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

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A B S T R A C T

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.

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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
expression of a silent gene in the pathway. Golden Rice 2 was created by the ectopic expression of maize PSY genes and caroten 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 overexpression vectors, CHY-β-wild type (IbCHY-β), and IbCHY-β 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 potato 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 pl 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.tulbera.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) (TAA674_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 (At5g16760), 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 (CxxCxxGxxGxx) 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 H2O2, was 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 IbOr-Ins) with high expression of IbOr were selected for further study. IbOr-Wt transgenic calli displayed a light orange color, whereas IbOr-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).
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-b*. The *Ptf* 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,
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 H₂O₂. The results were determined by qualitative and quantitative analysis of H₂O₂ (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
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

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**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.
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. 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.
carotenoids resulting from increased expression of the \textit{IbOr} gene clearly demonstrates the close relationship with salt stress tolerance. The \textit{Arabidopsis} \textit{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. \textit{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. \textit{IbOr} gene expression was significantly different in ABA- and ethephon-treated leaves and roots (data not shown). These results suggest that \textit{IbOr} was differentially regulated by these hormones in sweetpotato tissues. \textit{IbOr} expression was slightly increased in response to treatment with PEG and NaCl. Thus, we anticipate that \textit{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 \textit{IbOr} were higher than those in the controls (Fig. 5). The increased antioxidant activity and salt stress tolerance in transgenic sweetpotato calli expressing \textit{IbOr} correlated with increased levels of \(\beta\)-carotene and total carotenoids. These transgenic studies demonstrate that \textit{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 [\textit{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\(\pm\)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\(\pm\)1°C in the dark for further use.

4.2. Cloning, construction of expression vectors, and transformation of \textit{IbOr}

Total RNA was isolated from the storage roots of sweetpotato (\textit{I. batatas} cv. Sinhwangmi) using the RNeasy Mini Kit by QIAGEN. The first-strand cDNA was synthesized by SuperScript III First-
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'-AAATCCCCGAACCCTAAGCTG-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'-CTTGCGATATCTACTCCAAACCTAGACCAAGCCTCGAGAAGTC-3'; IbOr-Ins reverse, 5'-TGGTTAAGATTTTGAATTTTGGTTTCTCC-3'. PCR products were used for the second PCR reaction with primers containing attB sites (5'-GGGGCAAGTTTGTACAAAAAAGCACA-3' and 5'-AGATTGGGAACCATATTTTTCAG-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

<table>
<thead>
<tr>
<th>Target name</th>
<th>Sequence</th>
<th>Direction</th>
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<td>CRTISO (JX393307)</td>
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<td>CAACATTTGACACACATAC</td>
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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'-ACCTTTGTCCTCTCCTCCTCCAGAAGCC-3') and IbOr reverse (5'-GGGTGCGCTCATGCTGCTGCCAGCCG-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
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 CaCl₂, 0.5 mM MgCl₂, 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 mm² 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 T₁ 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.

4.5. 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 H₂O₂ 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, Na₂SO₄, and NaHCO₃. The sample was ultrasonicated twice with 15 mL of acetone (0.01% BHT) for 10 min, and then centrifuged at 3075 g 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 C30 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 tᵣ (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. 1-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. H₂O₂ assays

To measure cellular levels of H₂O₂, 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 H₂O₂ 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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References


