Muhammad Zubair Khan, Miho Takemura, Takashi Maoka, Motoyasu Otani and Norihiko Misawa*

Carotenoid analysis of sweetpotato *Ipomoea batatas* and functional identification of its lycopene β- and ε-cyclase genes

DOI 10.1515/znc-2016-0150
Received July 24, 2016; revised August 2, 2016; accepted August 2, 2016

Abstract: Sweetpotato *Ipomoea batatas* is known as a hexaploid species. Here, we analyzed carotenoids contained in the leaves and tubers of sweetpotato cultivars 'White Star' (WS) and W71. These cultivars were found to contain several carotenoids unique to sweetpotato tubers such as β-carotene-5,6,5′,8′-diepoxide and β-carotene-5,8-epoxide. Next, we isolated two kinds of carotene cyclase genes that encode lycopene β- and ε-cyclases from the WS and W71 leaves, by RT-PCR and subsequent RACE. Two and three lycopene β-cyclase gene sequences were, respectively, isolated from WS, named *IbLCYb1*, *2*, and from W71, *IbLCYb3*, *4*, *5*. Meanwhile, only a single lycopene ε-cyclase gene sequence, designated *IbLCYe*, was isolated from both WS and W71. These genes were separately introduced into a lycopene-synthesizing *Escherichia coli* transformed with the *Pantoea ananatis crtE*, *crtB* and *crtI* genes, followed by HPLC analysis. β-Carotene was detected in *E. coli* cells that carried *IbLCYb1-4*, indicating that the *IbLCYb1-4* genes encode lycopene β-cyclase. Meanwhile, the introduction of *IbLCYe* into the lycopene-synthesizing *E. coli* led to efficient production of δ-carotene with a monocyclic ε-ring, providing evidence that the *IbLCYe* gene codes for lycopene ε-(mono)cyclase. Expression of the β- and ε-cyclase genes was analyzed as well.

***Corresponding author: Norihiko Misawa,** Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi-shi, Ishikawa 921-8836, Japan, Phone: +81-76-227-7525, Fax: +81-76-227-7557, E-mail: n-misawa@ishikawa-pu.ac.jp
Muhammad Zubair Khan, Miho Takemura and Motoyasu Otani: Research Institute for Bioresources and Biotechnology, Ishikawa Prefectural University, 1-308 Suematsu, Nonoichi-shi, Ishikawa 921-8836, Japan
Takashi Maoka: Research Institute for Production Development, Shimogamo-morimotocho, Sakyo-ku, Kyoto 606-0805, Japan

Keywords: α-carotene; β-carotene; carotenoid; *Ipomoea batatas*; lycopene cyclase; sweetpotato.

Dedication: This work is dedicated to the memory of Professor Peter Böger. He kindly provided the opportunity for NM to work in his laboratory on the generation of bleaching herbicide-resistant plants, and encouraged NM to work on carotenoid biosynthesis.

1 Introduction

Sweetpotato [*Ipomoea batatas* (L.) Lam], also described as sweet potato, which belongs to the family Convolvulaceae, occupies the seventh position among the food crops of the world [1]. It can be used as food supply to combat malnutrition in the developing nations, since the tuberous roots (tubers) are enriched with starch and dietary fiber, along with carotenoids, anthocyanin, ascorbic acid, potassium, calcium, iron, and other bioactive ingredients [2–5]. Sweetpotato also possesses a potential for bioenergy production as it can adapt to growth on marginal lands [6]. For people of South-east Asia and Africa, this crop is the main source of β-carotene [7]. Sweetpotato is a hexaploid species ($2n = 6x = 90$) that has a basic chromosome number of 15, and genetic studies on this species are exhausting, since it is difficult to generate seeds and to evaluate the effects of polyploidy on the genome [8, 9]. Its tubers exhibit various colors such as white, yellow, orange, and purple among different cultivars, and all species include β-carotene as well as other carotenoids, while orange-fleshed lines were shown to contain β-carotene as the predominant carotenoid [2, 4, 5, 10, 11]. The tubers of the Japanese cultivar "Benimasari" were found to accumulate unique carotenoids such as β-carotene-5,8,5′,8′-diepoxide (40.5% of the total carotenoids), β-carotene-5,8-epoxide (6.5%) and ipomoeaxanthin A (3.2%), in addition to β-carotene (10.5%) [12].

Although sweetpotato is highly important as a valuable source of carotenoids including β-carotene, very little research has been done on molecular biological aspects of its carotenoid biosynthesis [6, 7, 9]. 'White Star' (WS) and

W71, producing white- and orange-fleshed tubers, respectively, are important sweetpotato cultivars, since they are amenable to *Agrobacterium*-mediated transformation ([13, 14] and our unpublished results). Chemical analysis of the carotenoids of these two cultivars has not been performed so far. The present study elucidates carotenoid biosynthesis in sweetpotato cultivars WS and W71 through chemical analysis of their carotenoids as well as functional and expression analyses of their carotene cyclase genes.

2 Experimental

2.1 Plant materials and growth

We used two cultivars of sweetpotato [*I. batatas* (L.) Lam], WS and W71, which generate white- and orange-fleshed tuberous roots (tubers), respectively. Both cultivars were bred in the USA [14, 15]. The vines (ca. 25 cm in length) of each cultivar were planted into Wagner pots (159 mm in diameter and 190 mm in height) containing culture soil [Engeibaido 1 (Nihon Hiryo, Tokyo, Japan)] in a greenhouse. After 5 months of growth, tubers were harvested from each cultivar.

2.2 Extraction of total RNA from sweetpotato leaves and cDNA synthesis

Leaf samples from the two cultivars were ground in liquid nitrogen. Total RNA was isolated from 100 mg of leaf powder from each cultivar, as described [14]. Total RNA extracts were then treated with DNase I to remove any contaminating genomic or foreign DNA. Using 1 μg of total RNA, first strand cDNAs were synthesized with an oligo-dT primer in a reaction catalyzed by reverse transcriptase (RT), ReverTra Ace (Toyobo, Osaka, Japan). Incubations were first at 42 °C for 20 min, swiftly shifted to 99 °C for 5 min, and at the end cooled down at 4 °C (and stored at –30 °C until the next use).

