Overexpression of the *IbMYB1* gene in an orange-fleshed sweet potato cultivar produces a dual-pigmented transgenic sweet potato with improved antioxidant activity

Sung-Chul Park, Yun-Hee Kim, Sun Ha Kim, Yu Jeong Jeong, Cha Young Kim, Joon Seol Lee, Ji-Yeong Bae, Mi-Jeong Ahn, Jae Cheol Jeong, Haeng-Soon Lee and Sang-Soo Kwak

*Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRRIB), Daejeon, Korea*

*Department of Green Chemistry and Environmental Biotechnology, University of Science & Technology (UST), Daejeon, Korea*

*Department of Biology Education, College of Education, and Institute of Agriculture and Life Science, Gyeongsang National University, Jinju, Korea*

*Infection Control Material Research Center, Bio-materials Research Institute, KRRIB, Jeongeup, Korea*

*Bioenergy Crop Research Center, National Institute of Crop Science, RDA, Muan, Korea*

*College of Pharmacy and Research Institute of Life Sciences, Gyeongsang National University, Jinju, Korea*

The R2R3-type protein IbMYB1 is a key regulator of anthocyanin biosynthesis in the storage roots of sweet potato (*Ipomoea batatas* (L.) Lam). Previously, we demonstrated that *IbMYB1* expression stimulated anthocyanin pigmentation in tobacco leaves and Arabidopsis. Here, we generated dual-pigmented transgenic sweet potato plants that accumulated high levels of both anthocyanins and carotenoids in a single sweet potato storage root. An orange-fleshed cultivar with high carotenoid levels was transformed with the *IbMYB1* gene under the control of either the storage root-specific sporamin 1 (*SPO1*) promoter or the oxidative stress-inducible peroxidase anionic 2 (*SWPA2*) promoter. The *SPO1-MYB* transgenic lines exhibited higher anthocyanin levels in storage roots than empty vector control (EV) or *SWPA2-MYB* plants, but carotenoid content was unchanged. *SWPA2-MYB* transgenic lines exhibited higher levels of both anthocyanin and carotenoids than EV plants. Analysis of hydrolyzed anthocyanin extracts indicated that cyanidin and peonidin predominated in both overexpression lines. Quantitative reverse transcription-polymerase chain reaction analysis demonstrated that *IbMYB1* expression in both *IbMYB1* transgenic lines strongly induced the upregulation of several genes in the anthocyanin biosynthetic pathway, whereas the expression of carotenoid biosynthetic pathway genes varied between transgenic lines. Increased anthocyanin levels in transgenic plants also promoted the elevation of proanthocyanidin and total phenolic levels in fresh storage roots. Consequently, all *IbMYB1* transgenic plants displayed much higher antioxidant activities than EV plants. In field cultivations, storage root yields varied between the transgenic lines. Taken together, our results indicate that overexpression of *IbMYB1* is a highly promising strategy for the generation of transgenic plants with enhanced antioxidant capacity.

**Abbreviations** - ANS, anthocyanidin synthase; AsA, L-ascorbic acid; bHLH, basic helix-loop-helix; CHI, chalcone isomerase; CHS, chalcone synthase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; DW, dry weight; EV, empty vector; F3H, flavonoid 3-hydroxylase; HPLC, high-performance liquid chromatography; LMO, living modified organism; PAL, phenylalanine ammonia-lyase; PAP1, PRODUCTION OF ANTHOCYANIN PIGMENT1; PDS, phytoene desaturase; RT-qPCR, quantitative reverse transcription-polymerase chain reaction; SHM, sinhwangmi; SPO1, sporamin 1; SWPA2, sweet potato anionic peroxidase 2; SZM, sinzami; TF, transcription factor; UV, ultraviolet.
Introduction

Sweet potato [Ipomoea batatas (L.) Lam] is one of the most nutritionally important root crops worldwide, especially in parts of Asia and Africa. It is widely used not only as a major food source but also as an important industrial raw material for the production of animal feed, alcohol and various antioxidants such as anthocyanin and carotenoids (Oki et al. 2002, Teow et al. 2007). The sweet potato flesh color depends on pigmentation from carotenoids or anthocyanin. These antioxidants are of particular interest because they perform protective roles in combating oxidative stress, which is implicated in aging and the development of a number of diseases, including cancer (Chen et al. 2005, Luceri et al. 2008). As a consequence, recent attention has focused on orange- or purple-fleshed sweet potato cultivars that have naturally high levels of carotenoids or anthocyanins, respectively, and are popular among consumers.

