from 0.2 g of finely ground lyophilized storage roots as described by Chu et al. (2013). Briefly, lyophilized samples were extracted with 1 ml of acidic methanol containing 1% HCl (v/v) for 18 h at room temperature with moderate shaking. Subsequently, 500 μl of the supernatant was mixed with 500 μl of MilliQ H_2O and 300 μl of chloroform to remove carotenoids and other lipophilic compounds. The supernatant (water–methanol phase) was determined spectrophotometrically at 530 and 657 nm. Cyanidin (Sigma–Aldrich, St. Louis, MO) was used as a standard for identification.

2.6. Field production of transgenic sweetpotato plants

Sweetpotato field production was conducted in 2012 at the genetically modified organism (GMO) field of the Bioenergy Crop Research Center, National Institute of Crop Sciences, RDA (Muan, Korea). Fourteen transgenic lines were grown for 1 month in a seedling bed. Stems were then cut to 15 cm lengths, and 40 stems per line were transplanted to the field by hand, with 25 cm spacing between plants and 60 cm between rows. The experimental design comprised a randomized complete block with three replications. Nitrogen (55 kg ha^{-1}), phosphorus (63 kg ha^{-1}) and potassium (156 kg ha^{-1}) fertilizers were incorporated into the soil before transplanting. Thereafter, the plots were mulched with black polyethylene films. Other cultural management practices were performed in accordance with the standard sweetpotato cultivation methods defined by RDA, Korea. The chlorophyll contents and efficiency of photosystem II (Fv/Fm) were measured with SPAD (Minolta Camera Co., Ltd., Japan) and Handy PEA (Hansatech Instruments, King's Lynn, Norfolk, UK), respectively, 1 month after planting. At harvest time, the fresh weights of the above-ground shoots and storage roots were recorded.

2.7. Statistical analysis

Experimental data were analyzed by one-way ANOVA. Subsequent multiple comparisons were examined by using the Dunnett test. All statistical analyses were performed using the Statistical Package for Social Sciences (SPSS 12), and statistical significance was set at $^{*}P < 0.05$ and $^{**}P < 0.01$.

Table 1
Carotenoid contents in the storage roots of transgenic and wild-type sweetpotato plants (μg g^{-1} DW).

	Lutein	Zeaxanthin	Cryptoxanthin	α-carotene	13Z-β-carotene	β-carotene	9Z-β-carotene	Total carotenoids
WT	N.D.	0.2 ± 0.1	0.1 ± 0.0	N.D.	N.D	0.4 ± 0.2	0.1 ± 0.0	0.8 ± 0.4
Or-103	N.D.	2.4 ± 0.4**	0.4 ± 0.1**	0.4 ± 0.4**	0.7 ± 0.2**	0.7 ± 0.0**	1.1 ± 0.1**	5.8 ± 1.3**
Or-201	N.D.	1.5 ± 0.5**	0.3 ± 0.1**	N.D.	N.D.	0.7 ± 0.0**	0.8 ± 0.2**	3.4 ± 0.8**
Or-210	1.0 ± 0**	1.9 ± 0.2**	0.3 ± 0.0**	N.D.	0.6 ± 0.1**	0.7 ± 0.0**	0.8 ± 0.1**	4.3 ± 0.4**

**$P < 0.01$.
N.D.: Not detected.

Table 2
Primer sequences used for the expression analysis of sweetpotato genes in this study.

Gene	Forward	Reverse	GenBank accession
PSY	AAGTTCTTCGACGAGGCTGA	GCAGTTTCTTTGGCTTGCTT	HQ828092
PDS	GTACAAAACCGTGCCAGGAT	TCCTTTAAAATCGCCTGTGC	HQ828091
ZDS	ATAGCATGGAAGGAGCAACG	AGCTCATCAGATACAGCAGCAG	HQ828088
CHY-β	CAAGAGAAGGACCGTTCGAG	GACGAACATGTAGGCCATCC	HQ828095
LCY-ε	ACCAGTTGGAGGATCATTGC	CACCCATACCAGGACTTCGT	HQ828096
LCY- β	ATGGTGTGACGATTCAAGCA	GCCAATCCATGAAAACCATC	HQ828094
ZEP	TGGTACTTGGATCACCGACA	GCTGCCTGCAAAACTTTCAT	BJ828089
Pftf	TTTGACGATGTTGCTGGTGT	ACCAGCTATGGCTTTGGCTA	JX177356
NCED	GGGAAGATCCCGGAGTGTAT	GGACCAATCTATGCGTCTCC	BJ563195
CRTISO	AGGAGGTGGGGTCACCTAAG	ACCGCTATGACACCTTGACC	JX393307
CCD1	TGCTGAACCAGAGACTGGAA	TCCGGTGTTCTCATCGTGTA	JX393308
CCD4	CGGTGGAGAAACTCACGATT	TCCCGATATCTTCGGCATAG	AB499059
F3H	CAAGCCGATTATGGAAGAGC	GCTCCTTTGCGAACTTCTTG	FJ478181.1
DFR	CATCCCAAAGCAGAAGGAAG	ACCACAGGCAAGTCCTTTTC	EU402466.1
UF3GT	ACATGGCGCTAAAGTTATCC	AGTTGTGGTCATCGGAGATG	GU989254.1
ANS	TCATCCTCCACAACATGGTC	TGCTCTTGTACTTGCCGTTG	EF187730.1
ARF	CTTTGCCAGAAGGAGATGC	TCTTGTCCTGACCACCAACA	JX177359.1