2.3 Isolation of full length cDNAs for carotene cyclase genes and phylogenetic analysis

3′and 5′-RACE were carried out by SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the supplier's instructions. We designed primers for RACE-PCR based on the partial sequences of an *I. batatas* lycopene β-cyclase gene (*IbLCYb*; accession no. GQ283003) and an *I. batatas* lycopene ε-cyclase gene (*IbLCYe*; accession no. HQ828090) in the Genbank database. As the first PCR primers for 3′-RACE of *IbLCYb* and *IbLCYe*, IbLCYbF1 (5′-GCGTATGGCATTCTGGCAGAAGTGG-3′) and IbLCYeF1 (5′-CGAAGTCCTGGTATGGGTGATATGC-3′) were used, respectively. For the nested PCR amplification in 3′-RACE, respective nested gene-specific primers for IbLCYbF2 (5′-CATGGATTGGCGAGACTCTCACC-3′) and IbLCYeF2 (5′-CAAGCTTGGGAAACGCTTTGGCC-3′) were used. Similarly, for the amplification of the *IbLCYb* and *IbLCYe* 5′-ends, IbLCYbR1 (5′-AAGCCAGTTGCATCGAGAACCACAG-3′) and IbLCYeR1 (5′-TGAATACCCTGTAGCGGGATGAACC-3′) were used in the first PCR, respectively. For 5′-RACE, IbLCYbR2 (5′-CATGGATTGGCGAGACTCTCACC-3′) and IbLCYeR2 (5′-CAAGCTTGGGAAACGCTTTGGCC-3′) were used as the nested PCR primers. Each RACE product was subcloned into the pGEM T-easy vector (Promega, Madison, WI, USA), followed by DNA sequencing. Full length cDNA sequences of *IbLCYb* and *IbLCYe* were obtained through the above PCRs. The full length cDNAs of the *IbLCYb* and *IbLCYe* genes were isolated by PCR amplification with a pair of primers: IbLCYbF3 (5′-CGTCGACATGGATACTCTGCTAAA-3′) and IbLCYbR3 (5′-GGCGGCCGCTTAATCTATATCCTGTAAC-3′), and IbLCYeF3 (5′-CGGATCCATGGAGTGCATCGGAGCTC-3′) and IbLCYeR3 (5′-CGTCGACTTACAGAGTGAGATAAGT-3′), respectively.

DNA sequences were analyzed using DNASIS DNA analysis software (Hitachi Solutions, Tokyo, Japan). Homology search was performed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Amino acid sequences were aligned by CLUSTAL W (http://www.clustal.org/), and a neighbor-joining tree was constructed with a 500 bootstrap replication support using MEGA7 software. Transit peptides of the gene products were predicted by ChloroP (http://www.cbs.dtu.dk/services/ChloroP/).

2.4 Functional analysis of isolated carotene cyclase genes

The coding regions of the *IbLCYb* and *IbLCYe* genes were amplified by PCR and cloned into the pETDuet vector (Merck Millipore, Darmstadt, Germany), yielding plasmids pETD-IbLCYb and pETD-IbLCYe, respectively. For complementary expression of the two *IbLCYb* and *IbLCYe* genes, they were cloned simultaneously into pETDuet and named pETD-IbLCYb/IbLCYe. Each plasmid was introduced into a lycopene-producing *Escherichia coli* [BL21 (DE3)], which carried the plasmid pACCRT-EIB for the

expression of the *crtE*, *crtB*, and *crtI* genes from the bacterium *Pantoea ananatis* [16]. Each transformed *E. coli* was grown in 2YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) with 100 mg/L of ampicillin and 30 mg/L of chloramphenicol at 37 °C. When an optical density of 0.8–1.0 had been reached, 0.05 mM of IPTG was added, and cultivation continued at 21 °C for 2 days.

Extraction of carotenoids from recombinant *E. coli* was carried out by the method described [17]. Recombinant *E. coli* cultures were centrifuged and cell pellets were extracted with methanol (MeOH) using a mixer for 5 min. Tris-HCl (50 mM, pH 7.5) (containing 1 M NaCl) was added and mixed. Chloroform was then added to the mixture and the emulsion centrifuged for 5 min. The chloroform phase was collected and dried by centrifugal evaporation. Dried residues were re-suspended in ethyl acetate and applied to HPLC in a Waters Alliance 2695–2996 (PDA) system (Milford, CT, USA). HPLC was performed according to the method described [18] using a TSKgel ODS-80Ts column (4.6 × 150 mm, 5 μm; Tosoh, Tokyo, Japan) at a flow rate of 1.0 mL/min at 25 °C with solvent A (water-MeOH, 5:95, v/v) for 5 min, followed by a linear gradient from solvent A to solvent B (tetrahydrofuran: MeOH, 3:7, v/v) for 5 min, and solvent B alone for 8 min. Individual carotenoids were identified by comparing retention times and absorption spectra with those of the authentic standards [19]. To separate α- and β-carotene, an isocratic method was applied. HPLC was performed on a Nova-pak HR 6 μ C18 column (3.9 × 300 mm; Waters) at a flow rate of 1.0 mL/min at 25 °C with the solvent [acetonitrile (AcCN)-MeOH-2-propanol, 90: 6:4, v/v/v] for 80 min.

2.5 Carotenoid analysis

Extraction of carotenoids from the leaves and tubers of the two sweetpotato cultivars and subsequent chromatographic and spectroscopic analyses were carried out as follows: Total carotenoids were extracted from leaves and tubers with acetone at room temperature. The extract was concentrated to a small volume in vacuo and partitioned with *n*-hexane-diethyl ether (1:1, v/v) and water. The organic layer was evaporated to dryness and submitted to LC/MS analysis. The total carotenoid contents were calculated employing an extinction coefficient of $E_{cm}^{1\%} = 2400$ at λ max. The ether-hexane solution was evaporated. In the case of leaf carotenoid analysis, chlorophylls were removed by treatment with 5% (w/v) KOH/MeOH. The residue was subjected to analysis in a LC/photodiode-array detection (PDA)/MS system. The LC/PDA/MS analysis of carotenoids was carried out using a Waters Xevo G2S QTof mass spectrometer equipped with an Acquity UPLC system. UV-Visible (UV-VIS) absorption spectra were recorded from 200 to 600 nm by PDA. The electro-spray ionization (ESI) time-of-flight (TOF) MS spectra were acquired by scanning from *m/z* 100 to 1500 with a capillary voltage of 3.2 kV, cone voltage of 40 eV, and source temperature of 120 °C. Nitrogen was used as a nebulizing gas at a flow rate of 30 L/h. An Acquity 1.7 μm BEH UPLC C18 column (Waters) was used as a stationary phase with the mobile phase of: AcCN-water (85:15, v/v) → AcCN-MeOH (65:35), with a column temperature of 40 °C, at the flow rate of 0.4 mL/min.