In higher plants, natural purple, orange and green pigmentations are controlled by the relative quantities of anthocyanins, carotenoids and chlorophyll, respectively. These compounds are not only necessary for maintaining plant health and performance but are also considered to be phytonutrients that are beneficial for human health (Tanaka et al. 2008). Plant anthocyanins are elevated in response to developmental, environmental and stress stimuli, including temperature, nutrient status, wounding, pathogen infection, water stress and various light conditions (Christie et al. 1994, Chalker-Scott 1999, Steyn et al. 2002). Anthocyanins have antioxidant properties and free radical-scavenging capacities and can inhibit the growth of tumor cells (Chen et al. 2005). Anthocyanin biosynthesis is transcriptionally regulated by a combination of three components: an R2R3-MYB protein, a basic helix-loop-helix (bHLH, MYC) protein and WD40-type co-regulators (WD40) (Ramsay and Glover 2005). MYB transcription factors (TFs) play particularly pivotal roles in the transcriptional regulation of secondary metabolism in plant flavonoid biosynthetic pathways. For example, maize purple plant (Cone et al. 1993), Arabidopsis PRODUCTION OF ANTHEROCYANIN PIGMENT 1 (PAP1)/AtMYB75 and PAP2/AtMYB90 (Borevitz et al. 2000), grape VIMYBA (Kobayashi et al. 2002) and apple MdMYB10 (Espley et al. 2007, Espley et al. 2013) all control anthocyanin biosynthesis. The overexpression of PAP1 in transgenic Arabidopsis stimulated the upregulation of a number of genes in the anthocyanin biosynthesis pathway, including phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS) and dihydroflavonol 4-reductase (DFR) (Borevitz et al. 2000, Tohge et al. 2005).

The apple MdMYB10 gene was found to control anthocyanin biosynthesis in apple fruit skin and in the flesh of a red-fleshed cultivar (Espley et al. 2007). In sweet potato, Mano et al. (2007) reported that the R2R3-type IbMYB1 from a purple-fleshed sweet potato variety (cv. Ayamurasaki) was responsible for purple pigmentation in the flesh of storage roots. IbMYB1 exhibits an expression pattern that correlates with the expression of anthocyanin pathway genes and is able to activate the promoters of a number of these genes (Mano et al. 2007).

Plants do not generally accumulate carotenoids in their roots; however, the storage roots of sweet potato and carrot are exceptions that serve as good sources of carotenoids, especially β-carotene. In plants, carotene is a precursor for abscisic acid biosynthesis and is also essential for photosynthetic processes. In animals, carotene provides the major dietary source of provitamin A; this is essential, as animals and humans are unable to synthesize vitamin A (Tanaka et al. 2008). Vitamin A deficiency is a serious global problem, especially in Africa and South-East Asia. The World Health Organization estimates that vitamin A deficiency affects an estimated 190 million preschool children and 19.1 million pregnant women worldwide. Furthermore, night blindness is estimated to affect approximately 15 million preschool children and pregnant women (WHO 2009).

Transgenic approaches, aimed at providing dietary improvement, have been used to increase successfully anthocyanins or flavonoids in useful crop plants such as tomato (Butelli et al. 2008) and apple (Espley et al. 2013). Transgenic MYB10 apple exhibited increased levels of anthocyanin, flavonoids and proanthocyanidins through increased transcription of related genes, and β-carotene content also increased in the peel and cortex of MYB10 fruit (Espley et al. 2013). Overexpression of the maize TFs Lc (bHLH) and colorless1 (MYB) in tomato fruit produced elevated flavonoid levels but no increase in anthocyanin levels (Bovy et al. 2002). Another transgenic tomato, in which the DET1 gene was downregulated, exhibited increased flavonoid and carotenoid contents (Davuluri et al. 2005). Moreover, a DET1 knockout mutant had increased anthocyanin content in light conditions (Mustilli et al. 1999). A black-colored (peel and flesh) anthocyanin-rich tomato was developed through overexpression of the Delila (bHLH) and Rosea1 (MYB) TFs from Antirrhinum majus (Butelli et al. 2008). Although sweet potato is recognized as an important healthful and useful root crop plant, the metabolic engineering of various pigment antioxidants in transgenic plants has not been substantially investigated to date.
In a previous study, we demonstrated that expression of *IbMYB1* under the control of a storage root-specific sporamin 1 (*SPO1*) promoter or an oxidative stress-inducible peroxidase anionic 2 (*SWPA2*) promoter regulated phenylpropanoid pathway genes such as *PAL, CHS, chalcone isomerase (CHI), flavonoid 3-hydroxylase (F3H), DFR* and anthocyanidin synthase (*ANS*) in tobacco leaves and Arabidopsis (Kim et al. 2010, Chu et al. 2013). In this study, we generated dual-pigmented transgenic sweet potato plants by overexpressing the *IbMYB1* gene, under the control of the sweet potato *SPO1* or *SWPA2* promoters, in a β-carotene-rich orange-fleshed sweet potato. Expression of the *IbMYB1* gene caused activation of anthocyanin synthesis genes in the transgenic sweet potato, thereby conferring high levels of antioxidant activity in the storage roots.