3. Results

3.1. Phenotypes of transgenic sweetpotato plants

Transgenic sweetpotato plants expressing *IbOr-Ins* under the control of the *CaMV 35S* promoter (referred to as *IbOr* plants) were successfully generated by *Agrobacterium*-mediated transformation. *IbOr-Ins* encodes IbOr and seven additional amino acids (KSPNPNL) inserted between residues of the original IbOr protein (Fig. 1A) (Kim et al., 2013a). We generated 50 putative transgenic plantlets from 200 of fresh embryogenic calli. 42 positive plantlets were selected by genomic PCR with *HPT* selection marker gene (data not shown). Only fourteen transgenic plants produced storage roots from 42 transgenic plants in growth chambers for 5 months. Fourteen transgenic plants confirmed by genomic PCR were grown in a GMO field for 4 months to obtain storage roots. Three transgenic lines, *IbOr*-103, -201 and -210, were selected based on the expression level of *IbOr* (Fig. S1). *IbOr* plants exhibited different color densities in their storage roots from those of the WT (Fig. 1B). *IbOr*-201 storage roots were a darker purple than those of the WT, whereas those of the other lines (*IbOr*-103 and *IbOr*-210) exhibited a pale purple color. The phenotypes of leaves and petioles were not significantly different between *IbOr* and WT plants (Fig. 1B). All *IbOr* lines exhibited significantly higher expression of *IbOr* in both their storage roots and leaves than WT plants. The basal *IbOr* transcript levels in the leaves of WT plants were slightly higher than those of the storage roots (Fig. 1C).

3.2. Increased carotenoid contents in transgenic plants

To assess the effects of increased *IbOr* expression on the regulation of carotenoid biosynthesis, we investigated the carotenoid contents in the storage roots of *IbOr* plants by HPLC analysis (Table 1). The total carotenoid levels in the storage roots of *IbOr*-103 (5.8 μg g^{-1} DW), *IbOr*-201 (3.4) and *IbOr*-210 (4.3) were 4.25–7.25 times higher than those of the WT (0.8 μg g^{-1} DW) (Table 1). The contents of zeaxanthin, β-cryptoxanthin, β-carotene and 9Z-β-carotene were also higher in all *IbOr* lines. Only *IbOr*-210 and *IbOr*-103 plants contained lutein (0.1 μg g^{-1} DW) and α-carotene (0.4), respectively. We detected 13Z-β-carotene in *IbOr*-103 (0.7) and *IbOr*-210 (0.6) but not in the WT or *IbOr*-201 lines. The levels of zeaxanthin and β-carotene, two major carotenoids, in WT plants comprised 25% (0.2) and 50% (0.4) of total carotenoids, respectively. Interestingly, the major carotenoid in the *IbOr* plants was zeaxanthin, which comprised approximately 41–44% of total carotenoids.

3.3. Differential expression of carotenoid pathway genes in transgenic plants

We performed qRT-PCR to compare the expression levels of carotenoid metabolic pathway genes in the WT versus *IbOr* plants (Fig. 2A). The transcription profiles of carotenoid pathway genes in transgenic and WT plants showed positive correlations with carotenoid contents. The expression levels of carotenoid biosynthetic genes such as *PDS, ZDS, LCY-β, CHY-β, ZEP* and *plastid fusion/translocation factor (Pftf)* in the storage roots of *IbOr* plants were higher than those of the WT; however, the expression of *PSY* was not significantly altered in the transgenic plants, and the expression of *LCY-ε* was reduced in lines *IbOr*-103 and *IbOr*-201. The expression of *NCED* (which is associated with ABA biosynthesis) was ~3 times higher in transgenic plants than in WT plants (Fig. 2B). The expression of genes encoding CCDs was also significantly higher in all transgenic lines than in the WT; the average transcript levels of *CCD1* and *CCD4* in these *IbOr* lines were approximately 1.6- and 5.6 times higher, respectively, than those of the WT (Fig. 2B). These results suggest that ectopic expression of *IbOr* affects the expression of both carotenoid biosynthesis and carotenoid degradation genes in sweetpotato.

3.4. Different levels of anthocyanins in transgenic plants

We examined the anthocyanin levels in the storage roots of transgenic and WT plants using spectroscopy. The total anthocyanin contents in *IbOr* plants varied from 2815 to 4976 μg g^{-1} DW depending on the line (Fig. 3A). The total anthocyanin contents in *IbOr*-201 were 1.54 times higher than that of the WT, whereas *IbOr*-210 exhibited slightly reduced anthocyanin contents compared to WT plants. The transcription profiles of anthocyanin pathway genes such as *flavanone-3-hydroxylase* (*F3H*), *dihydroflavonol 4-reductase* (*DFR*), *UDP glucose: flavonoid-3-O-glucosyltransferase* (*UF3GT*), and *anthocyanidin synthase* (*ANS*) in transgenic and WT plants were analyzed by quantitative RT-PCR analysis (Fig. 3B). The transcript levels of most of these genes in *IbOr* plants were different from those of WT plants. *IbOr*-103 and *IbOr*-203 exhibited higher expression of *F3H* and *ANS* than WT plants, while only *IbOr*-210

