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Research article

Cloning and characterization of an *Orange* gene that increases carotenoid accumulation and salt stress tolerance in transgenic sweetpotato cultures

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ABSTRACT

The *Orange* (*Or*) gene is responsible for the accumulation of carotenoids in plants. We isolated the *Or* gene (*IbOr*) from storage roots of orange-fleshed sweetpotato (*Ipomoea batatas* L. Lam. cv. Sinhwangmi), and analyzed its function in transgenic sweetpotato calli. The *IbOr* gene has an open reading frame in the 942 bp cDNA, which encodes a 313-amino acid protein containing a cysteine-rich zinc finger domain. *IbOr* was strongly expressed in storage roots of orange-fleshed sweetpotato cultivars; it also was expressed in leaves, stems, and roots of cultivars with alternatively colored storage roots. *IbOr* transcription increased in response to abiotic stress, with gene expression reaching maximum at 2 h after treatment. Two different overexpression vectors of *IbOr* (*IbOr-Wt* and *IbOr-Ins*, which contained seven extra amino acids) were transformed into calli of white-fleshed sweetpotato [cv. Yulmi (Ym)] using *Agrobacterium*. The transgenic calli were easily selected because they developed a fine orange color. The expression levels of the *IbOr* transgene and genes involved in carotenoid biosynthesis in *IbOr-Wt* and *IbOr-Ins* transgenic calli were similar, and both transformants displayed higher expression levels than those in Ym calli. The contents of β-carotene, lutein, and total carotenoids in *IbOr-Ins* transgenic lines were approximately 10, 6, and 14 times higher than those in Ym calli, respectively. The transgenic *IbOr* calli exhibited increased antioxidant activity and increased tolerance to salt stress. Our work shows that the *IbOr* gene may be useful for the biotechnological development of transgenic sweetpotato plants that accumulate increased carotenoid contents on marginal agricultural lands.

1. Introduction

Sweetpotato (*Ipomoea batatas* Lam.) is one of the most important industrial food crops that produce starch and useful components. It is rich in secondary metabolites and antioxidants such as carotenoids, anthocyanins, and vitamin C [1–3]. Orange-fleshed sweetpotato cultivars with high levels of carotenoids are popular with health-minded consumers. In higher plants, carotenoids are essential in photosynthetic processes, function as precursors for ABA biosynthesis, and provide a primary dietary source of provitamin A in human diets [4]. Carotenoids are synthesized in plastids and accumulate as red, orange, and yellow pigments in flowers, fruit, and roots. Carotenoids such as β-carotene, lycopene, and lutein are important in the food and oil industries because of their powerful antioxidant activities, and sweetpotato carotenoids are important dietary sources of nutrients and antioxidants. Therefore, a complete understanding of carotenoid metabolism and accumulation is crucial to improve the nutritional value of agriculturally important sweetpotato cultivars. The metabolic engineering of carotenoid biosynthesis is a strategic approach to manipulate secondary metabolic content in food crops.

Metabolic manipulation to regulate carotenoid biosynthesis in plants generally involves either the modification of key enzymes or

Abbreviations: ABA, abscisic acid; CaMV, cauliflower mosaic virus; CHY-β, β-carotene hydroxylases; CRTISO, carotenoid isomerase; DPPH, 2,2-diphenyl-1-picrylhydrazyl; GFP, green fluorescent protein; Hm, Shinhwangmi; HPLC, high-performance liquid chromatography; PSY, phytoene synthase; LCY-β, lycopene β-cyclase; NCED, 9-cis-epoxycarotenoid dioxygenase; NT, non-transgenic; Pftf, plastid fusion/translocation factor; ROS, reactive oxygen species; Ym, Yulmi; Zm, Shinzami.

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expression of a silent gene in the pathway. Golden Rice 2 was created by the ectopic expression of maize *PSY* genes and carotene desaturase (*CrtI*) of *Erwinia uredovora* in a mini-carotenoid biosynthetic pathway. In the rice endosperm of Golden Rice 2, the amount of β-carotene is increased up to 31 μg/g at the dry weight. It is the recommended level of vitamin A for a day [5]. In potato (*Solanum tuberosum*. L.), down regulation of *LCY-β*, *CHY-ε*, and *ZEP* resulted in increased zeaxanthin, β-carotene, and total carotenoid contents [6–9]. The overexpression of *PSY*, *LCY-β*, and *CHY-β* in tomato (*Lycopersicon esculentum*) resulted in gibberellic acid (GA) depletion and dwarfism, and increased the levels of lycopene, β-carotene, and zeaxanthin [10–13]. In our previous studies on sweetpotato culture cells, the metabolic engineering of carotenoids by silencing *IbCHY-β* or *IbLCY-ε* resulted in increased contents of β-carotene and total carotenoids [3,14]. Previous work on metabolic engineering of carotenoids focused on manipulating carotenoid biosynthetic genes in several plants. Orange-fleshed sweetpotato contains high levels of carotenoids; however, the biosynthesis of carotenoids in sweetpotato is still poorly understood. This knowledge gap limits the possibilities of using genetic engineering approaches to manipulate carotenoid levels.

Lu et al. reported that an *Orange* (*Or*) gene was isolated from an orange cauliflower mutant (*Brassica oleracea* var. *botrytis*) that has a high level of β-carotene accumulation in the curd and stem tissues, due to the differentiation of non-colored plastids into chromoplasts that accumulate carotenoids [15]. The *Or* gene, highly conserved in many plant species, encodes a DnaJ cysteine-rich zinc binding domain-containing protein. Like the Or protein of cauliflower, the low molecular weight chaperones such as heat shock proteins (HSPs) is a member of the regulators for the plastid development. For example, HSP21 from tomato protects photosystem II from oxidative stress and promotes the conversion of chloroplasts into chromoplasts, which in turn leads to carotenoid accumulation [16]. Transgenic potato tubers expressing *Or* accumulated increased levels of carotenoids, and had continuously increased β-carotene contents during long-term cold storage [15,17,18]. A concomitant increase in sink capacity and the catalytic activity of carotenoid biosynthetic pathways may provide a promising strategy for increasing carotenoid levels in food crops.

Here, we report the isolation and characterization of the *Or* gene (*IbOr*) from orange-fleshed sweetpotato (cv. Sinhwangmi). For functional analysis of the *IbOr* gene, we constructed two *IbOr* overexpression vectors, *IbOr*-wild type (*IbOr-Wt*), and *IbOr-Wt* that was modified by inserting an additional 21 nucleotides (*IbOr-Ins*). The overexpression vectors were transformed into sweetpotato calli using *Agrobacterium*. We measured the carotenoid content, antioxidant capacity, and salt stress tolerance in transgenic sweetpotato cultured cells and control cells. Our results indicate that overexpression of the *IbOr* gene induces increased carotenoid accumulation and promotes salt stress tolerance.