**Materials and methods**

**Plant materials and expression vectors**

Wild-type sweet potato cultivars Sinzami (SZM) and Sinhwangmi (SHM) were obtained from the National Institute of Crop Sciences, Rural Development Administration, Korea. SZM produces high amounts of anthocyanin (approximately 3.7 mg g⁻¹ dry weight [DW]) but a low level of total carotenoids (approximately 10 μg g⁻¹ DW). The SHM cultivar has 18× higher carotenoid levels than SZM but no detected anthocyanin in the storage root flesh (see Fig. S1). We isolated the *IbMYB1* gene from the anthocyanin-rich sweet potato SZM cultivar and constructed vectors as previously described (Kim et al. 2010).

**Sweet potato transformation and regeneration**

Embryogenic calli from an orange-fleshed sweet potato (SHM) were used for Agrobacterium-mediated transformation, as described previously (Lim et al. 2004). The transformed cells were selected on MS (Murashige and Shoog) medium containing 400 mg l⁻¹ cefotaxime and 100 mg l⁻¹ kanamycin and were subcultured to fresh medium at 3 week intervals. Regenerated plantlets were transferred to pots for further experiments.

**Sample preparation for experiments**

All storage root samples used in this study were obtained in 2013 from field-cultivated plants. On the day of harvest, approximately 200 g of storage roots (five roots from each line) were washed, towel dried, peeled and sliced and immediately frozen at −80°C, before lyophilization.

Lyophilized sweet potato samples were ground into a fine powder and stored at −80°C until required for further experiments. All analyses were conducted in biological triplicate.

**Analysis of anthocyanins**

Anthocyanins were extracted from 0.2 g of finely ground plant material, 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 H₂O₂ (Millipore, Bedford, MA, USA) and 300 μl of chloroform to remove carotenoids. The supernatant (water–methanol phase) was determined spectrophotometrically at 530 and 657 nm. The acidic methanol extraction (100 μl) was hydrolyzed as described by Chu et al. (2013). The sample was added to 900 μl of solvent [95:5 (v/v), n-butanol (100%):HCl (36%)], and the mixture was boiled for 2 h to release core anthocyanidins. The sample was then dried in a speed vacuum at room temperature, the residue was dissolved in 100 μl of 0.1% HCl–methanol solvent [0.1% HCl in high-performance liquid chromatography (HPLC)-grade methanol] and the sample was centrifuged at 14 500 g for 10 min. Core anthocyanidin molecules were identified from the supernatant by HPLC analysis, as described by Chu et al. (2013). All chromatograms were monitored at 520 nm. Cyanidin and hydrolyzed peonidin-3-O-glucoside chloride (Sigma-Aldrich, St. Louis, MO) were used as standards for identification.

