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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

Sun Ha Kim [a], Young Ock Ahn [a], Mi-Jeong Ahn [b], Jae Cheol Jeong [a], Haeng-Soon Lee [a,**], Sang-Soo Kwak [a,*]

[a] *Plant Systems Engineering Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), 125 Gwahak-ro, Yuseong-gu, Daejeon 305-806, Republic of Korea*

[b] *College of Pharmacy and Research Institute of Life Sciences, Gyeongsang National University, Jinju 660-751, Republic of Korea*

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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.

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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

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.

* Corresponding author. Tel.: +82 42 860 4432; fax: +82 42 860 4608.
** Corresponding author.
E-mail addresses: hslee@kribb.re.kr (H.-S. Lee), sskwak@kribb.re.kr (S.-S. Kwak).

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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).

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