Plant Cell Reports (2019) 38:1393–1402
https://doi.org/10.1007/s00299-019-02448-4

ORIGINAL ARTICLE

A single amino acid change at position 96 (Arg to His) of the sweetpotato Orange protein leads to carotenoid overaccumulation

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Received: 26 April 2019 / Accepted: 19 July 2019 / Published online: 25 July 2019

Abstract

Key message **IbOr-R96H resulted in carotenoid overaccumulation and enhanced abiotic stress tolerance in transgenic sweetpotato calli.**

Abstract The Orange (Or) protein is involved in the regulation of carotenoid accumulation and tolerance to various environmental stresses. Sweetpotato IbOr, with strong holdase chaperone activity, protects a key enzyme, phytoene synthase (PSY), in the carotenoid biosynthetic pathway and stabilizes a photosynthetic component, oxygen-evolving enhancer protein 2-1 (PsbP), under heat and oxidative stresses in plants. Previous studies of various plant species demonstrated that a single-nucleotide polymorphism (SNP) from Arg to His in Or protein promote a high level of carotenoid accumulation. Here, we showed that the substitution of a single amino acid at position 96 (Arg to His) of wild-type IbOr (referred to as IbOr-R96H) dramatically increases carotenoid accumulation. Sweetpotato calli overexpressing *IbOr-WT* or *IbOr-Ins* exhibited 1.8- or 4.3-fold higher carotenoid contents than those of the white-fleshed sweetpotato Yulmi (Ym) calli, and *IbOr-R96H* overexpression substantially increased carotenoid accumulation by up to 23-fold in sweetpotato calli. In particular, IbOr-R96H transgenic calli contained 88.4-fold higher levels of β-carotene than those in Ym calli. Expression levels of carotenogenesis-related genes were significantly increased in IbOr-R96H transgenic calli. Interestingly, transgenic calli overexpressing *IbOr-R96H* showed increased tolerance to salt and heat stresses, with similar levels of malondialdehyde to those in calli expressing *IbOr-WT* or *IbOr-Ins*. These results suggested that IbOr-R96H is a useful target for the generation of efficient industrial plants, including sweetpotato, to cope with growing food demand and climate change by enabling sustainable agriculture on marginal lands.

Keywords Carotenoid · IbOr · IbOr-R96H · Metabolic engineering · Sweetpotato

Abbreviations

CCD	Carotenoid cleavage dioxygenases
H_2O_2	Hydrogen peroxide
IbOr	*Ipomoea batatas* orange
MDA	Malondialdehyde
Or	Orange
PSII	Photosystem II
PSY	Phytoene synthase

Communicated by Salim Al-Babili.

So-Eun Kim and Ho Soo Kim contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s00299-019-02448-4) contains supplementary material, which is available to authorized users.

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ROS Reactive oxygen species
Ym Yulmi

Introduction

Carotenoids are valuable molecules for plant growth, human health, and animal survival. They are precursors of provitamin A and reduce some reactive oxygen species (ROS)-mediated disorders as antioxidants (Fiedor and Burda 2014). In plants, carotenoids are crucial components of light-harvesting mechanisms and protect the photosynthetic apparatus and membrane by absorbing blue–green light (Domonkos et al. 2013). Phytohormones, abscisic acid (ABA) and strigolactones are synthesized by cleavage of carotenoids (Auldridge et al. 2006; Walter and Strack 2011). Carotenoids are synthesized and stored in the plastids of plant cells. Despite extensive studies of the carotenoid biosynthesis pathway in plants (Hirschberg 2001), the regulatory mechanism controlling carotenoid accumulation is not well characterized.