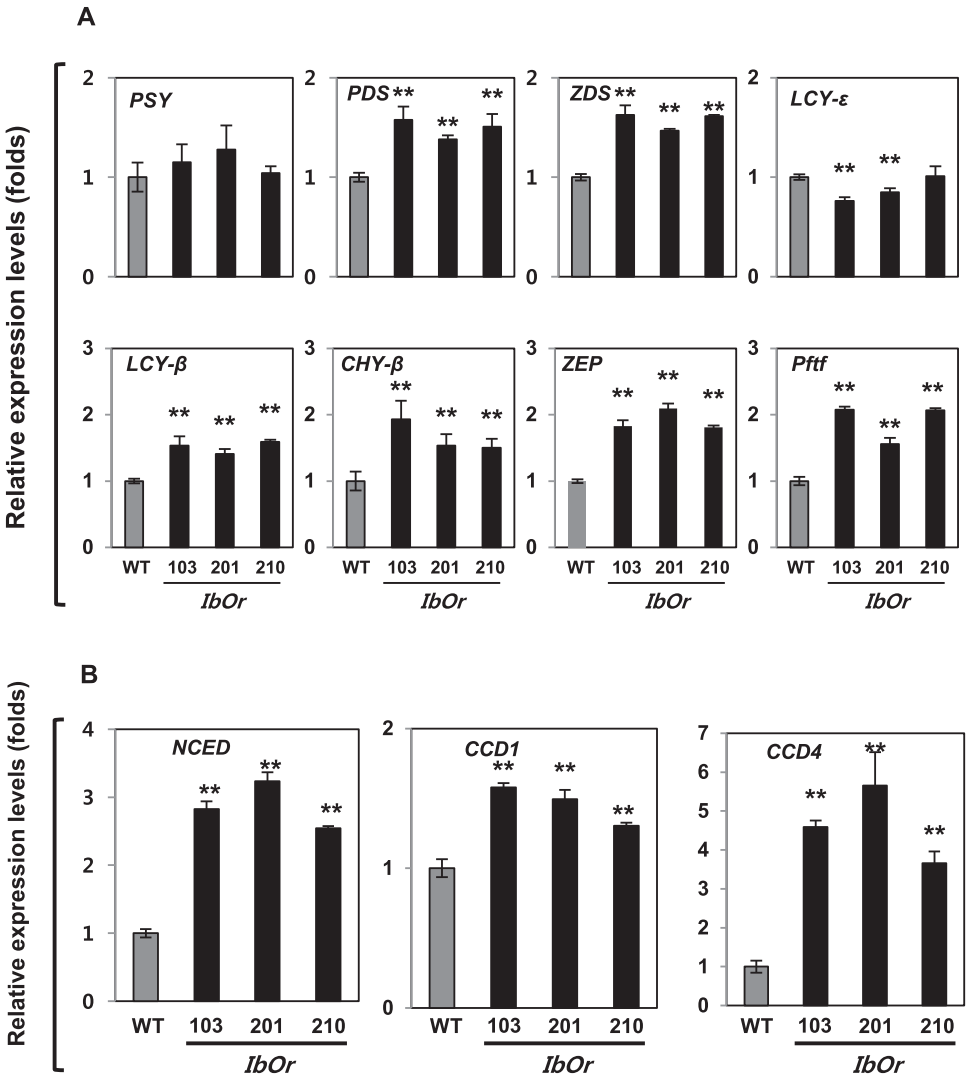

Fig. 2. Expression of carotenoid biosynthesis and degradation-related genes in storage roots of *IbOr* transgenic and wild-type (WT) plants. (A) Carotenoid biosynthesis-related genes in storage roots. (B) Carotenoid degradation-related genes. Data are the means ± SD of three technical qRT-PCR replicates from three individual storage roots.

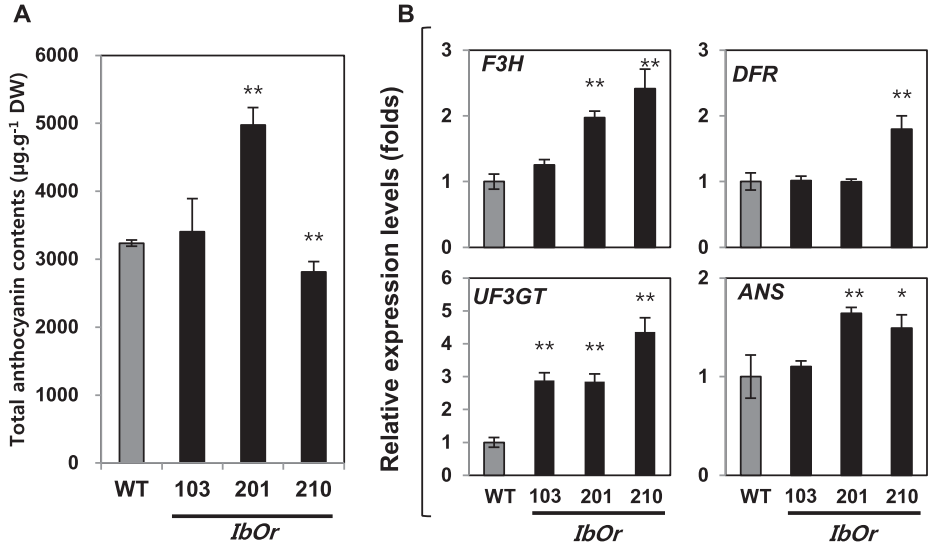

Fig. 3. Anthocyanin contents and expression levels of anthocyanin biosynthesis-related genes in storage roots of *IbOr* transgenic and wild-type (WT) plants. (A) Analysis of total anthocyanin contents. (B) Expression of anthocyanin biosynthesis-related genes. Data are means ± SD of three replicates from three individual storage roots.

exhibited increased expression of *DFR*. All transgenic plants had higher transcripts levels of *UF3GT* than the WT. These results suggest that overexpression of *IbOr* affects the anthocyanin contents and expression of anthocyanin biosynthetic genes in sweetpotato storage roots.