2. Results

2.1. Isolation and sequence analysis of IbOr

We isolated the *IbOr* cDNA coding for the *Or* gene from the storage roots of orange-fleshed sweetpotato (cv. Sinhwangmi) (accession no. HQ828087). The *Or* gene had a length of 942 bp, which encoded 313 amino acid residues. The Or protein had an estimated molecular mass of 34.3 kDa and pI of 8.46 (Fig. 1A). The genetic similarity between the sequences of *IbOr* and *Or* genes of various plant species was determined by using BLAST X and CLUSTAL W analysis (http://www.ualberta.ca/~stothard/javascript/color_align_cons.html). The phylogenetic tree showed that *IbOr* was most closely related to the *Or* gene of morning glory (*Ipomoea nil*) (TA6874_35883) and shared a 97% identity at the amino acid level (Fig. 1B). *IbOr* showed 73–80% sequence homology with several plant *Or* genes, including the putative *Or* genes of tomato (*L. esculentum*), grape (*Vitis vinifera*), *Arabidopsis thaliana* (At5g61670), and cauliflower (*B. oleracea* var. *botrytis*) (Fig. 1C). The deduced IbOr protein was predicted to contain two transmembrane domains, a plastid-targeting transit sequence, and a motif with repeating cysteines (CxxCxGxGx) that is characteristic of a DnaJ protein known as a chaperone.

2.2. Subcellular localization of IbOr

To determine the subcellular localization of IbOr, the coding region of *IbOr* was fused to green fluorescent protein (GFP) and transformed into *Arabidopsis* plants. Wild-type *Arabidopsis* protoplasts lacked green fluorescence throughout the cell (Fig. 1D). Transgenic protoplasts expressing the IbOr::GFP fusion protein displayed green fluorescence that strongly accumulated in the nucleus (Fig. 1D). This result indicates that IbOr is a nuclear-localized protein.

2.3. IbOr gene expression in various tissues and in response to abiotic stress

The expression pattern of *IbOr* was investigated in leaf, stem, fibrous root, and storage root of three different cultivars (Fig. 2A). The *IbOr* transcript was detected in all cultivars tested, including a white-fleshed sweetpotato (cv. Yulmi, Ym), a purple-fleshed sweetpotato (cv. Sinzami, Zm), and an orange-fleshed sweetpotato (cv. Sinhwangmi, Hm). The *IbOr* gene was strongly expressed in storage roots of the orange-fleshed sweetpotato cultivar, and was expressed in leaves, stems, fibrous roots, and storage roots of all cultivars irrespective of the storage root pigmentation.

The expression pattern of the *IbOr* gene in response to abiotic stresses, including NaCl, PEG, and H_2O_2, was continually monitored from the start of treatment to 48 h after treatment in leaves of the Ym cultivar (Fig. 2B). The *IbOr* transcript level increased sharply at 2 h after the start of treatment for each abiotic stress tested, and then the transcript level subsequently decreased. These results show that *IbOr* gene expression responds to abiotic stress.

2.4. Molecular characterization of transgenic sweetpotato calli expressing IbOr

To determine the function of *IbOr*, we constructed two overexpression vectors by fusing the C-terminal translocation signal containing a FLAG epitope to *IbOr-Wt* or *IbOr-Ins*. *IbOr-Wt* is the full-length *IbOr*. *IbOr-Ins* contains seven additional amino acids (KSPNPNL) inserted between residues 131–142 of IbOr-Wt (Fig. 3A). Two transgenic calli were generated by *Agrobacterium*-mediated transformation of the sweetpotato cultivar YM to express either *IbOr-Wt* or *IbOr-Ins*. Transgenic calli were selected on medium containing hygromycin B (HPT II). Integration of the *IbOr* gene expression cassette into the genome of the transformed calli was determined by PCR analysis using HPT II-specific primers (data not shown). More than 10 independent transgenic calli expressing each vector were selected. Four lines (#8 and #18 for *IbOr-Wt*, #17 and #33 for *InOr-Ins*) with high expression of *IbOr* were selected for further study. *IbOr-Wt* transgenic calli displayed a light orange color, whereas *InOr-Ins* transgenic calli displayed a dark orange color (Fig. 3B). The expression level of the *IbOr* transcript was clearly increased in all transgenic lines compared to non-transformed (Ym) calli (Fig. 3B, C). The transgenic calli expressing *IbOr* were further analyzed to confirm the expression of IbOr protein by western blot analysis using the FLAG tag antibody. The results confirmed that transgenic IbOr protein was detected in the transgenic lines but was not present in the non-transformed Ym controls (Fig. 3D).