2.6 Expression analysis of cloned carotene cyclase genes

Expression analysis of the two genes was performed by real-time PCR. Total RNA was extracted from sweetpotato leaves and tubers using an RNeasy Plant mini kit (Qiagen, Hilden, Germany), and treated with DNase I. One microgram each of total RNA was reverse-transcribed with oligo-dT primer using PrimeScript RT Master Mix (Takara, Shiga, Japan). Amplification in real-time PCR was performed using SYBR Premix DimerEraser (Takara), and data analysis was carried out using the ABI 7300 Real Time PCR System (Life Technologies). The primers used in real-time PCR are shown in Table 1. The expression levels of the various genes were normalized by the actin gene *IbACT7* as reference gene for the internal control.

2.7 Accession number of cloned carotene cyclase genes

The nucleotide sequences of the sweetpotato carotene cyclase genes cloned in this work were submitted to DDBJ under accession numbers LC164788 (*IbLCYb1*), LC164789 (*IbLCYb2*), LC164790 (*IbLCYb3*), LC164791 (*IbLCYb4*), LC164792 (*IbLCYb5*), and LC164793 (*IbLCYe*).

Table 1: Primers used in real-time PCR.

IbLCYbRTF1	5′-AGCGTTGTGTTATCCCAATGG-3′
IbLCYbRTR1	5′-TACCGCCAATTCCAACAACTC-3′
IbLCYeRTF1	5′-TCATTCCATGCAGGCTAGTTACTG-3′
IbLCYeRTR1	5′-CCCAACCTCATACTGCAACAATT-3′
IbACT7F1	5′-CCAAGAGCAGTGTTTCCCAGTAT-3′
IbACT7R1	5′-TCTGTCCCATCCCAACCATAA-3′

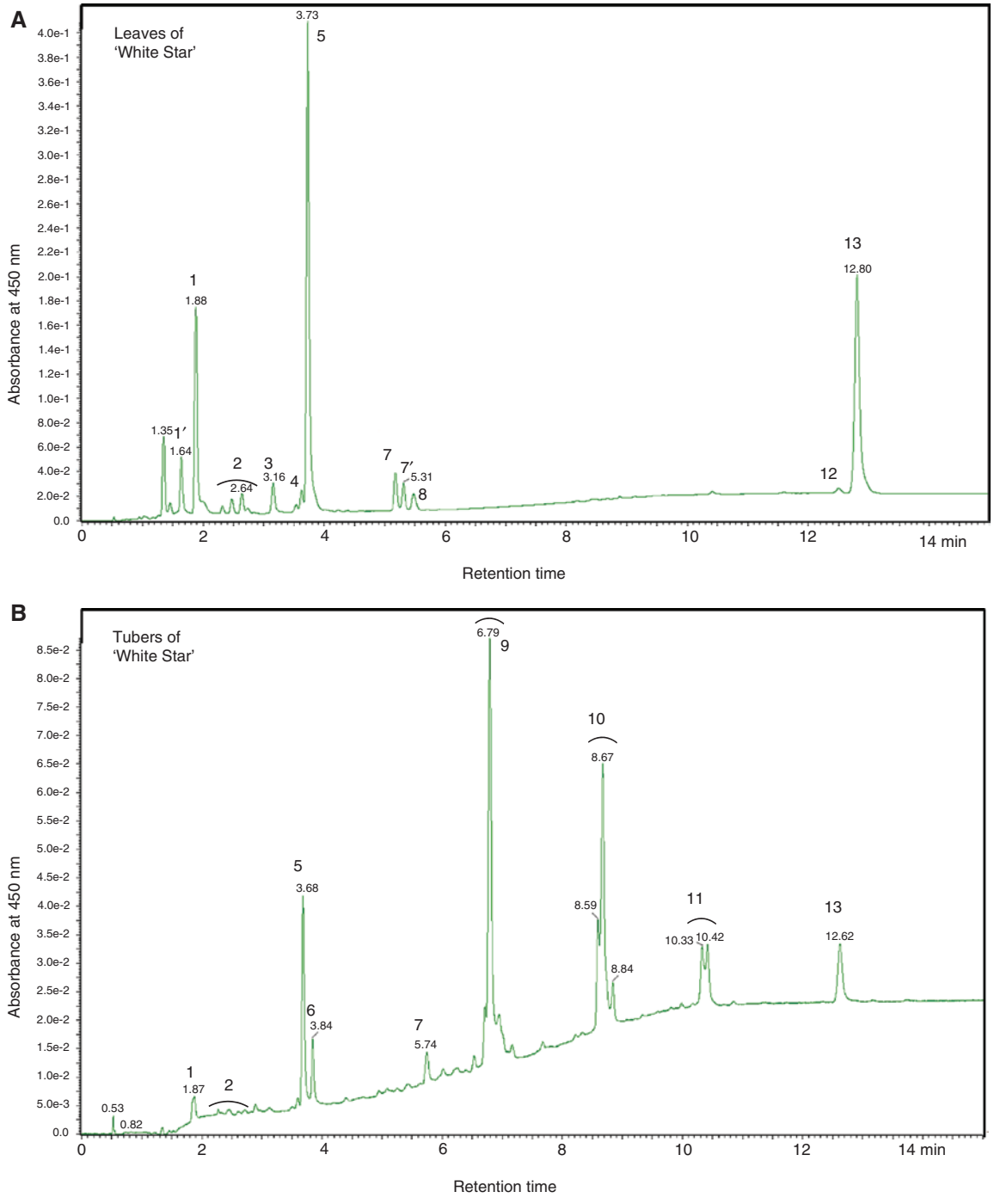

Figure 1: HPLC chromatograms of carotenoids extracted from the leaves (A) and tubers (B) of sweetpotato (*Ipomoeoa batatas*) cultivar WS. An Acquity 1.7 μm BEH UPLC C18 column (Waters) was used, as described in Section 2.5 of the Experimental section. Identification of peaks: 1′, neochrome; 1, neoxanthin; 2, violaxanthin (including auroxanthin); 3, antheraxanthin; 4, zeaxanthin; 5, lutein; 6, ipomoeaxanthin A; 7, 9-*cis*-lutein, 7′, 13-*cis*-lutein; 8, β-cryptoxanthin; 9, β-carotene-5,6,5′,8′-diepoxide (stereoisomers); 10, β-carotene-5,8,5′,8′-diepoxide (stereoisomers); 11, β-carotene-5,8-epoxide (stereoisomers); 12, α-carotene; 13, β-carotene. Neochrome (peak 1′) and auroxanthin (some in peak 2) are considered to be formed from neoxanthin and violaxanthin, respectively, during the extraction process. Peaks 9, 10 and 11 are individually composed of several peaks, which are thought to contain stereoisomers, and sometimes geometrical isomers generated during the extraction process.