3.5. Yield of storage roots under field conditions

To evaluate whether overexpression of *IbOr-Ins* affects photosynthesis, we measured the chlorophyll contents and maximum efficiency of photosystem II (Fv/Fm) in the transgenic lines. The chlorophyll contents and Fv/Fm were not statistically different between WT and *IbOr* transgenic plants under field conditions (Fig. 4B and C). Moreover, the yields of the storage roots and aerial parts were not significantly different in *IbOr* versus WT plants (Fig. 4D), although some transgenic plants exhibited a slight increase in yield compared to WT plants. The average yield of the storage roots in WT plants was 238 kg per are (a), whereas *IbOr*-103, *IbOr*-201 and *IbOr*-210 produced 250, 272.7 and 278.6 kg a^{-1}, respectively. The average yields of aerial parts ($kg.a^{-1}$) ranged from 285.6 (*IbOr*-210) to 383.3 (*IbOr*-201), while that of WT plants was 287.5 (Fig. 4E).

4. Discussion

Increasing the levels of health-promoting antioxidants such as anthocyanin and carotenoids in edible staple crops may help improve human health and enhance crop survival under harsh conditions. Increasing carotenoid levels in crops may reduce vitamin A deficiency, which affects 190 million preschool-aged children and 19.1 million pregnant women worldwide (WHO, 2009). In addition, the intake of anthocyanin-rich foods may help reduce the incidence of cardiovascular disease, cancer, hyperlipidemias and other chronic diseases (Pascual-Teresa and Sanchez-Ballesta, 2008). In this study, we generated transgenic sweetpotato plants overexpressing *IbOr-Ins* in a purple-fleshed cultivar to increase the levels of carotenoids (a lipophilic antioxidant) as well as anthocyanin (a hydrophilic antioxidant that is highly abundant in purple-fleshed sweetpotato). Foods rich in both membrane-associated and soluble antioxidants are thought to offer the best protection against oxidative stresses derived from free radicals under stress conditions (Yeum et al., 2004).

To increase the levels of carotenoids in edible crops, most previous studies have focused on engineering specific carotenoid biosynthetic genes (Sandmann et al., 2006). In addition, recent studies have suggested the possibility of increasing carotenoid levels through overexpression of *Orange* (*Or*), a non-biosynthetic pathway gene involved in the accumulation of carotenoids. The *Or* gene, originally isolated from a cauliflower orange curd mutant, functions in the differentiation of non-colored plastids into chromoplasts for carotenoid accumulation (Lu et al., 2006; Paolillo et al., 2004). When *Or* was introduced into potato plants and rice calli, the carotenoid content increased in both potato tubers and rice calli through increased chromoplast differentiation (Bai et al., 2014; Lu

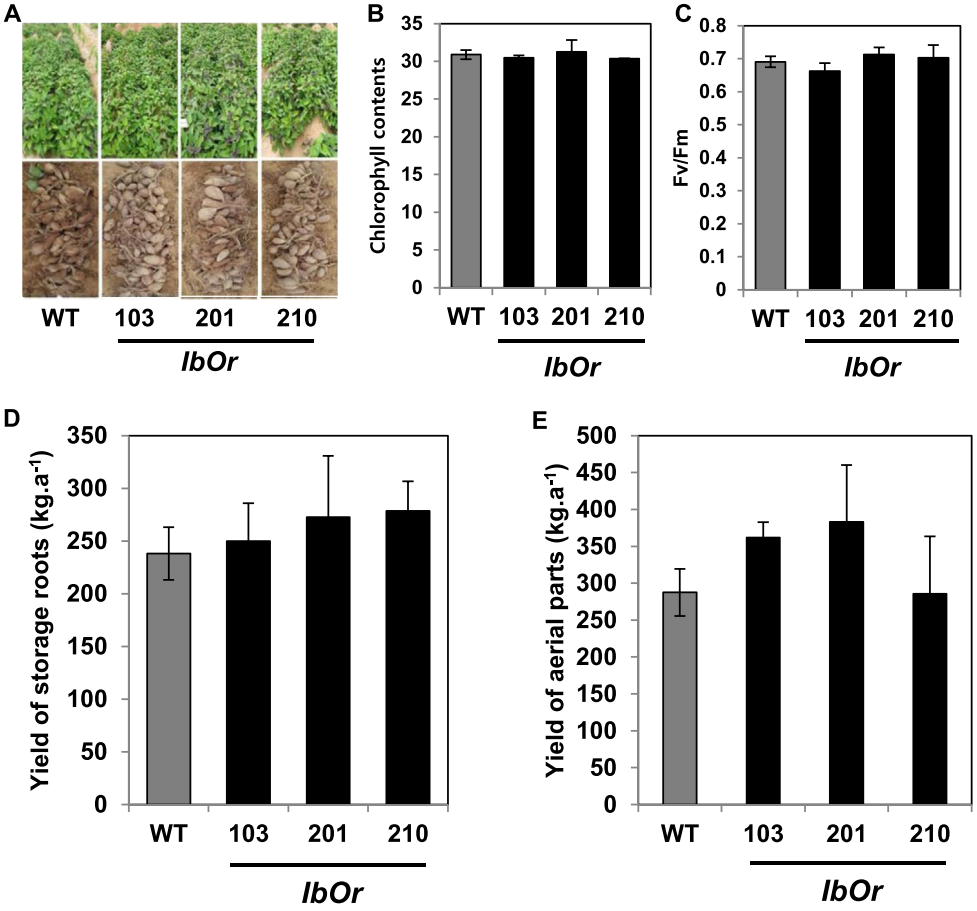

Fig. 4. Growth-related features of *IbOr* transgenic and wild-type (WT) sweetpotato plants under field conditions. (A) Photograph of aerial plant parts and storage roots. (B) Chlorophyll contents. (C) PSII activity (as Fv/Fm values). (D) Average yields of storage roots. (E) Average yields of aerial plant parts. Data are means ± SD of three row replicates (40 individual plants were planted per line).

et al., 2006). Recently, we demonstrated that overexpression of sweetpotato *Orange*-insertion type (*IbOr-Ins*) in calli derived from white-fleshed sweetpotato increases the accumulation of carotenoids due to the slight induction of carotenoid biosynthesis pathway genes (Kim et al., 2013a). In the current study, we showed that overexpression of *IbOr-Ins* increased the carotenoid content in the storage roots of transgenic plants through higher expression of carotenoid biosynthetic genes, except for *PSY* and *LCY-ε* (Table 1 and Fig. 2). These results confirm the notion that *IbOr-Ins* is involved in the accumulation of carotenoids in sweetpotato storage roots.