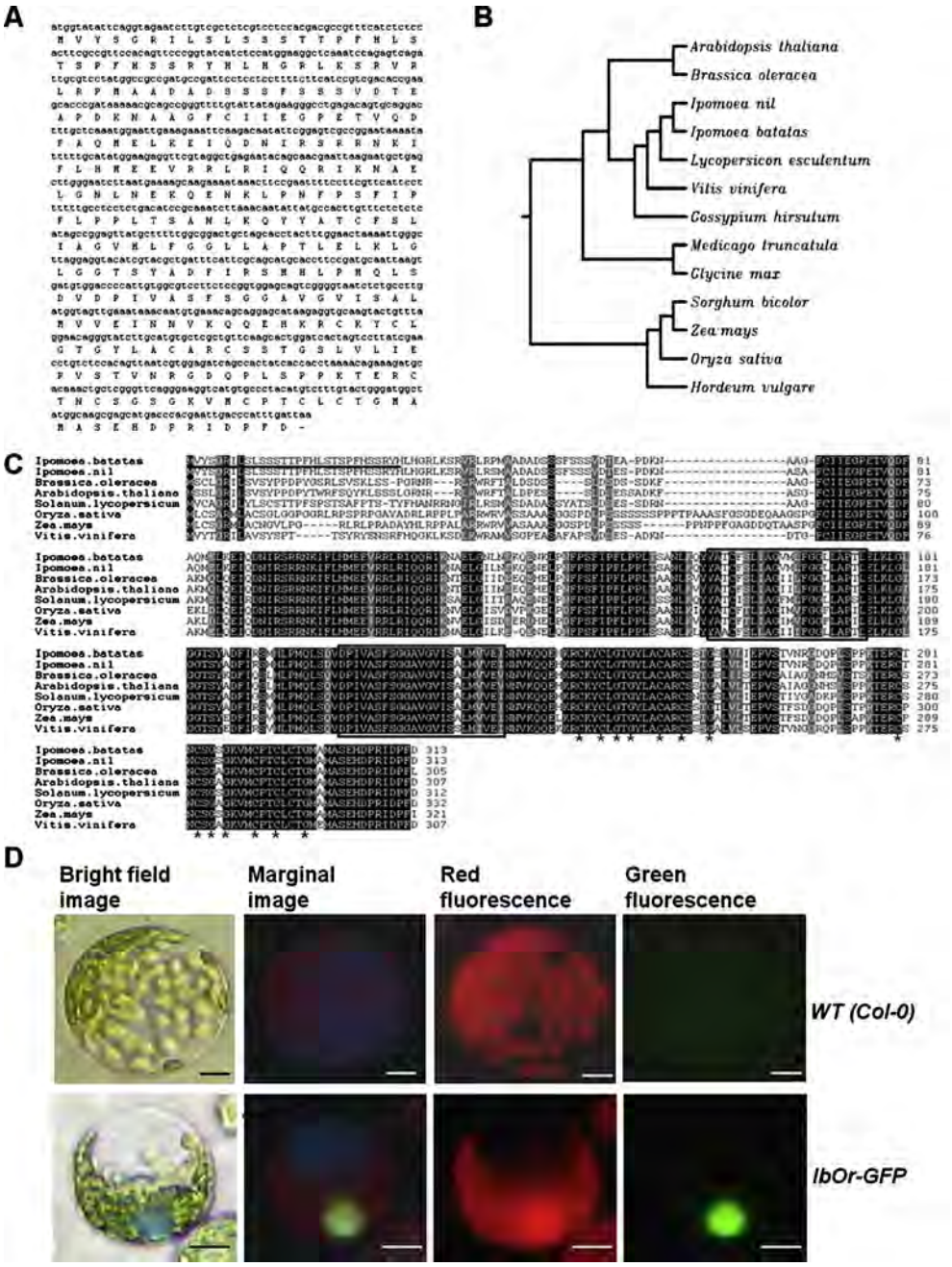

Fig. 1. Characterization and structural analysis of *IbOr*. (A) Nucleotide and deduced amino acid sequences of the open reading frame of *IbOr*. (B) Phylogenetic analysis of *IbOr* and other *Or* genes. The alignment of amino acid sequences was performed using the CLUSTAL W (1.82) multiple sequence alignment program (http://align.genome.jp/). (C) Amino acid sequences were deduced from *Or* genes of *Ipomoea batatas* (accession no. HQ828087), *I. nil* (accession no. TA6874_35883), *Brassica oleracea* var. Botrytis (accession no. ABH07405), *Vitis vinifera* (accession no. CAO62305), *Zea mays* (TA16877_4577), *Oryza sativa* (NP_001047593), *L. esculentum* (TA6503_4081), and *Arabidopsis thaliana* (accession no. NP_200975). The *Or* genes of *I. nil*, *Z. mays* and *L. esculentum* deposited in the TIGR Plant Transcript Assemblies database. Two predicted transmembrane domains are represented as boxes. The plastid-targeting signal peptide is underlined. The stars denote repeating cysteine residues in the putative DnaJ domain. (D) Subcellular localization of IbOr::GFP in transgenic *Arabidopsis* expressing *IbOr*. These images represent multiple analyses of three independent transgenic lines. Bars = 20 μm.

To investigate whether *IbOr* expression affected the carotenoid biosynthesis pathway, we performed RT-PCR to compare the expression levels of six key genes involved in the carotenoid metabolic pathway in non-transgenic and transgenic calli (Fig. 4). The results show that the expression levels of most of the tested genes increased in *IbOr-Wt* and *InOr-Ins* transgenic calli compared to the levels in Ym calli. The expression level of *NCED*, which is involved in ABA biosynthesis, also increased strongly. However, there was no significant increase in the expression levels of *PSY* and *CHY-β*. The *Pftf* gene, which is involved in chromoplast differentiation, showed a significant increase in transcript levels in both transgenic lines.

2.5. Increased carotenoid contents in transgenic sweetpotato calli expressing IbOr

To confirm that the color changes observed in transgenic calli expressing *IbOr* were due to increased carotenoid accumulation,

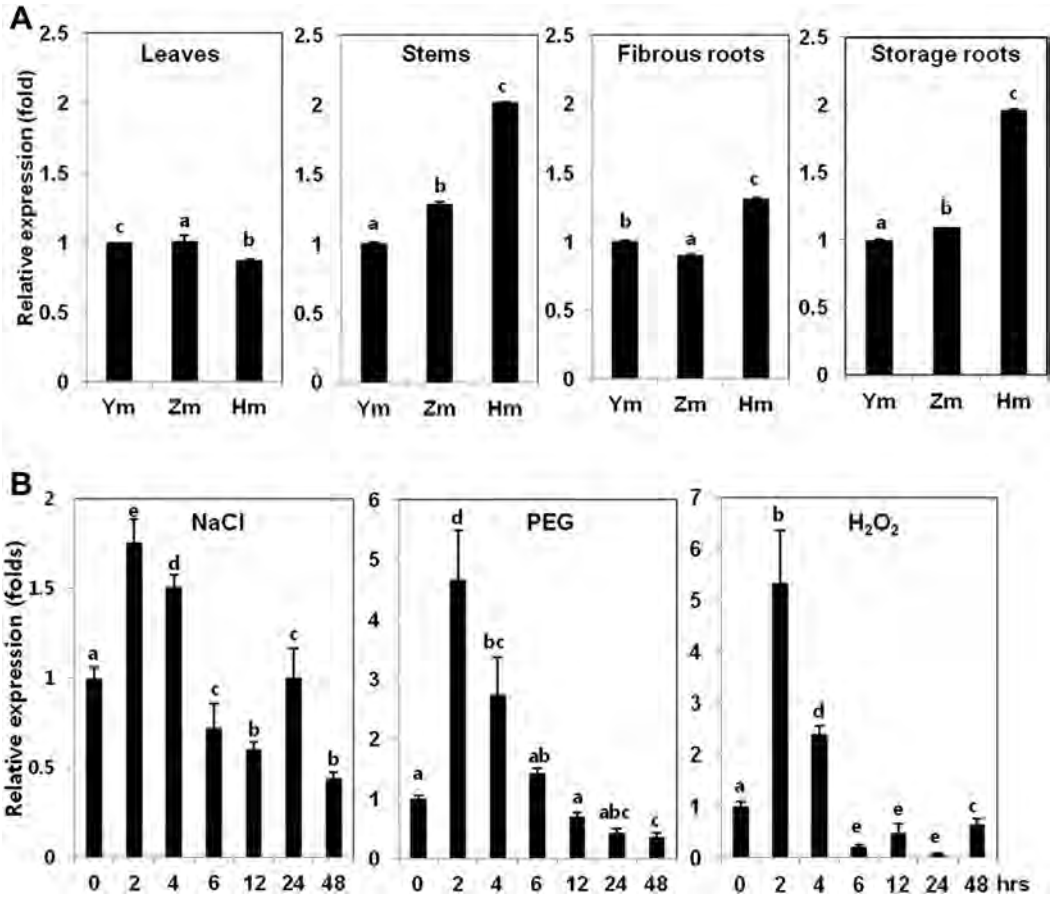

Fig. 2. *IbOr* expression analysis using RT-PCR. (A) Distribution of the *IbOr* gene in different cultivars and expression patterns of the *IbOr* gene in various tissues of sweetpotato plants. Different letters indicate statistically significant differences between the means ($p < 0.05$) for Ym. (B) Expression patterns of the *IbOr* gene under various abiotic stresses in sweetpotato leaves. Different letters indicate statistically significant differences between the means ($p < 0.05$) for time 0.

the carotenoid contents in transgenic callus lines were quantitatively analyzed by high-performance liquid chromatography (HPLC) (Fig. 5). The *IbOr* transgene markedly affected the accumulation of carotenoids and the composition of molecular species comprising the carotenoid pool in transgenic lines. The carotenoid contents in *IbOr-Ins* transgenic calli with dark orange color were much higher than those in *IbOr-Wt* transgenic calli with light orange color. The average total carotenoid contents in *IbOr-Ins* and *IbOr-Wt* transgenic calli lines were approximately 13.37 and 3.97 times higher, respectively, than those in Ym callus.