3 Results and discussion

3.1 Carotenoids in sweetpotato cultivars WS and W71

We selected two sweetpotato (*I. batatas*) cultivars, WS and W71, as representative genotypes, exhibiting white and orange tuberous roots (tubers), respectively, since they have also been used in *Agrobacterium tumefaciens*-mediated transformation ([13, 14], and our unpublished results). Carotenoid pigments were extracted from the two cultivars and subjected to HPLC-PDA-HRMS analysis. Figure 1 shows HPLC chromatograms of carotenoids extracted from the leaves (A) and tubers (B) of cultivar WS. Table 2 contains the spectroscopic data of the individual carotenoids appearing in Figure 1. Table 3 shows the carotenoid composition in the leaves and tubers of sweetpotato cultivars WS and W71, which was calculated based on the HPLC chromatograms (Figure 1 for WS, and data not shown for W71). Carotenoid composition in the leaves of both sweetpotato cultivars was substantially the same as that of other higher plant leaves mediating

Table 2: Spectroscopic data of the individual carotenoids in Figure 1.

Number[a]	Carotenoid	UV-VIS nm	ESI TOF MS *m/z*
1′	Neochrome	405, 428, 450	601.4232 (M^+, $C_{40}H_{57}O_4$)
1	Neoxanthin	414, 439, 469	601.4232 (M^+, $C_{40}H_{57}O_4$)
2	Violaxanthin	414, 439, 469	601.4232 (M^+, $C_{40}H_{57}O_4$)
2	Auroxanthin	376, 399, 423	601.4232 (M^+, $C_{40}H_{57}O_4$)
3	Antheraxanthin	425, 446, 473	568.4299 (M^+ $C_{40}H_{56}O_2$), 551.4246 (M^+-H_2O)
4	Zeaxanthin	(425), 451, 477	568.4299 (M^+ $C_{40}H_{56}O_2$)
5	Lutein	425, 446, 473	568.4299 (M^+ $C_{40}H_{56}O_2$), 551.4246 (M^+-H_2O)
6	Ipomoeaxanthin A	425, 446, 473	586.4410 (M^+ $C_{40}H_{58}O_3$)
7	9-*cis*-Lutein	422, 441, 468	568.4299 (M^+ $C_{40}H_{56}O_2$), 551.4246 (M^+-H_2O)
7′	13-*cis*-Lutein	420, 439, 465	568.4299 (M^+ $C_{40}H_{56}O_2$), 551.4246 (M^+-H_2O)
8	β-Cryptoxanthin	(425), 451, 477	552.4327 (M^+ $C_{40}H_{56}O$)
9	β-Carotene-5,6, 5′,8′-diepoxide	405, 426, 451	569.4348 (MH^+ $C_{40}H_{57}O_2$)
10	β-Carotene-5,8, 5′,8′-diepoxide	379, 399, 424	569.4348 (MH^+ $C_{40}H_{57}O_2$)
11	β-Carotene-5,8-epoxide	400, 426, 451	552.4327 (M^+ $C_{40}H_{56}O$)
12	α-Carotene	425, 446, 473	536.4383 (M^+ $C_{40}H_{56}$)
13	β-Carotene	(425), 451, 477	536.4383 (M^+ $C_{40}H_{56}$)
	β-Cryptoxanthin-5′,6′-epoxide[b]	425, 446, 473	568.4299 (M^+ $C_{40}H_{56}O_2$)

[a]Numbers correspond to those used in Figure 1. [b]β-Cryptoxanthin-5′,6′-epoxide was detected only in cultivar W71.

Table 3: Carotenoid content and composition in sweetpotato cultivars WS and W71.

	WS leaf	W71 leaf	'White Star' tuber	W71 tuber
Total carotenoid (μg/g FW)	34	170	0.5	4.0
Carotenoid composition	%	%	%	%
β-Carotene	30.7	31.1	7.3	59.3
α-Carotene	0.6		nd	nd
β-Cryptoxanthin	2.4			3.2
Zeaxanthin	0.7			trace
Lutein	43.2	46.8	13.7	2.0
cis-Lutein	3.3	3.3		
Antheraxanthin	1.8			
Violaxanthin (+ Auroxanthin)	12.3	3.5	0.9	
Neoxanthin (+ Neochrome)	3.6	6.4	1.5	
β-Carotene-5,8-epoxide	nd	nd	9.4	4.6
β-Carotene-5,6,5′,8′-diepoxide	nd	nd	35.7	9.2
β-Carotene-5,8,5′,8′-diepoxide	nd	nd	25.1	13.8
β-Cryptoxanthin-5′,6′-epoxide	nd	nd	nd	2.2
Ipomoeaxanthin A	nd	nd	4.5	nd
Others	1.4	8.9	1.9	5.7

nd, not detected.