Interestingly, the levels of zeaxanthin were significantly elevated in the storage roots of *IbOr* transgenic lines (to levels approximately 12 times higher than those of WT plants (Table 1). Recent *Or* overexpression studies were performed on crops that could not accumulate the β-ring of β-carotene (Bai et al., 2014; Lopez et al., 2008). Normal purple-fleshed sweetpotato accumulates small amounts of β-carotene (0.4 μg g^{-1} DW for the SZM cultivar in this study). Both WT rice calli and potato tubers mainly accumulate the ε-branch-specific pathway carotenoid lutein (Bai et al., 2014; Lopez et al., 2008). Overexpression of *ZmPSY* and *AtOr* significantly increases β-carotene levels in rice calli (Bai et al., 2014), and, furthermore, cauliflower *Or*-overexpressing potato accumulates high levels of β-carotene (Lopez et al., 2008); however, we previously showed that the β-cryptoxanthin and zeaxanthin levels were dramatically higher in *IbOr* transgenic calli than in WT calli. A similar phenomenon was previously reported for sweetpotato calli with suppressed expression of *LCY-ε*, which creates one ε-ring and increases the synthesis of α-carotene. These calli also contained much higher levels of β-branch-specific pathway carotenoids such as β-carotene, β-cryptoxanthin, zeaxanthin and violaxanthin, whereas the level of lutein, an end product of the ε-branch-specific carotenoid pathway, was reduced (Kim et al., 2013b). In the current study, we observed slightly reduced expression of *LCY-ε* in *IbOr* plants, whereas other carotenoid biosynthetic genes were up-regulated (Fig. 2). *IbOr* plants exhibited increased levels of zeaxanthin, the metabolite in the next step of β-carotene synthesis in the β-branch-specific carotenoid pathway; however, overexpression of *Or* in rice calli and potato tubers primarily affects the production of metabolites up to the β-carotene stage in the β-branch-specific pathway. According to these results, we speculate that *IbOr-Ins* creates a metabolic sink to provide a pulling force that mainly affects the β-branch-specific carotenoid pathway in the storage roots of sweetpotato.

CCDs generate apocarotenoids via degradation of carotenoids (Walter and Strack, 2011). It is thought that *CCD* genes are related to the maintenance of carotenoid homeostasis in plants, with some members of the CCD family also controlling the synthesis of the phytohormones ABA and strigolactone (Tanaka and Ohmiya, 2008; Walter and Strack, 2011). The expression of *CCD1* and *CCD4* is negatively correlated with carotenoid accumulation in a number of plant species such as chrysanthemum flowers (Tanaka and Ohmiya, 2008), potato (Campbell et al., 2010; Zhou et al., 2011) and strawberry (García-Limones et al., 2008). Moreover, the loss of either *CCD1* or *CCD4* activity in seeds results in a significant increase in seed carotenoids, indicating that both enzymes can utilize carotenoids as substrate (Gonzalez-Jorge et al., 2013). Interestingly, in the current study, *NCED*, *CCD1* and *CCD4* were up-regulated in *IbOr-Ins* overexpressing sweetpotato storage roots despite the fact that the carotenoid contents of these roots were up to 7-fold that of the WT (Table 1). Together with previous reports, our results suggest that the carotenoid accumulation rate, which is controlled by *IbOr-Ins*, is greater than the increased rate of degradation of carotenoids due to the presence of *CCD* genes. Furthermore, IbOr protein not only activated *CCD* expression, but may have also increased the catalytic activities of CCDs via certain mechanisms. Therefore, we speculate that silencing of *CCD1* and/or *CCD4* in *IbOr* plants might increase the accumulation of carotenoids more than overexpression of *IbOr-Ins*. The metabolites of *CCD1* and *CCD4* in *IbOr* plants and the interaction between IbOr protein and CCDs will be studied in detail in terms of their effects on carotenoid homeostasis.

Interestingly, overexpression of *IbOr-Ins* in transgenic sweetpotato plants seemed to have modified the anthocyanin contents in one of the three transgenic events and transcription. *Or* is a specific regulator of the carotenoid pathway but does not appear to be linked to anthocyanin biosynthesis. There are several reports demonstrating that manipulating plants to increase the contents of either carotenoids or anthocyanin simultaneously increases the accumulation of both of these compounds in transgenic crops such as tomato and apple. Down-regulation of the photomorphogenesis regulatory gene *DET1* increases both the carotenoid and flavonoid contents of tomato fruits (Davuluri et al., 2005; Enfissi et al., 2010). In addition, transgenic apple overexpressing *MYB10*, encoding an anthocyanin biosynthesis-activating transcription factor, exhibits increased levels of both anthocyanin and carotenoids and increased expression of carotenoid biosynthetic genes such as *PDS* and *PSY* (Espley et al., 2013). While direct cross-talk between carotenoid and anthocyanin biosynthesis processes should be evaluated in detail to explain how these processes are co-regulated, the current results suggest that overexpression of *IbOr-Ins* increases the levels of both health-promoting phytochemicals in a single sweetpotato storage root. It remains to be determined whether *IbOr-Ins* directly regulates anthocyanin biosynthetic genes at the transcriptional and/or post-transcriptional levels. A larger number of samples will however be needed to account for possible epigenetic changes due to regeneration in vitro from embryogenic calli. Further characterization of the molecular function of *IbOr-Ins* might reveal links between these pathways.