There are two biosynthetic pathways of carotenoids. The α-branch pathway produces lutein from α-carotene. The β-branch pathway produces neoxanthin from β-carotene, which is produced from lycopene. The contents of α-carotene and lutein in *IbOr-Ins* transgenic calli were approximately 4.45 and 7.42 times higher, respectively, than the levels in Ym calli. The contents of α-carotene and lutein in *IbOr-Wt* transgenic calli were 2.83 and 0.9 times higher, respectively, than the levels in Ym calli. The β-carotene contents in the *IbOr-Wt* and *IbOr-Ins* transgenic calli lines were 4.7 and 10.8 times higher, respectively, than the levels in Ym calli. The β-cryptoxanthin content in the *IbOr-Ins* transgenic callus was 14.2–16.5 μg g^{-1} dry weight, a level that was approximately 300-fold higher than that in the control callus. The level of zeaxanthin in transgenic *IbOr-Ins* lines was 17.5–19.2-fold higher than that in the control Ym line (Fig. 5). These results indicate that carotenoid contents are higher in *IbOr-Ins* lines than in *IbOr-Wt* lines. There was no significant difference in ABA contents in *IbOr* transgenic lines and Ym calli (data not shown).

2.6. Increased antioxidant activity and salt stress tolerance in IbOr transgenic calli

To investigate the antioxidant activity in *IbOr* transgenic sweetpotato calli, we analyzed the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity. Both *IbOr* transgenic lines displayed higher DPPH radical-scavenging activity than that detected in Ym calli under normal conditions (Fig. 6A). This result indicates that increased carotenoid contents may correlate with increased antioxidant activity.

To assess the effects of *IbOr* overexpression on tolerance to oxidative stress induced by salt, 2-week-old calli were treated with 150 or 200 mM NaCl for 24 h. Salt-induced oxidative stress in calli was visualized with DAB staining, which turns to a dark brown color when oxidized by H_2O_2. The results were determined by qualitative and quantitative analysis of H_2O_2 (Fig. 6B, C). *IbOr* transgenic calli retained a yellow color, indicating that oxidized species and DAB reactants were reduced. The control calli (Ym) were a dark brown color, indicating the accumulation of oxidized species and peroxides. These results show that *IbOr* expression conferred an increased tolerance to NaCl stress in transgenic calli (Fig. 6B, C), and indicate that *IbOr* overexpression is involved in mediating tolerance to salt stress induced oxidation states.

3. Discussion

Recent work reported the isolation of the *Orange* (*Or*) gene from a cauliflower mutant, and showed that the *Or* gene was responsible

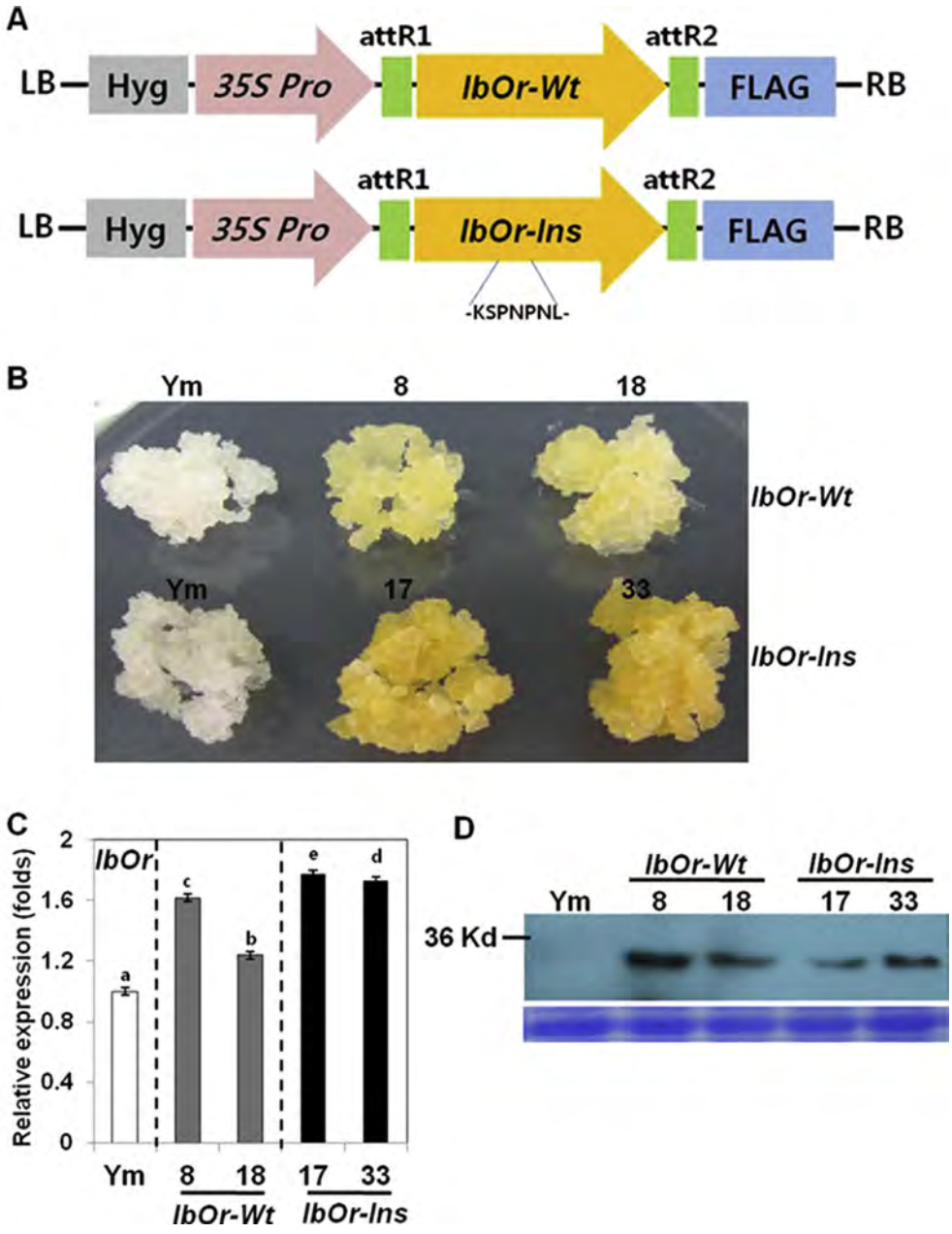

Fig. 3. Characterization of transgenic sweetpotato calli overexpressing *IbOr*. (A) Diagram of the pGWB11-*IbOr-Wt* and pGWB11-*IbOr-Ins* constructs. (B) Phenotypes of transgenic sweetpotato calli overexpressing *IbOr*. (C) Transcript levels of *IbOr* genes in the transgenic sweetpotato calli. Different letters indicate statistically significant differences between the means ($p < 0.05$) for Ym. (D) Protein immunoblot of transgenic sweetpotato calli overexpressing IbOr::FLAG. The same amount of protein (45 μg) was loaded on each lane. The introduced IbOr protein was detected with the FLAG antibody. No band is present in the wild-type lane due to the absence of the FLAG tag in non-transgenic Ym calli.

for the observed carotenoid accumulation and chromoplast differentiation in this mutant [15]. Here, we showed that the *Or* gene isolated from orange-fleshed sweetpotato (*IbOr*) was involved in the increased accumulation of carotenoids via an increased expression of carotenoid biosynthetic genes. *IbOr* also conferred increased tolerance to salt-mediated oxidative stress in transgenic sweetpotato calli. Therefore, these results suggest that *IbOr* will be a useful gene target for the metabolic engineering of carotenoids in agronomically important crops.