**Analysis of carotenoids**

Carotenoids were extracted from 100 mg of peeled sweet potato storage roots and analyzed using the Agilent 1100 HPLC system (Hewlett-Packard, Palo Alto, CA, USA), as previously described (Kim et al. 2012, Kim et al. 2013). The HPLC-DAD system was operated using CHEMSTATION software (Hewlett-Packard). Carotenoids were quantified by an external calibration method. The β-carotene, β-cryptoxanthin, lutein, violaxanthin 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: violaxanthin (peak: 11.5), lutein (peak: 23.3), zeaxanthin (peak: 26.6), β-cryptoxanthin (peak: 33.5) and β-carotene (peak: 39.2). Carotenoid contents were measured in a minimum of three different storage roots. All content levels were expressed as the mean (the average content in g DW) ± SD (the standard deviation) of two independent determinations from three individual storage roots.
Analysis of proanthocyanidin

Quantification of total proanthocyanidin from the empty vector (EV) line and *IbMYB1* transgenic plants was performed using the vanillin–HCl method, as described previously (Price et al. 1978). Lyophilized storage root flesh (100 mg) was extracted using 10 ml of absolute methanol for 20 min, with shaking. Samples were centrifuged at 2500 g for 10 min, and the supernatant was used for subsequent analysis. Supernatant (1 ml) was mixed with 5 ml of 1% (v/v) vanillin and 4% HCl solution in methanol and added to 5 ml of 4% HCl volumetric mix. Mixtures were incubated for 20 min at 30°C in a water bath. Absorbance of samples and blanks was assessed at 500 nm using an ultraviolet (UV)/Vis spectrophotometer. Proanthocyanidin concentrations were determined in triplicate.

Analysis of total phenolics

Total phenolic contents were spectrophotometrically analyzed according to the method of Ainsworth and Gillespie (2007). Total phenolic acid was extracted with 2 ml of ice-cold 95% (v/v) methanol from 20 mg of lyophilized storage root flesh. Following centrifugation at 13,000 g for 5 min, 100 μl of the supernatant was mixed with 200 μl of 10% (v/v) Folin–Ciocalteu reagent, and 800 μl Na2CO3 (700 mM) was added. The mixture was incubated at room temperature for 2 h, after which absorbance was determined at 765 nm. Total phenolics were calculated as chlorogenic acid equivalents using the regression equation between chlorogenic acid standards and absorbance at 765 nm.

Analysis of radical-scavenging activity

The radical-scavenging activity of 2,2-diphenyl-1-picrylhydrazyl (DPPH) was analyzed as described by Kim et al. (2013). Four *IbMYB1* overexpression sweet potato storage roots (approximately 200 g) were prepared by boiling in water for 20 min and then cooling to room temperature. Boiled sweet potatoes were peeled and whole flesh lyophilized. Extracts were prepared in 95% methanol from unboiled and boiled storage root flesh from *IbMYB1* overexpression lines and EV control lines. Absorbance of the extract was determined at 517 nm. L-Ascorbic acid (AsA) was used as a standard for the calibration curve, and the DPPH radical-scavenging activities were calculated as AsA equivalents per gram of tested samples.

Gene expression analysis

Total RNA was extracted from the lyophilized storage root samples with Trizol reagent (Invitrogen, Carlsbad, CA, USA) and then treated according to the manufacturer's instructions with DNase I (Takara, Kyoto, 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, Mountain View, CA, 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 were as indicated in Table S2. Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis was carried out in 96-well plates with a CFX real-time PCR system and CFX system software (Bio-Rad, Hercules, CA, USA) using the EvaGreen-based PCR assay. Each reaction (final volume of 20 μl) contained 1 μl diluted cDNA, 10 μl of EvaGreen PCR Master Mix (Solgent, Daejeon, 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 was used as the reference gene for quantification as its transcript level is constant throughout root and leaf tissues.

Field cultivation of transgenic sweet potato plants

Sweet potato field cultivation was conducted in 2013 at the living modified organism (LMO) field of the National Institute of Crop Science, Mokpo, Korea. Twenty-five transgenic lines were grown for 1 month. Stems were then cut at 15 cm lengths, and 40 stems of each line were transplanted by hand in the field with 25 cm 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, plots were mulched with black polyethylene films. Other cultural management practices were performed in accordance with the standard sweet potato cultivation methods defined by the Rural Development Administration, Korea. At harvest time, the fresh weights of the above-ground shoots and storage roots were recorded.