The *IbOr*-201 line showed the highest anthocyanin content; however, the *IbOr*-210 line showed the highest expression of anthocyanin biosynthesis-related genes (Fig. 3). Based on previous studies of apple, we suggest that this phenomenon may depend on the developmental stage of the sweetpotato storage root. Anthocyanin and anthocyanin biosynthetic genes were analyzed in red-fleshed apple during the five stages of fruit development at 40, 67, 102, 130 and 146 days after full bloom (DAFB). Anthocyanin content increased during early developmental stages, peaked at 130 DAFB, and decreased at 146 DAFB. However, anthocyanin biosynthetic gene products were most abundant at 102 DAFB (Espley et al., 2007). In this study, we speculate on the basis of storage root weight that the developmental stage of the *IbOr*-210 line might be earlier stage than that of the *IbOr*-103 and −201 lines. The anthocyanin content and expression of *IbOr* and anthocyanin biosynthetic genes during storage root formation of sweetpotato will be investigated in more detail in a future study.

In this study, we used the *CaMV 35S* promoter to increase the levels of carotenoids in the storage roots of sweetpotato. Recently, Diretto et al. (2007) used constitutive *CaMV 35S* and tuber-specific patatin promoters to increase carotenoids in potato tubers. Expression of *CrtI, CrtB, CrtY* genes, under tuber-specific promoter control, results in tubers with ~114.4 μg g^{-1} DW contents of total carotenoids, whereas three genes, *CaMV 35S* promoter control, results in tubers with ~16.5 μg g^{-1} DW. These results indicate that appropriate choice of promoter is important to accumulate carotenoids in specific tissue of transgenic plants. In sweetpotato, the *sporamin* promoter is highly expressed in the storage roots; its expression is induced in leaves, stems and petioles by exogenous application of sucrose and in response to wounding stress (Hattori et al., 1991). The oxidative stress-inducible *SWPA2* promoter is strongly induced in response to various abiotic stresses including wounding, chilling, sulfur dioxide and ozone exposure, as well as

UV irradiation (Kim et al., 1999). In a previous study, transgenic *Arabidopsis* harboring *SWPA2-IbMYB1* accumulated high levels of anthocyanin in both 8-day-old seedlings and 4-week-old rosette leaves. Eight-day-old *Sporamin-IbMYB1* lines accumulated approximately 3-fold higher levels of anthocyanin than *CaMV 35S-IbMYB1* lines (Chu et al., 2013). Furthermore, we recently demonstrated that the storage roots of transgenic sweetpotato overexpressing *IbMYB1* under the control of the *sporamin* promoter accumulated more anthocyanin than that under the control of the *SWPA2* promoter (Park et al., 2014), suggesting that the storage root-dominant *sporamin* promoter will be more useful than the *SWPA2* promoter for developing sweetpotatoes that produce higher levels of carotenoids in their storage roots. Transgenic plants such as sweetpotato, potato and alfalfa overexpressing *IbOr-Ins* under the control of various promoters are currently under development for use in sustainable agriculture on marginal lands worldwide.

To evaluate whether the expression of *IbOr-Ins* affects yields, we measured the yields of storage root and aerial parts under field condition. The results show that the yields of the storage roots and aerial parts of plants were not significantly different between WT and *IbOr* plants, although some *IbOr* plants had slightly higher yields than WT plants (Fig. 4). The maximum efficiency of photosystem II (Fv/Fm) and the chlorophyll contents were also similar in WT and *IbOr* plants (Fig. 4B and C). Further characterization of *IbOr* plants should be performed to determine their abiotic stress tolerance under harsh conditions.

In conclusion, anthocyanin-rich transgenic sweetpotato plants overexpressing *IbOr-Ins* were successfully generated and characterized in terms of carotenoid contents, transcription levels of carotenoid biosynthetic and degradation genes, and antioxidant activity in storage roots. The results indicate that overexpression of *IbOr-Ins* can increase the carotenoid contents of sweetpotato storage roots. The production of transgenic plants overexpressing *IbOr-Ins* under the control of the proper promoter is expected to contribute to human health by increasing the contents of value-added carotenoids in these crops and by increasing their tolerance to environmental stresses, which would be beneficial for sustainable agriculture on marginal lands worldwide.

Author contributions

S.S. Kwak and S.C Park conceived and designed the experiments. S.C. Park, S.H. Kim, S. Park, H.U. Lee, J.Y. Bae and Y.H. Kim performed the experiments. J.C. Jeong, H.S. Lee, J.S. Lee and M.J. Ahn analyzed the data. S.S. Kwak, H.S. Lee and J.S. Lee contributed reagents/materials/analysis tools. S.C. Park and S.S. Kwak wrote the paper.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.plaphy.2014.11.017.

References

Auldridge, M.E., McCarty, D.R., Klee, H.J., 2006. Plant carotenoid cleavage oxygenases and their apocarotenoid products. Curr. Opin. Plant Biol. 9, 315–321.

Bai, C., Rivera, S.M., Medina, V., Alves, R., Vilaprinyo, E., Sorribas, A., Canela, R., Capell, T., Sandmann, G., Christou, P., Zhu, C., 2014. An *in vitro* system for the rapid functional characterization of genes involved in carotenoid biosynthesis and accumulation. Plant J. 77, 464–475.

Campbell, R., Ducreux, L.J.M., Morris, W.L., Morris, J.A., Suttle, J.C., Ramsay, G., Bryan, G.J., Hedley, P.E., Taylor, M.A., 2010. The metabolic and developmental roles of carotenoid cleavage dioxygenase4 from potato. Plant Physiol. 154, 656–664.