The cauliflower *Or* gene had a structural alteration of the four alternatively spliced transcripts, including *Or-WT* (915 bp), *Or-Ins* (954 bp insertion), *Or-Del* (897 bp deletion), and *Or-LDel* (810 bp large deletion) [15]. Wild-type cauliflower (with white curd) predominantly expressed only one *Or-WT*. However, orange cauliflower (an orange-curd mutant) expressed the four alternatively spliced transcripts (*Or-WT*, *Or-Ins*, *Or-Del*, and *Or-LDel*). These results suggested that more than one alternatively spliced transcript of the *Or* gene may be involved in carotenoid accumulation in cauliflower. In sweetpotato, at least one *IbOr* transcript is required for carotenoid accumulation.

The cauliflower Or protein was located in plastids and in nuclei [15,19]. The sweetpotato IbOr protein contained a putative plastid-targeted transit peptide. However, the IbOr protein was localized in the nucleus (Fig. 1D). Lu et al. (2006) reported that the Or protein was targeted to plastids in leaf epidermal cells of Or-expressing transgenic Arabidopsis. A recent report showed that Or was localized in the nucleus of young bud cells [19]. Thus, Or protein (including IbOr) might be targeted to nuclei and plastids, and the

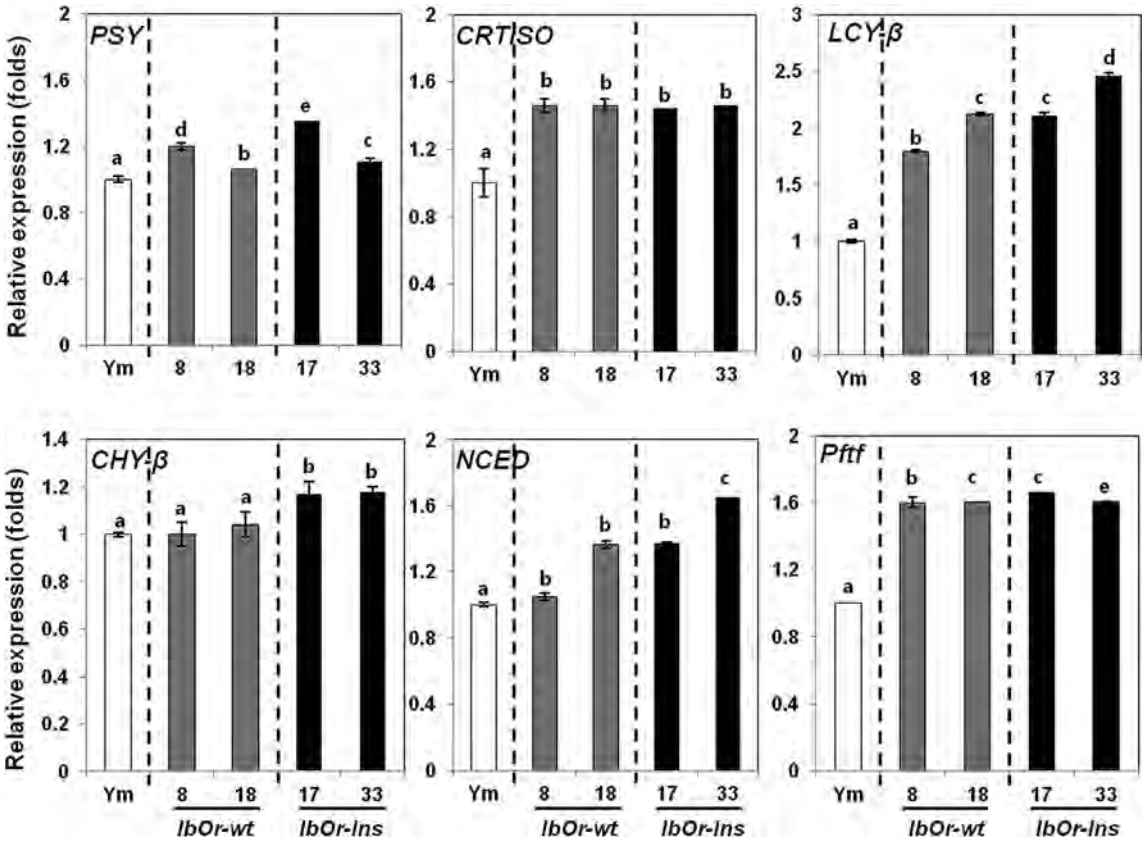

Fig. 4. Expression pattern of various genes involved in carotenoid biosynthesis in transgenic sweetpotato calli expressing *IbOr*. Different letters indicate statistically significant differences between the means ($p < 0.05$) for Ym. *PSY*, phytoene synthase; *CRTISO*, carotenoid isomerase; *LCY-β*, lycopene β-cyclase; *CHY-β*, β-carotene hydroxylase; *NCED*, 9-*cis*-epoxycarotenoid dioxygenase; *Pftf*, plastid fusion/translocation factor.

preferred target sites might depend on the developmental stage and stress conditions. A few plant proteins, such as *why1*, *CEBP*, and *SWIB*-domain protein, were localized in both nuclei and plastids during flower development and senescence [20–22]. The Or protein contains a class of DnaJ cysteine-rich zinc finger domain that is highly specific to molecular chaperones; however, its molecular functions are unknown in plants [15]. The chaperones belong to heat shock protein families. The presence of a conserved DnaJ cysteine-rich domain in *IbOr* suggests that the IbOr protein may function in association with HSPs. Transgenic *Arabidopsis* plants overexpressing the *IbOr* gene are undergoing evaluation for their tolerance to heat stress. Current data indicate that overexpression of *IbOr* in transgenic *Arabidopsis* confers tolerance to 47 °C heat shock (Park et al., unpublished data). Both the cauliflower *Or* and the sweetpotato *IbOr* affect β-carotene accumulation. Therefore, further investigation is needed to isolate proteins that interact with *IbOr* and are associated with β-carotene accumulation.