In a variety of plant species, carotenogenesis-related genes have been modified by genetic engineering to improve carotenoid contents. For example, transgenic plants overexpressing *phytoene synthase* (*PSY*) show increased levels of total carotenoids in carrot roots (Hauptmann et al. 1997), tomato fruits (Fraser et al. 2002), rice (Paine et al. 2005), and potato tubers (Ducreux et al. 2005). Carotenoid levels also increase in response to the overexpression of *PSY* in *Arabidopsis* seeds (Lindgren et al. 2003). In addition, the down-regulation of *lycopene ε-cyclase* (*LCY-ε*) increases β-carotene levels in potato (Diretto et al. 2006), canola (Yu et al. 2008), and tobacco (Shi et al. 2014). In sweetpotato, many genes encoding enzymes in the carotenoid metabolic pathway, including *PSY*, *phytoene desaturase* (*PDS*), *zeta-carotene desaturase* (*ZDS*), *carotenoid isomerase* (*CRTISO*), *lycopene β-cyclase* (*LCY-β*), *LCY-ε*, *beta-carotene hydroxylase* (*CHY-β*), *zeaxanthin epoxidase* (*ZEP*), 9-*cis-epoxycarotenoid dioxygenase* (*NCED*), and *carotenoid cleavage dioxygenases* (*CCD*), have previously been cloned and characterized (Kim et al. 2013a; Li et al. 2017; Kang et al. 2017b). Knockdown of *CHY-β*, *LCY-β*, or *LCY-ε* using RNAi increases carotenoid accumulation and resistance to abiotic stress such as heat, drought, and salt in transgenic sweetpotato plants and calli (Kim et al. 2012, 2013b, 2014; Lu et al. 2013; Kang et al. 2017a, 2018; Ke et al. 2019). Numerous studies, including those mentioned above, have altered the expression of key enzymes in the carotenoid pathway. Recently, an alternative strategy has been developed in which sink strength is enhanced by triggering chromoplast differentiation by cloning *Orange* (*Or*) from the orange curd cauliflower *Brassica oleracea botrytis* (*BoOr*) (Lu et al. 2006).

The *Or* gene encodes a DnaJ Cys-rich zinc finger motif-containing protein; this protein is highly conserved among various plant species (Lu et al. 2006). The ectopic-expression of *BoOr* mutant in both white cauliflower and potato causes the generation of orange tissues with increased carotenoid contents (Lu et al. 2006; Lopez et al. 2008). The enhanced carotenoid accumulation in the BoOr plants is involved in the differentiation from chloroplast to chromoplasts, which provide a metabolic sink for the storage of carotenoids in non-photosynthetic tissues (Lu et al. 2006; Lopez et al. 2008; Li et al. 2012). In sweetpotato, *IbOr*, a homolog of *BoOr*, was isolated from the storage roots of an orange-fleshed cultivar (cv. Sinhwangmi) (Kim et al. 2013a). Transgenic sweetpotato calli overexpressing *IbOr-WT* from white-fleshed sweetpotato calli exhibit elevated total carotenoid contents and a light yellow color. Furthermore, the overexpression of *IbOr-Ins*, which contains seven extra amino acids (KSPNPNL) in the N-terminal region of IbOr-WT, results in a dark-yellow color and a higher carotenoid content than that of tissues expressing IbOr-WT. Transgenic calli overexpressing both *IbOr-WT* and *IbOr-Ins* show enhanced tolerance to salt stress. IbOr transgenic sweetpotato plants exhibit increased levels of total carotenoids contents in the storage roots compared with those in non-transgenic plants (Park et al. 2015).

In addition, IbOr functions as a chaperone protein and contains a DnaJ-like domain. Consistent with *Arabidopsis* Or (AtOr), IbOr directly interacts with IbPSY in the chloroplast (Zhou et al. 2015; Yuan et al. 2015; Park et al. 2016). IbOr stabilizes IbPSY, a rate-limiting enzyme in the carotenoid biosynthetic pathway, via its chaperone activity under heat and oxidative stress conditions. Transgenic sweetpotato or *Arabidopsis* plants overexpressing IbOr display improved tolerance to heat and oxidative stresses (Park et al. 2016). In addition, IbOr interacts with IbPsbP, an important protein of the oxygen-evolving multi-complex of photosystem II (PSII), and the holdase chaperone activity of IbOr can also protect IbPsbP against heat-induced denaturation (Kang et al. 2017a). IbOr plays important roles in carotenoid accumulation and photosynthesis under abiotic stresses.