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.
Results

Generation of dual-pigmented transgenic sweet potato plants

To generate dual-pigmented sweet potato plants by genetic engineering, binary vectors containing the *IbMYB1* gene under the control of either the storage roots-specific *SPO1* promoter or the oxidative stress-inducible peroxidase anion 2 (*SWPA2*) sweet potato promoter were transformed into a β-carotene-rich sweet potato cultivar (SHM) (Fig. 1A). Transgenic sweet potato plants expressing *IbMYB1* under the control of the *SPO1* (referred to *SPO1-MYB* plants) or *SWPA2* promoter (*SWPA2-MYB* plants) and EV as control (EV) were successfully generated by Agrobacterium-mediated transformation. Each independent transgenic line was grown in a growth chamber and LMO field for 4 months. Putative transgenic sweet potato lines exhibited ectopic accumulation of anthocyanin that produced a deep or mild pigmentation in the storage roots. Ten of fifteen *SPO1-MYB* lines and three of five *SWPA2-MYB* lines accumulated anthocyanin in storage roots (see Fig. S2). Two transgenic lines each from *SPO1-MYB* (2 and 14) and *SWPA2-MYB* (1 and 3) were selected on the basis of their high anthocyanin and carotenoid levels and were used for further characterization (Fig. 1B, Table S3). Anthocyanin strongly accumulated in shoot xylem in *SWPA2-MYB* line 1, but only weak purple pigmentation was noted in the xylem of the other transgenic lines compared with EV control plants (Fig. 1B). *SPO1-MYB* leaves (both lines) were similar to those in EV plants, whereas the *SWPA2-MYB* leaves showed slightly higher anthocyanin accumulations than those of EV plants. Starch is the major storage carbohydrate of sweet potato (Bovell-Benjamin 2007). We measured starch contents to determine whether increased anthocyanin content affects starch levels in the storage roots of *IbMYB1* transgenic plants. Starch contents (23–25%) of wild-type (SHM), EV and *IbMYB1* transgenic plants were not statistically different (data not shown).

Increased levels of anthocyanins and transcripts of anthocyanin biosynthetic genes

Anthocyanin levels in *IbMYB1* transgenic and EV storage roots were spectroscopically assessed (Fig. 2A). Total average anthocyanin content varied from 176.5 μg g⁻¹ DW in *SWPA2-MYB* line 3 to 1545.4 μg g⁻¹ DW in *SPO1-MYB* line 14, whereas the EV line storage roots produced no anthocyanin. HPLC chromatograms indicated that cyanidin and peonidin were the predominant core anthocyanidins recovered from the *SPO1-MYB* and *SWPA2-MYB* storage roots (Fig. 2B). These data indicate that the overexpression of *IbMYB1* in transgenic sweet potato promoted accumulation of cyanidin and peonidin anthocyanidins.

Quantitative RT-PCR analysis was used to analyze the transcription profiles of anthocyanin pathway

![Fig. 1](https://example.com/fig1.png)
genes in *IbMYB1* transgenic and EV plants and confirmed significant differences in the transgenic plants that correlated with increases in anthocyanin (Fig. 3). Expression of *IbMYB1* was higher in *SPO1-MYB* lines than in *SWPA2-MYB* lines, and no *IbMYB1* transcript was detected in EV line. Expression of the anthocyanin biosynthetic genes *PAL*, cinnamate-4-hydroxylase (*C4H*), *CHS*, *CHI*, *F3H*, UDP glucose: flavonoid-3-O-glucosyltransferase, *DFR* and *ANS* increased in storage roots in parallel with *IbMYB1* overexpression. As with *IbMYB1*, expression of anthocyanin biosynthetic genes was lower in *SWPA2-MYB* lines than in the *SPO1-MYB* lines, which correlated with the lower levels of anthocyanin accumulation in the *SWPA2-MYB* transgenic plants. This reflects the relative activities of the two promoters, with the *SPO1* promoter driving expression in storage roots more strongly than the *SWPA2* promoter. The high *IbMYB1* transcript levels produced under the control of the *SPO1* promoter likely caused the abundant anthocyanin accumulation in the *IbMYB1* transgenic sweet potato lines. These results indicate that expression of *IbMYB1* induces anthocyanin production in the storage roots of sweet potato by elevating the transcript levels of multiple anthocyanin biosynthetic genes.