The high level of carotenoid accumulation conferred by the cauliflower *Or* gene is ascribed to an increase in sink strength rather than an increased expression of carotenoid biosynthesis genes [15]. In transgenic potato, the *Or* transgene conferred increased carotenoid accumulation and the formation of carotenoid-sequestering structures in chromoplasts, rather than increasing the expression of endogenous carotenoid biosynthesis genes [18,23]. The increased biosynthesis of β-carotene and other carotenoids in transgenic potato is likely associated with the Or-regulated stability of PSY protein during long-term cold storage [17]. These results suggest that Or-induced carotenoid accumulation is not directly related to the increased expression of carotenoid biosynthesis genes. By contrast, the accumulation of carotenoids in transgenic sweetpotato calli expressing *IbOr* likely resulted from increased expression of carotenoid biosynthesis genes. *CRTISO* and *LCY-β* were expressed strongly in the transgenic *IbOr* calli. The *Pftf* gene was known to be involved in chromoplast differentiation in red pepper [24]. The cauliflower homolog of *Pftf* was highly expressed in the *IbOr* transgenic calli (Fig. 4), suggesting that *IbOr* might be associated with chromoplast formation. Therefore, high levels of carotenoids in transgenic *IbOr* calli likely resulted from increased expression of carotenoid biosynthesis genes and increased sink strength of chromoplasts. In transgenic sweetpotato calli, *IbOr-Ins* lines exhibited higher carotenoid contents than *IbOr-Wt* lines (Fig. 5). This suggests that the mutant *IbOr-Ins* has an increased carotenoid biosynthesis activity, possibly due to an increase in its binding activity for its target protein. Lu et al. (2006) showed that the mutation in cauliflower Or altered the binding to its target proteins, or created a novel functionality for the protein, which caused the increased chromoplast biogenesis activity and carotenoid accumulation. Further research is underway to determine the molecular mechanisms induced by the mutation of *IbOr*.

Transgenic calli expressing *IbOr* exhibited increased tolerance to salt stress, although there was no significant difference in ABA content of transgenic calli compared to that in Ym (Fig. 6B). The increased tolerance of transgenic *IbOr* calli to salt stress appears to correlate with the increased levels of carotenoids. We reported that carotenoid biosynthetic intermediates act cooperatively with reactive oxygen species to reduce cellular damage under salt stress conditions [3,14]. *IbOr* transgenic calli displayed higher antioxidant activity than that in Ym calli (Fig. 6A). This evidence indicated that higher carotenoid content correlated with higher DPPH radical-scavenging activity. Taken together, the increased levels of

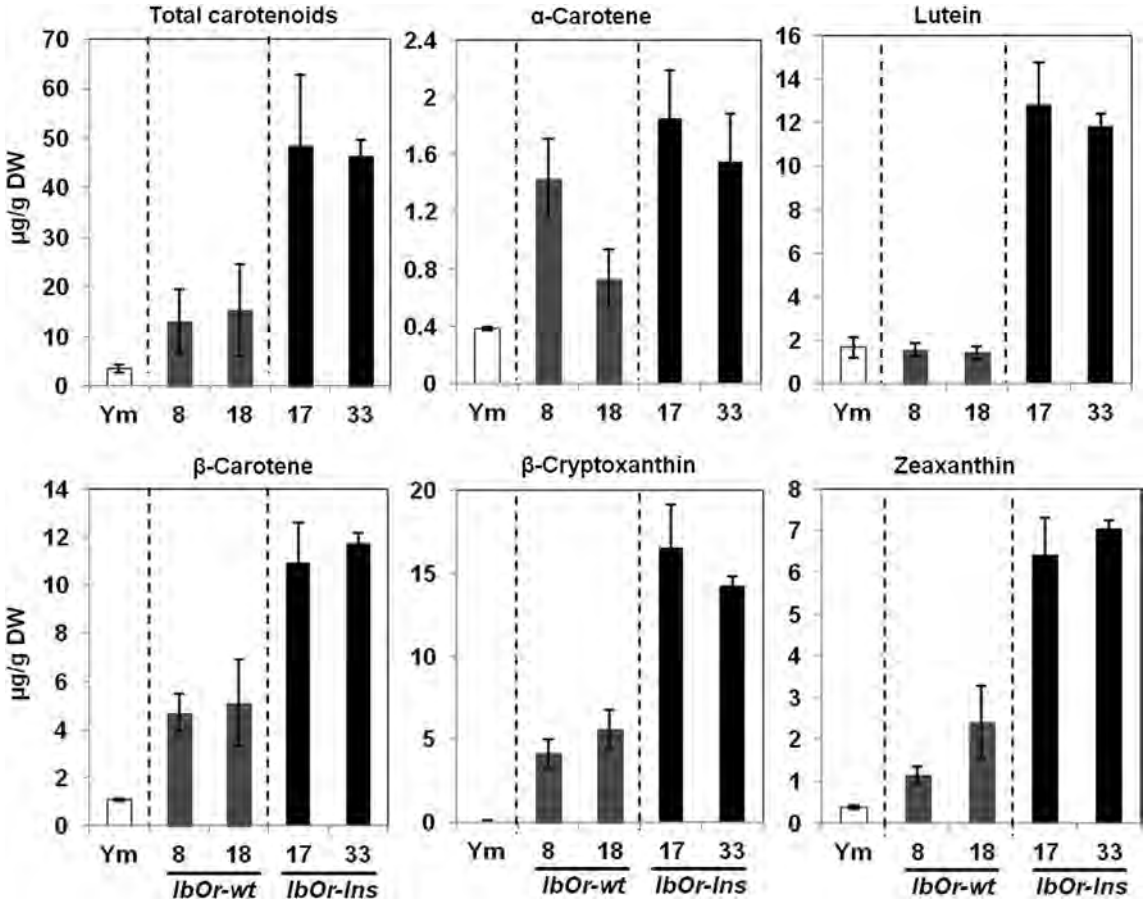

Fig. 5. Quantitative HPLC analysis of total carotenoid contents and carotenoid compounds in transgenic sweetpotato calli expressing *IbOr*. Each carotenoid was measured by HPLC using a minimum of five different calli. All levels are expressed as the mean (the average content in gram dry weight) ± SD of two independent determinations.

carotenoids resulting from increased expression of the *IbOr* gene clearly demonstrates the close relationship with salt stress tolerance. The *Arabidopsis Or* gene also appears to correlate with programmed cell death, heat stress, osmotic stress, and drought- and salt stress tolerance as determined by Genevestigator expression analysis (https://www.genevestigator.com/gv/). High soil salinity is a major environmental stress that limits crop productivity in arid and semi-arid regions around the world [25]. Evidence suggests that protection against oxidative stress improves resistance to many abiotic stresses, including salt stress [26]. Thus, it could be possible to generate transgenic plants with increased tolerance to diverse environmental stresses by manipulating the genes involved in carotenoid biosynthesis. *IbOr* gene expression showed diverse responses to treatment with stress-related compounds such as ABA, ethephon, and methyl jasmonate (MeJA); gene expression increased at 36 h and then decreased to basal levels. *IbOr* gene expression was significantly different in ABA- and ethephon-treated leaves and roots (data not shown). These results suggest that *IbOr* was differentially regulated by these hormones in sweetpotato tissues. *IbOr* expression was slightly increased in response to treatment with PEG and NaCl. Thus, we anticipate that *IbOr* will be a useful target for metabolic engineering to increase carotenoid biosynthesis and tolerance to abiotic stresses.