In melon (*Cucumis melo*), a single amino acid difference in Or determines fruit flesh color (Tzuri et al. 2015). A single-nucleotide polymorphism (SNP) in the gene encoding CmOr causes a transition from Arg to His, changing green-fleshed fruits to orange-fleshed fruits. The orange color is a result of massive β-carotene accumulation (Tzuri et al. 2015). In addition, single amino acid substitutions in CmOr in *Arabidopsis thaliana* (AtOR) and in sorghum (*Sorghum bicolor*; SbOR), AtOR^{His} (R90H), and SbOR^{His} (R104H) also promote a high level of carotenoid accumulation in *Arabidopsis* calli (Yuan et al. 2015).

This study focused on carotenogenesis in sweetpotato. As one of the most important edible crops, nutritional

fortification of the sweetpotato is of great significance. In this study, we performed site-directed mutagenesis of wild-type IbOr. The mutation target was based on the CmOr protein, where substitution of a single Arg to His is responsible for the orange-fleshed melon (Tzuri et al. 2015). Our results show that the overexpression of IbOr-R96H increases the total carotenoid content and substantially increases the biosynthesis of β-carotene in sweetpotato calli. We also investigated the ability of IbOr-R96H to tolerate abiotic stresses, including salt and heat, and monitored expression pattern of carotenogenesis-related genes.

Materials and methods

Plant materials

White-fleshed sweetpotato plants [*Ipomoea batatas* (L.) Lam. cv. Yulmi (Ym)] were used in this study. Non-embryogenic calli were induced from shoot meristems of sweetpotato Ym cultivar and cultured on MS1D (Murashige and Skoog 1962) media supplemented with 3% sucrose, 1 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D), and 0.4% Gelrite. Calli were propagated on MS1D media with 14 d subculture intervals and incubated at 25 °C in the dark.

Site-directed mutagenesis and transformation into the sweetpotato callus

Single amino acid substitutions in full-length GST-IbSPF1 were produced using the primers R96H 5′-GAA ATT CAA GAC AAT ATT CGG AGT CAC CGG AAT AAA ATA TTT TTG CA-3′ and 5′-TGC AAA AAT ATT TTA TTC CGG TGA CTC CGA ATA TTG TCT TGA ATT TC-3′ using Agilent QuikChange Primer Design. Site-directed mutagenesis of IbOr was performed using the QuikChange II XL Site-Directed Mutagenesis Kit following the manufacturer's protocol.

For transformation into the sweetpotato callus, we used the GV3101 strain of *Agrobacterium tumefaciens* harboring pGWB11/IbOr-R96H construct as described (Kim et al. 2012). Detailed methods are provided in the Supplemental Information.

Analysis of carotenoid contents

Carotenoids were extracted with acetone (0.01% BHT) from 2-week-old transgenic calli. A carotenoid analysis was performed using the Agilent 1260 HPLC (high-performance liquid chromatography) system (Hewlett-Packard, Waldbronn, Germany) following our previously reported method (Kim et al. 2012). All extraction procedures were conducted under dim light to avoid pigment degradation and loss. Contents are expressed as means (average content in μg g^{-1} DW) ± SD (standard deviation) of three independent determinations.

Abiotic stress tolerance assay

Transgenic calli and non-transgenic calli were grown in the dark at 25 °C and proliferated by subculture for the abiotic stress tolerance assay. For salt stress treatment, both calli were treated on MS1D media containing 150 mM NaCl for 24 h. For high-temperature stress, calli were treated at 47 °C for 15 h on MS1D media. Each treatment contained six calli of each line (Ym, IbOr-WT, IbOr-Ins, and IbOr-R96H) for triplicate experiments. Until further analysis, all stress-treated calli were frozen immediately in liquid nitrogen and stored at − 70 °C.