Cazzonelli, C., Cazzonelli, B., Pogson, 2010. Source to sink: regulation of carotenoid biosynthesis in plants. Trends Plant Sci. 15, 266–274.

Chen, P.-N., Chu, S.-C., Chiou, H.-L., Chiang, C.-L., Yang, S.-F., Hsieh, Y.-S., 2005. Cyanidin 3-glucoside and peonidin 3-glucoside inhibit tumor cell growth and induce apoptosis in vitro and suppress tumor growth in vivo. Nutr. Cancer 53, 232–243.

Chu, H., Jeong, J.C., Kim, W.-J., Chung, D.M., Jeon, H.K., Ahn, Y.O., Kim, S.H., Lee, H.-S., Kwak, S.-S., Kim, C.Y., 2013. Expression of the sweetpotato R2R3-type *IbMYB1a* gene induces anthocyanin accumulation in *Arabidopsis*. Physiol. Plant 148, 189–199.

Cunningham, F.X., Gantt, E., 1998. Genes and enzymes of carotenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 557–583.

Davuluri, G.R., van Tuinen, A., Fraser, P.D., Manfredonia, A., Newman, R., Burgess, D., Brummell, D.A., King, S.R., Palys, J., Uhlig, J., Bramley, P.M., Pennings, H.M.J., Bowler, C., 2005. Fruit-specific RNAi-mediated suppression of *DET1* enhances carotenoid and flavonoid content in tomatoes. Nat. Biotechnol. 23, 890–895.

Diretto, G., Al-Babili, S., Tavazza, R., Papacchioli, V., Beyer, P., Giuliano, G., 2007. Metabolic engineering of potato carotenoid content through tuber-specific overexpression of a bacterial mini-pathway. PLoS ONE 2, e350.

Enfissi, E.M.A., Barneche, F., Ahmed, I., Lichtlé, C., Gerrish, C., McQuinn, R.P., Giovannoni, J.J., Lopez-Juez, E., Bowler, C., Bramley, P.M., Fraser, P.D., 2010. Integrative transcript and metabolite analysis of nutritionally enhanced *DE-ETIOLATED1* downregulated tomato fruit. Plant Cell 22, 1190–1215.

Espley, R.V., Bovy, A., Bava, C., Jaeger, S.R., Tomes, S., Norling, C., Crawford, J., Rowan, D., McGhie, T.K., Brendolise, C., Putterill, J., Schouten, H.J., Hellens, R.P., Allan, A.C., 2013. Analysis of genetically modified red-fleshed apples reveals effects on growth and consumer attributes. Plant Biotechnol. J. 11, 408–419.

Espley, R.V., Hellens, R.P., Putterill, J., Stevenson, D.E., Kutty-Amma, S., Allan, A.,C., 2007. Red colouration in apple fruit is due to the activity of the MYB transcription factor, MdMYB10. Plant J. 49, 414–427.

García-Limones, C., Schnäbele, K., Blanco-Portales, R., Luz Bellido, M., Caballero, J.L., Schwab, W., Muñoz-Blanco, J., 2008. Functional characterization of *FaCCD1*: a carotenoid cleavage dioxygenase from strawberry involved in lutein degradation during fruit ripening. J. Agric. Food Chem. 56, 9277–9285.

Giuliano, G., 2014. Plant carotenoids: genomics meets multi-gene engineering. Curr. Opin. Plant Biol. 19, 111–117.

Gonzalez-Jorge, S., Ha, S.-H., Magallanes-Lundback, M., Gilliland, L.U., Zhou, A., Lipka, A.E., Nguyen, Y.-N., Angelovici, R., Lin, H., Cepela, J., Little, H., Buell, C.R., Gore, M.A., DellaPenna, D., 2013. *CAROTENOID CLEAVAGE DIOXYGENASE4* is a negative regulator of β-carotene content in *Arabidopsis* seeds. Plant Cell 25, 4812–4826.

Hattori, T., Fukumoto, H., Nakagawa, S., Nakamura, K., 1991. Sucrose-induced expression of genes coding for the tuberous root storage protein, sporamin, of sweetpotato in leaves and petioles. Plant Cell Physiol. 32, 79–86.

Kim, K.-Y., Huh, G.-H., Lee, H.-S., Kwon, S.-Y., Hur, Y., Kwak, S.-S., 1999. Molecular characterization of cDNAs for two anionic peroxidases from suspension cultures of sweetpotato. Mol. Gen. Genet. 261, 941–947.

Kim, S.-H., Hamada, T., 2005. Rapid and reliable method of extracting DNA and RNA from sweetpotato, *Ipomoea batatas* (L). Lam. Biotechnol. Lett. 27, 1841–1845.

Kim, S.H., Ahn, Y.O., Ahn, M.-J., Lee, H.-S., Kwak, S.-S., 2012. Down-regulation of β-carotene hydroxylase increases β-carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweetpotato. Phytochemistry 74, 69–78.

Kim, S.H., Ahn, Y.O., Ahn, M.-J., Jeong, J.C., Lee, H.-S., Kwak, S.-S., 2013a. Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and salt stress tolerance in transgenic sweetpotato cultures. Plant Physiol. Biochem. 70, 445–454.

Kim, S.H., Kim, Y.-H., Ahn, Y.O., Ahn, M.-J., Jeong, J.C., Lee, H.-S., Kwak, S.-S., 2013b. 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. Physiol. Plant 147, 432–442.

Kim, S.H., Jeong, J.C., Park, S., Bae, J.-Y., Ahn, M.-J., Lee, H.-S., Kwak, S.-S., 2014. Down-regulation of sweetpotato lycopene β-cyclase gene enhances tolerance to abiotic stress in transgenic culture cells. Mol. Biol. Rep. http://dx.doi.org/10.1007/s11033-014-3714-4.