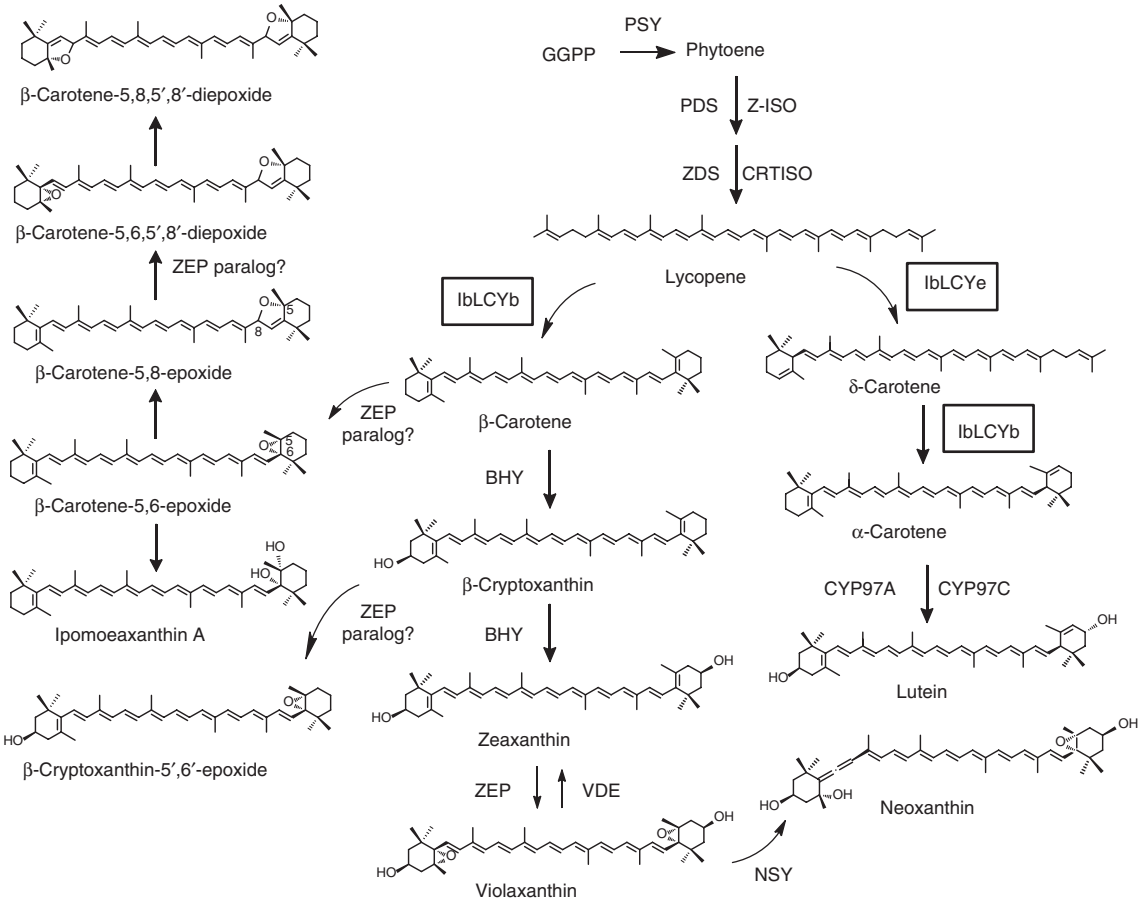

Figure 2: Proposed carotenoid biosynthetic pathway in the tubers of sweetpotato cultivars WS and W71.
Ipomoeaxanthin A and β-cryptoxanthin-5′,6′-epoxide were found only in the tubers of WS and W71, respectively. It was considered that β-carotene-5,8-epoxide was non-enzymatically produced from β-carotene-5,6-epoxide, which was not observed in either WS or W71. Carotenoids containing the 5,6-epoxy-β-ring are stereospecific compounds, while those containing the 5,8-epoxy-β-ring occur as stereoisomers (C-8). Carotene cyclases elucidated in this work are boxed. GGPP, geranylgeranyl diphosphate.

photosynthesis, i.e. their carotenoids contain approximately 45–50% lutein and approximately 30% β-carotene as the two predominant carotenoids, along with neoxanthin and violaxanthin, and often with small amounts of β-cryptoxanthin, antheraxanthin, zeaxanthin and α-carotene [20]. Meanwhile, the tubers of cultivars WS and W71 were found to contain unique carotenoids that were composed of β-carotene-5,8-epoxide (9.4% and 4.6% of the total carotenoids, respectively), β-carotene-5,6,5′,8′-diepoxide (35.7% and 9.2%), and β-carotene-5,8,5′,8′-diepoxide (25.1% and 13.8%). Moreover, the WS and W71 tubers were found to contain ipomoeaxanthin A (4.5%) and β-cryptoxanthin-5′,6′-epoxide (2.2%), respectively. Their chemical structures are shown in Figure 2. Such rare carotenoids had previously been identified as unique constituents of the tubers of the Japanese sweetpotato cultivar "Benimasari" [12]. It is likely that sweetpotato tubers generally contain rare carotenoids that include a 5,6-epoxy-β-ring and/or a 5,8-epoxy-β-ring in their structures, since WS and W71 (bred in USA) should be genetically distant from the Japanese cultivar "Benimasari".

The carotenoid biosynthetic pathway in sweetpotato tubers, proposed on the basis of the above results is shown in Figure 2. The tubers of the three cultivars WS, W71 and "Benimasari" thus contain sweetpotato-specific carotenoids with the 5,6-epoxy-β-ring, rather than the epoxy carotenoids common to higher plant leaves, i.e. violaxanthin and antheraxanthin, with the 3-hydroxy-5,6-epoxy-β-ring. Therefore, sweetpotato tubers are likely to contain a novel enzyme that converts carotenoids possessing a β-ring, such as β-carotene and β-cryptoxanthin, into metabolites with a 5,6-epoxy-β-ring, such as β-carotene-5,6-epoxide and β-cryptoxanthin-5′,6′-epoxide. Such a β-ring-epoxidation enzyme can be anticipated to have considerable homology to the ZEP (zeaxanthin epoxidase) enzyme, encoded by a ZEP paralog. A candidate gene has been cloned in our laboratory, awaiting functional analysis. Ipomoeaxanthin A is considered to be produced from

β-carotene-5,6-epoxide by an unknown oxidizing enzyme. Meanwhile, carotenoids with an 5,8-epoxy-β-ring can arise non-enzymatically from precursors having an 5,6-epoxy-β-ring through an epoxy-furan transposition reaction.

3.2 Isolation of carotene cyclase genes from sweetpotato cultivar WS and W71 and phylogenetic analysis

Two and three lycopene β-cyclase gene (*IbLCYb*) sequences were, respectively isolated from WS, named *IbLCYb1* and *IbLCYb2*, and from W71, designated *IbLCYb3*, *IbLCYb4* and *IbLCYb5*. *IbLCYb3* and *IbLCYb4* were found to encode the same deduced amino acid sequences. In the case of the lycopene ε-cyclase gene (*IbLCYe*), a single sequence, designated *IbLCYe*, was isolated from both WS and W71. Another putative lycopene ε-cyclase gene had previously been isolated from sweetpotato cultivar Nongdafu 14 [21] (albeit without functional identification of the gene product as ε-cyclase), which encoded a protein with a N-terminal region totally different from that of our IbLCYe, but the mature proteins were 97.9% identical.