This study showed that the total carotenoid contents in sweetpotato calli expressing *IbOr* were higher than those in the controls (Fig. 5). The increased antioxidant activity and salt stress tolerance in transgenic sweetpotato calli expressing *IbOr* correlated with increased levels of β-carotene and total carotenoids. These transgenic studies demonstrate that *IbOr* can be used as a novel molecular tool to enrich carotenoid contents, improve the nutritional value of crops, and increase tolerance to environmental stress on marginal agricultural lands.

4. Materials and methods

4.1. Plant materials

Three sweetpotato cultivars [*I. batatas* cv. Yulmi (Ym), Sinzami (Zm), Sinhwangmi (Hm)] were used in this study. Ym, Zm, and Hm were white-, purple-, and orange-fleshed sweetpotato, respectively. They were obtained from Bioenergy Crop Research Center, National Institute of Crop Science, Rural Development Administration, Korea. Plants were cultivated in a growth chamber in soil at 25 ± 1 °C under a photocycle of 16 h light/8 h dark. Calli were induced from shoot meristems of sweetpotato cultured on MS [27] medium supplemented with 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), 3% sucrose, and 0.4% Gelrite (MS1D). Calli were proliferated on MS1D media with subculture at 14 d intervals, and maintained at 25 ± 1 °C in the dark for further use.

4.2. Cloning, construction of expression vectors, and transformation of IbOr

Total RNA was isolated from the storage roots of sweetpotato (*I. batatas* cv. Sinhwangmi) using the RNeasy Mini Kit by QIAGEN. The first-strand cDNA was synthesized by SuperScript III First-

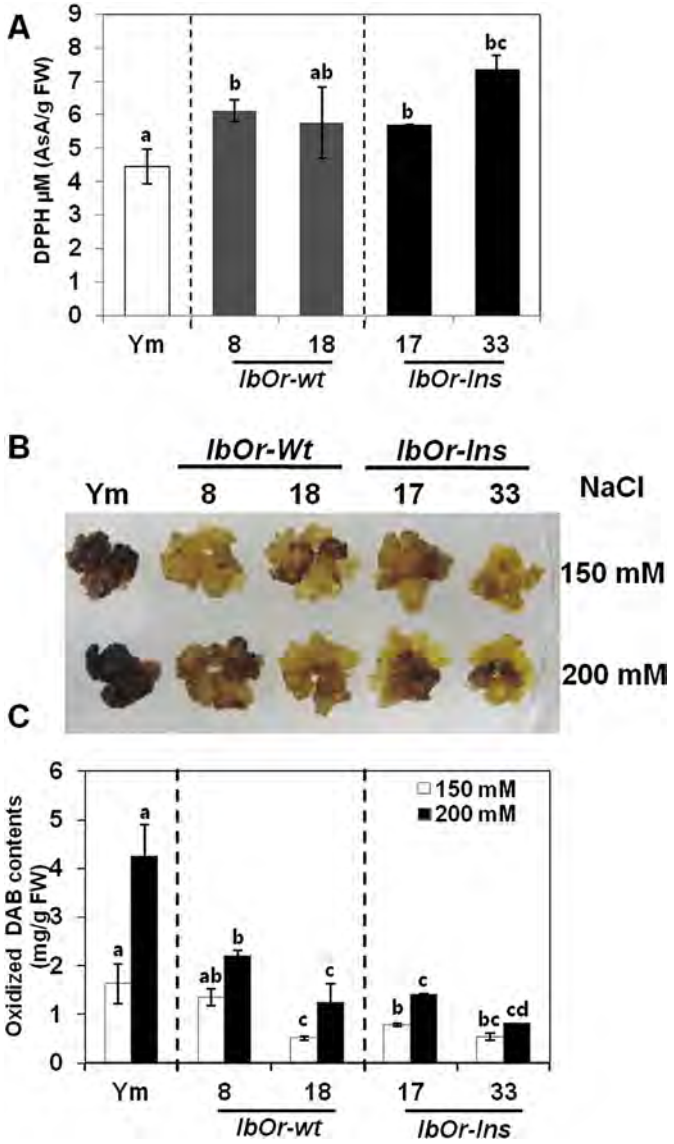

Fig. 6. Tolerance of transgenic sweetpotato calli expressing *IbOr* to salt-induced oxidative stress. (A) Analysis of DPPH radical-scavenging activity in transgenic sweetpotato calli expressing *IbOr*. Data are expressed as the mean values of three independent replicates ±SD. Different letters indicate statistically significant differences between the means ($p < 0.05$) for Ym. (B) H_2O_2 production after treatment with 150 and 200 mM NaCl for 24 h. Transgenic sweetpotato calli expressing *IbOr* were treated with a DAB–HCl solution. (C) Quantitative analysis of oxidized DAB content in transgenic sweetpotato calli in response to salt stress. Data are expressed as the mean values of three independent replicates ±SD. Different letters indicate statistically significant differences between the means ($p < 0.05$) for Ym.

Strand Synthesis System for RT-PCR (Invitrogen). To clone the *Or* gene from sweetpotato, a pair of forward and reverse primers was synthesized based on the sequence of a putative *Or* EST clone of morning glory flower (*I. nil*) published on the TIGR Plant Transcript Assemblies website (http://plantta.tigr.org/). PCR was performed using Advantage 2 polymerase (Clontech) with the first-strand cDNA synthesized with gene-specific primers for *IbOr* forward (5′-ATCTTGTCGCTCTCGTCCTCCACGACGCCG-3′) and *IbOr* reverse (5′-CGTGGGTCATGCTCGCTTGCCATAGCCATC-3′). The PCR conditions were as follows: an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 60 °C for 45 s, polymerization at 72 °C for 1 min, and a final extension at 72 °C for 10 min. The PCR product was cloned into the pGEM-T Easy vector (Promega, Madison, WI) and sequenced. The resulting cDNA was named *IbOr*.

To construct plant expression vectors, we used the Gateway® cloning system (Invitrogen California, USA). We constructed two overexpression vectors (*IbOr-Wt*, *IbOr-Ins*) according to methods in Lu et al. (2006). *IbOr-Wt* was a full-length clone of *IbOr*. *IbOr-Ins* contained seven additional amino acids (KSPNPNL; nucleotide sequence, 5′-AAATCCCCGAACCCTAACCTG-3′) inserted between residues 131–142 of *IbOr-Wt*. The two different cDNAs (*IbOr-Wt*, *IbOr-Ins*) were amplified using the template *IbOr* cDNA with the following primers: *IbOr-Ins* forward, 5′-CTTGGGATTCTTAATGAAAAGCAAGAAAATAAACTT-3′; *IbOr-Ins* reverse, 5′-TTGTTTAAGATTTCGAGTCAAGTTTATTTTCTTG-3′. PCR products were used for the second PCR reaction with primers containing *attB* sites (5′-GGGGACAAGTTTGTACAAAAAAGCAC-3′ and 5′-AGATTGGGGACCACTTTGTACAAGAA-3′). The linear fragments flanked by *attB* sequences were subjected to site-specific recombination with the entry vector pDONR207 (Invitrogen), which contained the *ccdB* gene flanked by *attP* sites; reactions were catalyzed by BP Clonase and yielded entry clones that were used to transform competent DH5α (*Escherichia coli*) cells. The pDONR207-*IbOr* clones were subjected to site-specific recombination [catalyzed by the LR Clonase enzyme mix (Invitrogen)] into a plant expression vector, pGWB11, which contained the cauliflower mosaic virus 35S promoter and the C-terminal FLAG epitope. The resulting expression constructs (pGWB11-*IbOr-Wt*, pGWB11-*IbOr-Ins*) were transformed into *Agrobacterium tumefaciens* strain GV3101 using the freeze-thaw method [28]. Sweetpotato calli (Ym) were transformed with *A. tumefaciens* strain EHA105 harboring the *IbOrs* expression vector according to the method described by Kim et al. [3,14].