### Different levels of carotenoids and transcripts of carotenoid biosynthetic genes

To assess the effects of altered *IbMYB1* expression on the regulation of carotenoid biosynthesis, the carotenoid content of transgenic lines was analyzed in sweet potato storage roots by HPLC analysis. As the major carotenoid, β-carotene consisted of approximately 75–80% of the total carotenoids. *SPO1-MYB* roots contained similar total carotenoid levels to EV line (170.4 μg g⁻¹ DW) (Fig. 4A), with 151.4 and 154.4 μg g⁻¹ DW observed in lines 2 and 14, respectively. *SWPA2-MYB* lines 1 (225.6 μg g⁻¹ DW) and 3 (259.7 μg g⁻¹ DW) harbored higher carotenoid levels than the EV line. Levels of β-carotene were also similar in the EV and *SPO1-MYB* lines but elevated in the *SWPA2-MYB* lines (Fig. 4B). We performed qRT-PCR to compare the expression levels of eight key carotenoid metabolic pathway genes in the EV and *IbMYB1* transgenic lines (Fig. 4C). Transcript levels mostly differed between the EV and *IbMYB1* transgenic plants. With the exception of β-carotene hydroxylase, all genes exhibited lower expression in *SPO1-MYB* lines than in the EV line. By contrast, most of the carotenoid biosynthesis-related genes, such as phytoene desaturase (*PDS*), zeta-carotene desaturase, lycopene β-cyclase and
lycopene c-cyclase, were expressed at slightly higher levels in at least one of the SWPA2-MYB lines than in the EV line. These results suggest that IbMYB1 expression might affect carotenoid biosynthesis-related gene expression and total carotenoid content.

**Increased antioxidant activity in transgenic plants**

In transgenic apple plants, an increase in anthocyanic color was accompanied by elevated concentrations of proanthocyanidins and various phenolic compounds (Espley et al. 2013). To evaluate whether similar effects were produced in transgenic sweet potato plants over-expressing IbMYB1, levels of proanthocyanidins and total phenolics were analyzed in the storage roots. As expected, proanthocyanidin and total phenolic levels increased significantly in response to IbMYB1 overexpression, and the increases correlated with increases in anthocyanin content (Fig. 5). Levels of proanthocyanidins and total phenolics were higher in SPO1-MYB lines than in SWPA2-MYB lines, and EV line had very low levels of both proanthocyanidins and total phenolics.

To investigate antioxidant activity in transgenic sweet potato, we analyzed DPPH radical-scavenging activity in both fresh and boiled sweet potato storage roots (Fig. 6). Fresh transgenic sweet potato lines exhibited approximately 3–5-fold more DPPH activity than the EV line, with the highest level observed in SPO1-MYB line 14. All transgenic boiled sweet potato lines also had stronger DPPH radical-scavenging activities than the EV line.

**Yield of IbMYB1 transgenic sweet potato under field conditions**

Field experiments were carried out from 2012 to 2013. In 2012, a preliminary experiment was conducted to evaluate dual-pigmented storage root phenotypes to select lines (data not shown). In 2013, the average yield of storage roots was assessed, and it varied between the transgenic lines. EV control plants produced 1083 kg per 10 ares (10 a−1), and SPO1-MYB line 2, SPO1-MYB line 14 and SWPA2-MYB line 1 produced similar yields of storage roots (Fig. 7A, B). In SWPA2-MYB line 3, the yield was significantly greater, at levels approximately 2.2-fold higher than the EV lines (Fig. 7B). The average yields of the aerial parts of the EV and IbMYB1 transgenic lines were similar in all lines (2083–2778 kg 10 a−1) (Fig. 7C).

**Discussion**

Dietary intervention with nutritionally enriched food has the potential to improve life expectancy. Increased public
**Fig. 4.** Carotenoid contents and expression levels of carotenoid biosynthesis-related genes in storage roots of *IbMYB1* transgenic and EV plants. (A) Analysis of total carotenoid contents. (B) Analysis of β-carotene content by HPLC. (C) Expression of various carotenoid biosynthesis-related genes. Data shown are means ± SD of two replicates from three individual storage roots.

Awareness of the nutritional benefits of antioxidants for human health has promoted scientific efforts to increase the levels of a variety of bioactive constituents in fruits and vegetables (Davies 2007, Pascual-Teresa and Sanchez-Ballesta 2008). While conventional breeding techniques may partially achieve this goal (Mayer et al. 2008, Whitty et al. 2013), the genetic diversity available within the sexually compatible species of any given crop limits the extent of improvement. The use of molecular breeding has great potential to increase the antioxidant and nutrient values of economically important crops and to allow development of nutritionally improved diets (Christou and Twyman 2004).