Figure 3 shows the phylogenetic analysis of the deduced proteins encoded by the isolated sweetpotato carotene cyclase genes, compared to other known carotene cyclases of higher plants. IbLCYb(1–5) and IbLCYe (in dotted boxes) individually formed single clades that were positioned close to the corresponding proteins from potato (*Solanum tuberosum*) and tomato (*Solanum lycopersicum*), which, like sweetpotato, belong to the order Solanales.

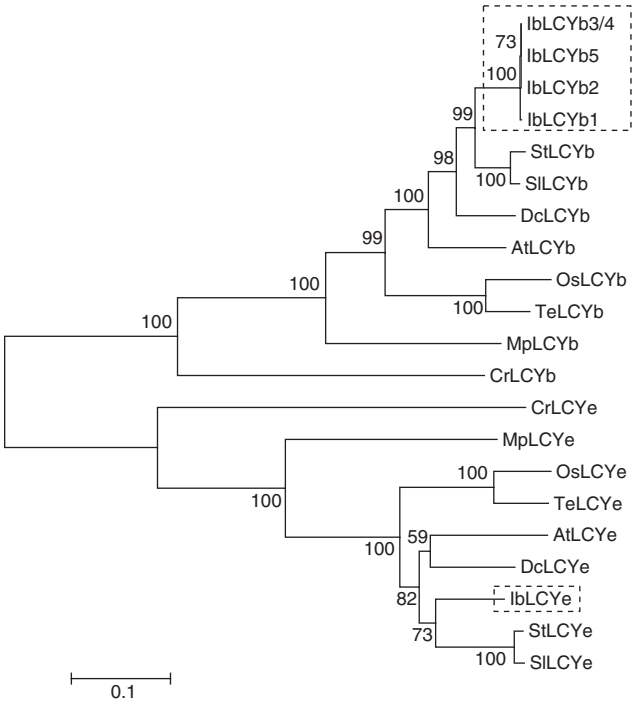

Figure 3: Phylogenetic tree of various lycopene β-cyclases (LCYb) and lycopene ε-cyclases (LCYe) including the newly isolated sweetpotato carotene cyclases.
The carotene cyclases elucidated in this paper are surrounded by dotted box. GenBank accession numbers of the aligned cyclases include: *Arabidopsis* LCYb (AtLCYb), U50739; carrot LCYb (DcLCYb), DQ192190; wheat LCYb (TaLCYb), JN622196.1; rice LCYb (OsLCYb), XP_015627235; tomato LCYb (SlLCYb1), EF650013;potato LCYb (StLCYb), XP_006364433; liverwort LCYb (MpLCYb),AB794089; *Chlamydomonas* LCYb (CrLCYb), AY860818; *Arabidopsis* LCYe (AtLCYe), U50738.1 carrot LCYe (DcLCYe), DQ192192.1; wheat LCYe (TaLCYe), EU649787; rice LCYe (OsLCYe), XP_015622198; tomato LCYe (SlLCYe), Y14387; potato LCYe (StLCYe), XP_006353544.1; liverwort LCYe (MpLCYe), AB794090; *Chlamydomonas* LCYe (CrLCYe), AY606130. The sweetpotato lycopene ε-cyclase reported by Ling et al. [21], is not shown in this figure, because its accession number was not available.

3.3 Functional analysis of the carotene cyclase genes identified in the sweetpotato cultivars WS and W71

When plasmids, pETD-IbLCYb1, pETD-IbLCYb2, and pETD-IbLCYb4, were separately introduced into the lycopene-producing *E. coli* cells, β-carotene was detected in all the extracts (Figure 4A–C). The enzymatic activity of IbLCYb4 (IbLCYb3) in the *E. coli* cells was weak compared with that of IbLCYb1 and IbLCYb2. In contrast, pETD-IbLCYb5 did not direct conversion of lycopene (Figure 4D). These results indicate that IbLCYb1, IbLCYb2, IbLCYb3, and IbLCYb4 possess lycopene β-cyclase activity, while IbLCYb5 is not functional. Therefore, only the *IbLCYb1-IbLCYb4* genes encode functional lycopene β-cyclase (Figure 2).

The introduction of plasmid pETD-IbLCYe led to efficient production of δ-carotene (Figure 4E), indicating that IbLCYe catalyzes the formation of δ-carotene by adding one monocyclic ε-ring to lycopene. Thus, this gene was shown to encode a lycopene ε-(mono)cyclase. MpLCYe derived from the liverwort *Marchantia polymorpha* was able to convert lycopene into ε-carotene with dicyclic ε-rings by way of δ-carotene [19], while IbLCYe appears to be strictly an ε-monocyclase (Figure 2).

The combined co-expression of *IbLCYb1* or *IbLCYb2* and *IbLCYe* (plasmid pETD-IbLCYb1/IbLCYe or pETD-IbLCYb2/IbLCYe) in the lycopene-producing *E. coli* resulted in α-carotene formation, along with a considerable amount of β-carotene (Figure 5), whereas co-expression of *IbLCYb4* and *IbLCYe* (plasmid pETD-IbLCYb4/IbLCYe) did not lead