Li, L., Yang, Y., Xu, Q., Owsiany, K., Welsch, R., Chitchumroonchokchai, C., Lu, S., Van Eck, J., Deng, X.-X., Failla, M., Thannhauser, T.W., 2012. The *Or* gene enhances carotenoid accumulation and stability during post-harvest storage of potato tubers. Mol. Plant 5, 339–352.

Lim, S., Yang, K.-S., Kwon, S.-Y., Paek, K.-Y., Kwak, S.-S., Lee, H.-S., 2004. *Agrobacterium*-mediated genetic transformation and plant regeneration of sweetpotato (*Ipomoea batatas*). J. Plant Biotechnol. 31, 267–271.

Lopez, A.B., Van Eck, J., Conlin, B.J., Paolillo, D.J., O'Neill, J., Li, L., 2008. Effect of the cauliflower *Or* transgene on carotenoid accumulation and chromoplast formation in transgenic potato tubers. J. Exp. Bot. 59, 213–223.

Low, J.W., Arimond, M., Osman, N., Cunguara, B., Zano, F., Tschirley, D., 2007. A food-based approach introducing orange-fleshed sweet potatoes increased vitamin A

intake and serum retinol concentrations in young children in rural Mozambique. J. Nutr. 137, 1320–1327.
Lu, S., Van Eck, J., Zhou, X., Lopez, A.B., O'Halloran, D.M., Cosman, K.M., Conlin, B.J., Paolillo, D.J., Garvin, D.F., Vrebalov, J., Kochian, L.V., Küpper, H., Earle, E.D., Cao, J., Li, L., 2006. The cauliflower *Or* gene encodes a DnaJ cysteine-rich domain-containing protein that mediates high levels of β-carotene accumulation. Plant Cell 18, 3594–3605.
Luceri, C., Giovannelli, L., Pitozzi, V., Toti, S., Castagnini, C., Routaboul, J.-M., Lepiniec, L., Larrosa, M., Dolara, P., 2008. Liver and colon DNA oxidative damage and gene expression profiles of rats fed *Arabidopsis thaliana* mutant seeds containing contrasted flavonoids. Food Chem. Toxicol. 46, 1213–1220.
Paine, J., Paine, C., Shipton, S., Chaggar, R., Howells, M., Kennedy, G., Vernon, S., Wright, E., Hinchliffe, J., Adams, A., Silverstone, R., Drake, 2005. Improving the nutritional value of Golden Rice through increased pro-vitamin A content. Nat. Biotechnol. 23, 482–487.
Paolillo Jr., D.J., Garvin, D.F., Parthasarathy, M.V., 2004. The chromoplasts of *Or* mutants of cauliflower (*Brassica oleracea* L. var. *botrytis*). Protoplasma 224, 245–253.
Park, S.-C., Kim, Y.-H., Kim, S.H., Jeong, Y.J., Kim, C.Y., Lee, J.S., Bae, J.Y., Ahn, M.J., Jeong, J.C., Lee, H.-S., Kwak, S.-S., 2014. Overexpression of the *IbMYB1* gene in an orange-fleshed sweet potato cultivar produces a dual-pigmented transgenic sweet potato with improved antioxidant activity. Physiol. Plant. http://dx.doi.org/10.1111/ppl.12281.
Park, S.-C., Kim, Y.-H., Ji, C.Y., Park, S., Jeong, J. c., Lee, H.-S., Kwak, S.-S., 2012. Stable internal reference genes for the normalization of real-time PCR in different sweetpotato cultivars subjected to abiotic stress conditions. PLoS One 7, e51502.
Pascual-Teresa, S., Sanchez-Ballesta, M., 2008. Anthocyanins: from plant to health. Phytochem. Rev. 7, 281–299.
Pons, E., Alquézar, B., Rodríguez, A., Martorell, P., Genovés, S., Ramón, D., Rodrigo, M.J., Zacarías, L., Peña, L., 2014. Metabolic engineering of β-carotene in orange fruit increases its *in vivo* antioxidant properties. Plant Biotechnol. J. 12, 17–27.
Sandmann, G., Römer, S., Fraser, P.D., 2006. Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. Metab. Eng. 8, 291–302.
Tanaka, Y., Ohmiya, A., 2008. Seeing is believing: engineering anthocyanin and carotenoid biosynthetic pathways. Curr. Opin. Biotechnol. 19, 190–197.
Teow, C.C., Truong, V.-D., McFeeters, R.F., Thompson, R.L., Pecota, K.V., Yencho, G.C., 2007. Antioxidant activities, phenolic and β-carotene contents of sweetpotato genotypes with varying flesh colours. Food Chem. 103, 829–838.
Walter, M.H., Strack, D., 2011. Carotenoids and their cleavage products: biosynthesis and functions. Nat. Prod. Rep. 28, 663–692.
World Health Organization (WHO), 2009. Global Prevalence of Vitamin A Deficiency in Populations at Risk 1995–2005. Available at: http://whqlibdoc.who.int/publications/2009/9789241598019_eng.pdf (accessed 15.06.14.).
Yeum, K.-J., Russell, R.M., Krinsky, N.I., Aldini, G., 2004. Biomarkers of antioxidant capacity in the hydrophilic and lipophilic compartments of human plasma. Arch. Biochem. Biophys. 430, 97–103.
Zhou, X., McQuinn, R., Fei, Z., Wolters, A.-M.A., Van Eck, J., Brown, C., Giovannoni, J.J., Li, L.I., 2011. Regulatory control of high levels of carotenoid accumulation in potato tubers. Plant Cell Environ. 34, 1020–1030.