Sweet potato is a valuable source of β-carotene, which is a key precursor of vitamin A, and is thus an excellent candidate for nutritional enrichment. Recent research demonstrated that β-carotene-rich sweet potato improved the vitamin A status of primary school children (van Jaarsveld et al. 2005). Recently, the Centre for Science in the Public Interest (CSPI) in the United States reported that its nutritional content makes sweet potato an extremely healthy food. The high dietary fiber and low saturated fat levels found in sweet potato, alongside the abundance of small molecule antioxidants such as vitamin C and carotenoids, might be beneficial to human health (CSPI 2009). Sweet potato is also considered to be moderately tolerant to abiotic stress due to its naturally high levels of various antioxidants. In this study, we generated dual-pigmented sweet potatoes that harbored increased levels of anthocyanin (a hydrophilic antioxidant) in addition to β-carotene (a lipophilic antioxidant highly abundant in orange-fleshed sweet potato).
Foods rich in both soluble and membrane-associated antioxidants are thought to offer the best protection against disease (Yeum et al. 2004). The important contribution of anthocyanin to the nutritional value of certain fruits and vegetables has led to attempts to induce or increase anthocyanin levels through metabolic engineering of the R2R3-MYB TF or anthocyanin biosynthesis pathway genes. In tomato, transgenic Del and Ros1 overexpression stimulated an increase in anthocyanin biosynthesis and lycopene levels. When this high anthocyanin (delphinidin- and petunidin-base) transgenic tomato was fed to cancer-susceptible Trp53 mice, the life span was extended by more than 1 month over the life span of mice fed with control tomatoes (Butelli et al. 2008). A transgenic apple overexpressing MYB10 showed increased anthocyanin (cyanidin-base) levels in the cortex (Espley et al. 2013). In this study, our dual-pigmented sweet potatoes exhibited increased anthocyanin content in storage roots in addition to the high natural carotenoid content (Figs 2 and 4). Our IbMYB1 transgenic sweet potatoes contained cyanidin- and peonidin-based anthocyanins (Fig. 2B). Previous studies examining transient expression of IbMYB1 in tobacco leaves and Arabidopsis seedlings found cyanidin to be the main anthocyanin produced (Kim et al. 2010, Chu et al. 2013). Cyanidin and peonidin were previously observed in purple-fleshed sweet potato cultivars, with the anthocyanin levels varying greatly between cultivars (Lee et al. 2013). Chen et al. (2005) reported that black rice-derived cyanidin and peonidin induced apoptosis in vitro and inhibited tumor cell growth in vitro and in vivo. Our IbMYB1 transgenic sweet potatoes also exhibited increased levels of proanthocyanidin and total phenolics, which led to high DPPH radical-scavenging activities in the fresh and boiled sweet potato storage roots (Figs 5 and 6). Taken together, these studies suggest that increased anthocyanin content in food might play a positive role in human health. Further work is required to assess the tolerance of the IbMYB1 transgenic plants generated in this study to various biotic and abiotic stresses in order to determine their adaptability to harsh conditions.

We generated twenty putative IbMYB1 transgenic lines. Among the transgenic plants, 5 of 15 SPO1-MYB lines and two of five SWPA2-MYB lines did not accumulate anthocyanin in storage roots (see Fig. S2), stems or leaves (data not shown). Different transgenic lines showed significantly different anthocyanin contents. We conducted Southern blot analysis to determine whether...
there was a correlation between the integrated gene copy number, the *IbMYB1* transcript level and anthocyanin accumulation. The SWPA2-MYB lines 1 and 3 and the *SPO1-MYB* line 14 had different band patterns, indicating multiple gene copy numbers, whereas the *SPO1-MYB* 2 line and the EV plant had single gene copy numbers (data not shown). This result suggested that gene copy number might affect anthocyanin accumulation in the storage roots of *IbMYB1* transgenic sweet potato.

Interestingly, the *SPO1-MYB* 14 line showed the highest anthocyanin content; however, the *SPO1-MYB* 2 line showed the highest expression of *IbMYB1* and anthocyanin biosynthesis-related genes (Figs 2 and 3). Based on previous studies of apple and pear, we suggest that this phenomenon may depend on the developmental stage of the sweet potato 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 (Espley et al. 2007). A similar phenomenon was previously observed in the skin of pear (cv. Aoguan). Maximum anthocyanin accumulation was observed at 16 DAFB (Days after full bloom); however, the *PyMYB10* transcript level peaked at 10 DAFB and gradually decreased at 22 and 28 DAFB (Feng et al. 2010). In this study, we speculate on the basis of storage root weight that the developmental stage of the *SPO1-MYB* 2 line might be earlier than that of the *SPO1-MYB* 14 line. The anthocyanin content and expression of MYB TF and anthocyanin biosynthetic genes during storage root formation of sweet potato will be investigated in more detail in a future study.