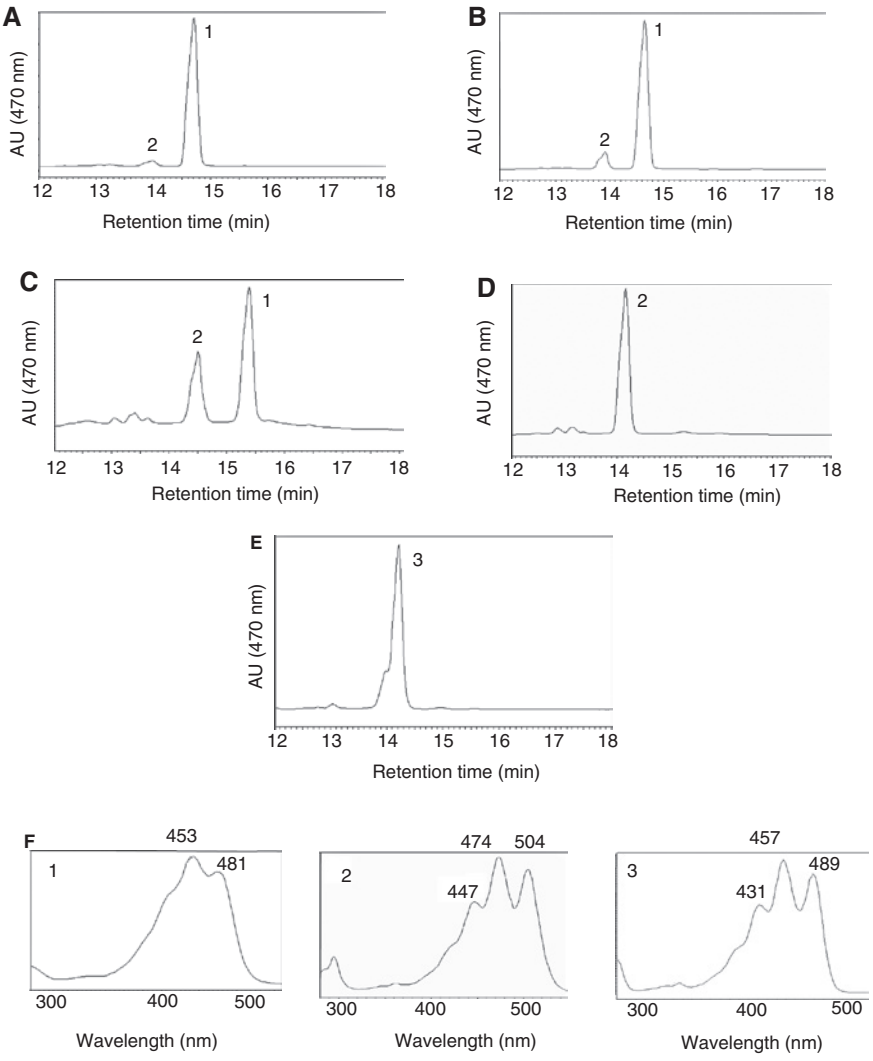

Figure 4: HPLC analysis of carotenoids formed in the lycopene-producing *E. coli* strain that had been transformed with the sweetpotato *IbLCYb* or *IbLCYe* genes.
A TSKgel ODS-80Ts column (4.6 × 150 mm, 5 μm) was used, as described in Section 2.4 of the Experimental section. (A–E) HPLC chromatograms of carotenoid extracts from *E. coli* that harbored pACCRT-EIB plus the respective sweetpotato gene: (A) pETD-IbLCYb1; (B) pETD-IbLCYb2; (C) pETD-IbLCYb4; (D) pETD-IbLCYb5; (E) pETD-IbLCYe. (F) Absorption spectra of individual peaks: 1, β-carotene; 2, lycopene; 3, δ-carotene.

to such an activity and only β-carotene was detected. It was therefore demonstrated that IbLCYb1 and IbLCYb2 can synthesize α-carotene in the presence of IbLCYe.

3.4 Expression analysis of the carotene cyclase genes isolated from sweetpotato cultivars WS and W71

Expression analysis of the carotene cyclase genes was performed in the leaves and tubers of the two sweetpotato cultivars (Figure 6). Both the *IbLCYb* and *IbLCYe* genes were highly expressed in the leaves of both cultivars, while in the tubers *IbLCYb* transcript levels were much higher than those of *IbLCYe*, providing an explanation why β-carotene and β-carotene-derived carotenoids occur abundantly in the tubers.

4 Conclusion

Chemical analysis of the carotenoids of the sweetpotato cultivars WS and W71 revealed that the tuberous roots (tubers) contain rare carotenoids unique to sweetpotato, which contain a 5,6-epoxy-β-ring and/or a 5,8-epoxy-β-ring in their structures. Lycopene β- and ε-cyclase gene

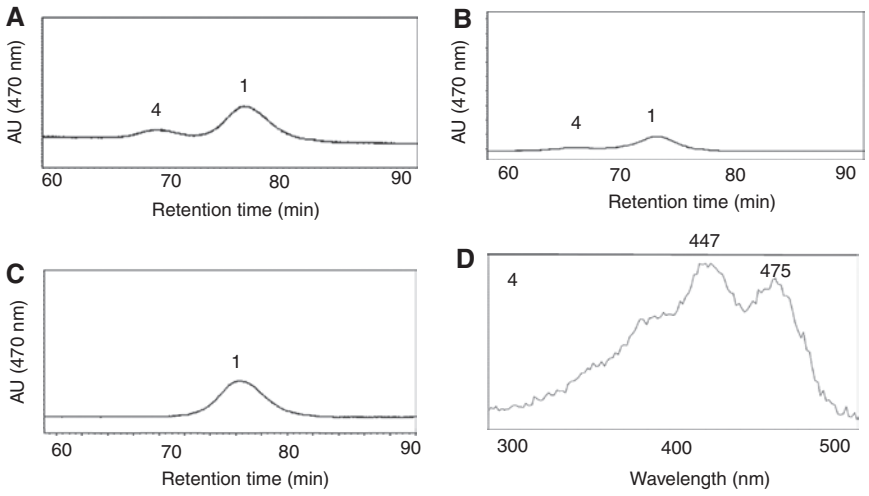

Figure 5: HPLC analysis of carotenoides formed in the lycopene-producing *E. coli* strain that had been transformed with both the sweetpotato *IbLCYb* and *IbLCYe* genes. A Nova-pak HR 6 μ C18 column (3.9 × 300 mm; Waters) was used, as described in Section 2.4 of the Experimental section.
(A–C) HPLC chromatograms of carotenoid extracts from *E. coli* that harbored pACCRT-EIB plus the respective sweetpotato genes:
(A) pETD-IbLCYb1/IbLCYe; (B) pETD-IbLCYb2/IbLCYe; (C) pETDuet-IbLCYb4/IbLCYe; (D) Absorption spectra of individual peaks: 4, α-carotene; 1, β-carotene (see Figure 4F).