4.3. Analysis of gene expression

Total RNA was extracted from sweetpotato plants and callus with the Easy-Spin™ total RNA extraction kit (iNtRON, Daejeon, Korea) according to the manufacturer's instructions. First-strand cDNA synthesis was performed from total RNA (1 μg) using M-MLV reverse transcriptase (MBI-Fermentas, St. Leon-Rot, Germany), according to the manufacturer's instructions. The expression levels of *IbOr* and genes involved in carotenoid biosynthesis were analyzed by semi-quantitative RT-PCR using the gene-specific primers listed in Table 1. The densitometry data corresponding to the band intensities of different sets of experiments were generated by analyzing the gel images on the Image J (Version 1.33, USA http://rsb.info.nih.gov/ij/).

Table 1
Primer sequences for genes involved in carotenoid biosynthesis in sweetpotato.

Target name (accession no.)	Sequence	Direction	Amplicon size (bp)
PSY (HQ828092)	TATTTACCTCAAGATGAATTAGCTC	FOR	399
	TCAGCTTCTTCAGTACAGTATTACA	REV	
CRTISO (JX393307)	TTAAGGCCGAGGTTCTACCA	FOR	518
	ATCAGCAGCAACACGATGAG	REV	
LCY-β (HQ828094)	TAGATATGAAGGATATTCAGGAAAG	FOR	358
	AGTAGAATATCCATACCAAAACAGA	REV	
CHY-β (HQ828095)	GTTTACTGTTTAGTCCTTTAAGTCG	FOR	334
	AACATCTCAGTATATGGAACTTCTC	REV	
NCED (BJ563195)	GGGAAGATCCCGGAGTGTAT	FOR	381
	GTGGTACGGCAAATCGTCTT	REV	
Pftf (JX177356)	TTCTGCTATCAAGGCGGTCT	FOR	402
	GGAGCATTTTCCTTGGCTTT	REV	
α-tubulin (DV037573)	CAACTACCAGCCACCAACTGT	FOR	220
	CAAGATCCTCACGAGCTTCAC	REV	

4.4. Isolation of protoplasts and localization of IbOr-GFP in Arabidopsis

Five grams of rosette leaves were cut from *Arabidopsis* plants and placed in a Petri dish containing 0.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM MES, 1.5% Cellulase RS, 0.03% Pectinase Y23, 0.25% BSA, pH 5.5. Leaves were chopped with a sterile razor blade into small squares of 5–10 mm^2 and incubated for 2 h at room temperature with gentle agitation in the dark until mesophyll cell protoplasts were released. The protoplast suspension was filtered through a nylon mesh (30 μm), washed several times, and resuspended with basic medium. Protoplasts derived from T_3 transgenic plants were mounted on glass slides under coverslips, and GFP fluorescence was visualized using excitation with the 488 nm line of an argon laser and a 505–530 nm band-pass emission filter.

4.5. Stress treatments

Sweetpotato plants were grown for 50 d in a growth chamber at 25 °C. The third or fourth leaves were detached from the end of the main stem of Ym. The leaves were treated with 250 mM NaCl, 30% PEG, or 400 mM H_2O_2 for 2, 4, 6, 12, 24, and 48 h. For NaCl treatments, calli derived from transgenic plants and wild-type Ym plants were grown for 2 weeks on MS1D liquid medium, and then treated with 150 or 200 mM NaCl for 24 h before analysis.

4.6. Analysis of carotenoid contents

Carotenoid contents of sweetpotato calli were analyzed by HPLC according to the method of Lim et al. (2009). All extraction procedures were carried out under dim light to avoid pigment degradation and loss. One hundred milligrams of lyophilized sweetpotato callus were placed in a pre-chilled mortar and pestle and homogenized with 3 mL acetone (0.01% butylated hydroxytoluene, BHT), sea sand, Na_2SO_4, and $NaHCO_3$. The sample was ultrasonicated twice with 15 mL of acetone (0.01% BHT) for 10 min, and then centrifuged at 3075× *g* at 4 °C for 5 min. The supernatant was concentrated *in vacuo* and resuspended with 200 μL of acetone and methylene chloride (200:1). The solution was filtered using a 0.45 μm membrane filter (Whatman, PTFE, 13 mm) and then subjected to HPLC analysis by an Agilent 1100 HPLC system (Hewlett–Packard, Palo Alto, CA, USA). Twenty microliters of standard or sample were injected directly onto a YMC C_{30} carotenoid column (3 μm, 4.6 × 250 mm). The eluent was detected at 450 nm using a UV–visible detector. Chemstation software (Hewlett–Packard) was used for operation of the HPLC-DAD system. For quantification of carotenoids, 1 mg of each standard was dissolved in 10 mL of dichloromethane containing 0.01% BHT. Working calibration solutions (0.02, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, and 50 μg mL^{-1}) were then prepared by diluting stock solutions of the external standards. Standards of carotenoids were purchased from CaroteNature (Lupsingen, Switzerland). Under these chromatographic conditions, standard carotenoids produced peaks at t_R (min) values of 11.5 for violaxanthin, 23.3 for lutein, 26.6 for zeaxanthin, 33.5 for β-cryptoxanthin, and 39.2 for β-carotene.

4.7. Analysis of radical-scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity of the sweetpotato calli was analyzed as described by Kim et al. [3,14]. The extracts of transgenic and Ym calli were prepared in MeOH. The absorbance of the solution was measured using a spectrophotometer and a wavelength of 517 nm. L-ascorbic acid (AsA, 0.015–0.125 mM) was used as the standard for the calibration curve, and the DPPH radical-scavenging activities were calculated as mole AsA equivalents per gram of tested samples.

4.8. H_2O_2 assays

To measure cellular levels of H_2O_2, each callus was placed in a 1 mg mL^{-1} solution of 3,3-diaminobenzidine (DAB)–HCl (pH 3.8) for 5 h at 25 °C under continuous light according to the methods of Chadwick et al. [29] and Kim et al. [3,14]. For H_2O_2 assays, the DAB solution was measured as the absorbance at 460 nm. Oxidized DAB concentrations were calculated from the standard curve of DAB.

4.9. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA). Subsequent multiple comparisons were examined based on the least significant difference (LSD) test. All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS 12; SPSS Inc., Chicago, IL) and statistical significance was set at either $P < 0.05$, according to Duncan's multiple range test.

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