In this study, we used two different promoters from sweet potato. The tissue-specific *sporamin* promoter was highly expressed in storage root. In addition, its expression is induced in leaves, stem and petiole by sucrose addition and in response to wounding stress (Hattori et al. 1991, Ohta et al. 1991, Yeh et al. 1997). The oxidative stress-inducible *SWPA2* gene encodes an anionic peroxidase in cell cultures of sweet potato, and its expression was strongly induced in response to various stresses, including wounding, chilling, sulfur dioxide...
and ozone exposure and UV irradiation (Kim et al. 1999, Kim et al. 2007). The transgenic tobacco plants showed that the SWPA2 promoter could be systemically induced by environmental stresses, including H2O2 exposure, wounding and UV irradiation (Kim et al. 2003). In our previous study, the SWPA2-IbMYB1 Arabidopsis line showed higher anthocyanin accumulation than SPO-IbMYB1 lines in 8-day seedlings and 4-week-old rosette leaves (Chu et al. 2013). Interestingly, the results of this study showed that SPO1-MYB lines accumulate more anthocyanins in storage roots than SWPA2-MYB lines (Fig. 2A). The anthocyanin accumulation results suggest that the storage root-specific sporamin promoter is applicable to sweet potato systems for storage root targeting in transgenic plants.

SWPA2-MYB lines harbored increased levels of total carotenoids and β-carotene. An interaction between the phenylpropanoid and carotenoid pathways was observed when the light-signaling pathway regulatory gene, DET1, was suppressed using RNA interference method (Davuluri et al. 2005). Subsequent analysis demonstrated that DET1 disruption had a widespread effect, which reflects the putative global nature of DET1 as a core regulator of essential plant processes (Enfissi et al. 2010). The light hyper-responsiveness observed in DET1 may result from the coordinated regulation of pathways related to high light conditions, such as those concerning carotenoid and flavonoid-type antioxidants. In a range of species, including Arabidopsis, MYB TFs were shown to be specific regulators of the flavonoid pathway, but without apparent links to carotenoid biosynthesis (Tohge et al. 2005).

Our study showed overexpression of the IbMYB1 gene in a carotenoid-rich sweet potato cultivar produced dramatic increases in the levels of storage root anthocyanin alongside the originally high carotenoid levels. To our knowledge, there have been no previous examples where metabolic engineering has produced simultaneously high levels of both hydrophilic and lipophilic antioxidants in a single storage root. Our study is the first report in which sweet potato plants with dual-pigmented storage roots were successfully generated by transgenic approaches. Achieving this combination of anthocyanin and carotenoid traits with tissue-specific or stress-inducible expression systems might be challenging with conventional breeding. Therefore, our dual-pigmented transgenic plants represent a new production technology for sweet potato that may contribute to human health, particularly in developing countries, allowing sustainable development on marginal lands.

Acknowledgements—This work was supported by grants from the National Center for GM Crops (PJ008097) and the Systems & Synthetic Agrobiotech Center (PJ009506), Biogreen21 Project for Next Generation, Rural Development Administration, Korea, and the KRIBB initiative program.

References


Lee MJ, Park JS, Choi DS, Jung MY (2013) Characterization and quantitation of anthocyanins in purple-fleshed...
sweet potatoes cultivated in Korea by HPLC-DAD and HPLC-ESI-QTOF-MS/MS. J Agric Food Chem 61: 3148–3158

Supporting Information
Additional Supporting Information may be found in the online version of this article:

Fig. S1. Phenotype of wild-type sweet potato and antioxidant contents.

Fig. S2. Phenotypes of transgenic sweet potato storage roots.

Table S1. Carotenoid and total anthocyanin contents in storage roots of transgenic and nontransgenic sweet potato plants.

Table S2. Primer sequences used for expression analysis of sweet potato genes in this study.

Edited by V. Shulaev