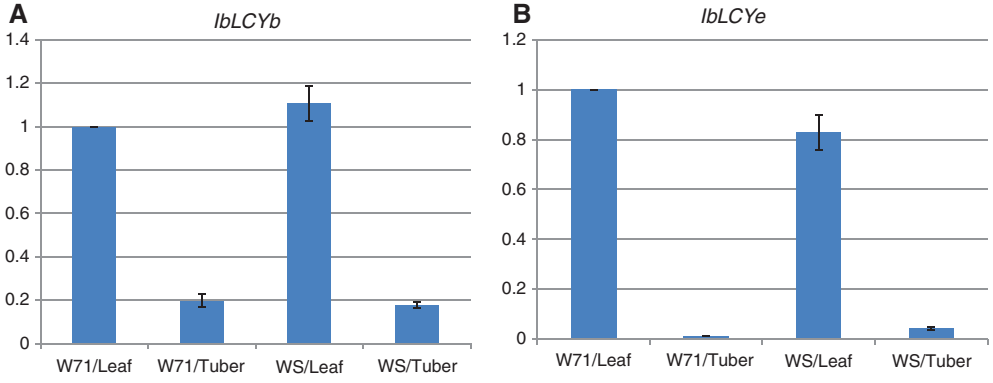

Figure 6: Transcript levels of the *IbLCYb* gene (A) and the *IbLCYe* gene (B) in the leaves and tubers of sweetpotato cultivars W71 and WS.

sequences, designated *IbLCYb1-4* and *IbLCYe*, were isolated from both cultivars, and the encoded proteins were functionally identified.

Acknowledgments: The authors are grateful to Mr. Mitsuo Hashimoto for his technical support in growing the sweetpotato plants.

References

1. Rodriguez-Bonilla L, Cuevas HE, Montero-Rojas M, Bird-Pico F, Luciano-Rosario D, Siritunga D. Assessment of genetic diversity of sweet potato in Puerto Rico. PLoS One 2014;9:e116184.
2. Purcell AE, Wallter WM. Carotenoids of centennial variety sweet potato, *Ipomoea batatas*. J Agric Food Chem 1968;16:769–70.
3. Yoshinaga M, Yamakawa O, Nakatani M. Genotypic diversity of anthocyanin content and composition in purple-fleshed sweet potato [*Ipomoea batatas* (L.) Lam]. Breeding Sci 1999;49:43–7.
4. Teow CC, Truong V-D, McFeeters RF, Thompson RL, Pecota KV, Yencho GC. Antioxidant activities, phenolic and β-carotene contents of sweetpotato genotypes with varying flesh colours. Food Chem 2007;103:829–38.
5. Grace MH, Yousef GG, Gustafson SJ, Truong V-D, Yencho GC, Lila MA. Phytochemical changes in phenolics, anthocyanins, ascorbic acid, and carotenoids associated with sweetpotato storage and impacts on bioactive properties. Food Chem 2014;145:717–24.
6. Kim SH, Ahn YO, Ahn M-J, Lee H-S, Kwak S-S. Down-regulation of β-carotene hydroxylase increases β-carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweetpotato. Phytochemistry 2012;74:69–78.
7. Liao Z, Chen M, Yang Y, Yang C, Fu Y, Zhang Q et al. A new isopentenyl diphosphate isomerase gene from sweet potato: cloning, characterization and color complementation. Biologia 2008;63:221–6.

8. Roullier C, Duputié A, Wennekes P, Benoit L, Fernández Bringas VM, Rossel G, et al. Disentangling the origins of cultivated sweet potato (*Ipomoea batatas* (L.) Lam.). PLoS One 2013;8:e62707.
9. Arizio CM, Costa SM, Tártara M, Manifesto M. Carotenoids gene markers for sweetpotato (*Ipomoea batatas* L. Lam): applications in genetic mapping, diversity evaluation and cross-species transference. Mol Genet Genomics 2014;289:237–51.
10. Holden JM, Eldridge AL, Beecher GR, Buzzard IM, Bhagwat AS, Davis CS, et al. Carotenoid content of U.S. Foods: An update of the database. J Food Comp Anal 1999;12:169–96.
11. Burns J, Fraser PD, Bramley PM. Identification and quantification of carotenoids, tocopherols and chlorophylls in commonly consumed fruits and vegetables. Phytochemistry 2003;62:939–47.
12. Maoka T, Akimoto N, Ishiguro K, Yoshinaga M, Yoshimoto M. Carotenoids with a 5,6-dihydro-5,6-dihydroxy-beta-end group, from yellow sweet potato "Benimasari", *Ipomoea batata*s Lam. Phytochemistry 2007;68, 1740–5.
13. Gama MI, Leite Jr RP, Cordeiro AR, Cantliffe DJ. Transgenic sweet potato plants obtained by *Agrobacteriun tumefaciens*-mediated transformation. Plant Cell Tissue Organ Cult 1996;46:237–44.
14. Takahata Y, Tanaka M, Otani M, Katayama K, Kitahara K, Nakayachi O, et al. Inhibition of the expression of the starch synthase II gene leads to lower pasting temperature in sweetpotato starch. Plant Cell Rep 2010;29:535–43.
15. Mullen MA, Jones A, Abrogast RT. Resistance of sweet potato lines to infestations of sweet potato weevil, Cylas *formicarius elegantulus* (Summers). HortSci 1981;16:539–40.
16. Misawa N, Satomi Y, Kondo K, Yokoyama A, Kajiwara S, Saito T, et al. Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. J Bacteriol 1995;177:6575–84.
17. Fraser PD, Elisabete M, Pinto S, Holloway DE, Bramley PM. Application of high-performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. Plant J 2000;24:551–8.
18. Yokoyama A, Mik, W. Composition and presumed biosynthetic pathway of carotenoids in the astaxanthin-producing bacterium *Agrobacterium aurantiacum*. FEMS Microbiol Lett 1995;128:139–44.
19. Takemura M, Maoka T, Misawa N. Carotenoid analysis of a liverwort *Marchantia polymorpha* and functional identification of its lycopene β- and ε-cyclase genes. Plant Cell Physiol 2014;55:194–200.
20. Goodwin TW (ed). Plant pigments. London: Academic Press, 1988.
21. Ling Y, Hong Z, Wei C, Shao-zhen H, Qing-chang L. Cloning and functional analysis of ε-cyclase (*IbLCYe*) gene from sweetpotato, *Ipomoea batatas* (L.) Lam. J Integrative Agric 2013;12:773–80.