Analysis of hydrogen peroxide (H_2O_2) levels

To visualize the oxidation levels from the reaction of DAB with H_2O_2, transgenic calli were incubated in a 1 mg mL^{-1} solution of 3,3-diaminobenzidine (DAB)-HCl (pH 3.8) for 5 h at 25 °C under light following previously described methods (Chadwick et al. 1995; Kim et al. 2013a, b).

Measurement of lipid peroxidation

Lipid peroxidation was measured using a modified thiobarbituric acid (TBA) method (Puckette et al. 2007) to calculate the concentration of MDA, as previously described (Wang et al. 2009). Full details are provided in the Supplemental Information.

RNA extraction and qRT-PCR analysis of carotenogenesis-related genes

Total RNA was isolated from sweetpotato calli using RNA extraction kit (GeneAll, Seoul, Korea) and extensively treated with RNase-free DNase I (Takara, Kyoto, Japan) to remove contaminating genomic DNA. Quantitative RT-PCR was performed in a CFX96 Touch™ Real-Time PCR (Bio-Rad, Hercules, USA) using EvaGreen fluorescent dye according to the manufacturer's instructions. The expression levels of sweetpotato genes were determined by quantitative RT-PCR with the gene-specific primers listed in Supplemental Table S1. Linear data were normalized against the mean CT value of the reference gene *Ubiquitin* (*UBI*), and relative expression values were calculated.

Results

IbOr-R96H transgenic sweetpotato calli exhibited a dark-orange color

According to our previous functional studies, IbOr maintains high levels of total carotenoids and β-carotene contents in sweetpotato (Kim et al. 2013a; Park et al. 2015). A single-nucleotide substitution in the gene encoding CmOr is correlated with the orange color fruit. This substitution results in an Arg to His change at the 108th amino acid of CmOr (Tzuri et al. 2015). In previous report, overexpression of AtOr and SbOr with mutations at the site corresponding to the mutation in CmOr (golden SNP altering Arg to His) resulted in high total carotenoid levels and β-carotene accumulation (Yuan et al. 2015). An amino acid sequences alignment of Or protein displayed that Arg is highly conserved among various plant species (Fig. 1). In a sequence alignment, we found that IbOr possessed Arg at the 96th amino acid, corresponding to the 108th position of CmOr and 90th position of AtOR (Fig. 1).

To examine whether the substitution of the conserved Arg to His in IbOr promoted carotenoid accumulation, site-directed mutagenesis of IbOr-WT was performed to generate IbOr-R96H. The 287th nucleotide position of *IbOr* was changed from G to A by mutagenesis, resulting in an alteration of CGC to CAC and from Arg to His in IbOr. An expression vector harboring IbOr-R96H was introduced into non-embryogenic calli of the white-fleshed sweetpotato cultivar Yulmi (Ym) via *Agrobacterium*-mediated transformation (Fig. 2a). To evaluate carotenoid accumulation in IbOr-R96H, we used previously generated transgenic calli overexpressing IbOr-WT and IbOr-Ins as positive controls (Kim et al. 2013a). The transcript levels of the IbOr variants (*IbOr-WT*, *IbOr-Ins*, or *IbOr-R96H*) were clearly higher in all transgenic lines than in untransformed (Ym) calli (Fig. 2b). As shown in Kim et al. (2013a), IbOr-WT-overexpressing calli displayed a light-yellow color and IbOr-Ins transgenic calli exhibited a dark-yellow color (Fig. 2c). IbOr-R96H transgenic calli had a dark-orange color (Fig. 2c). These results suggest that a single amino acid change in IbOr promotes carotenoid overaccumulation, as observed for $AtOr^{His}$ and $SbOr^{His}$.

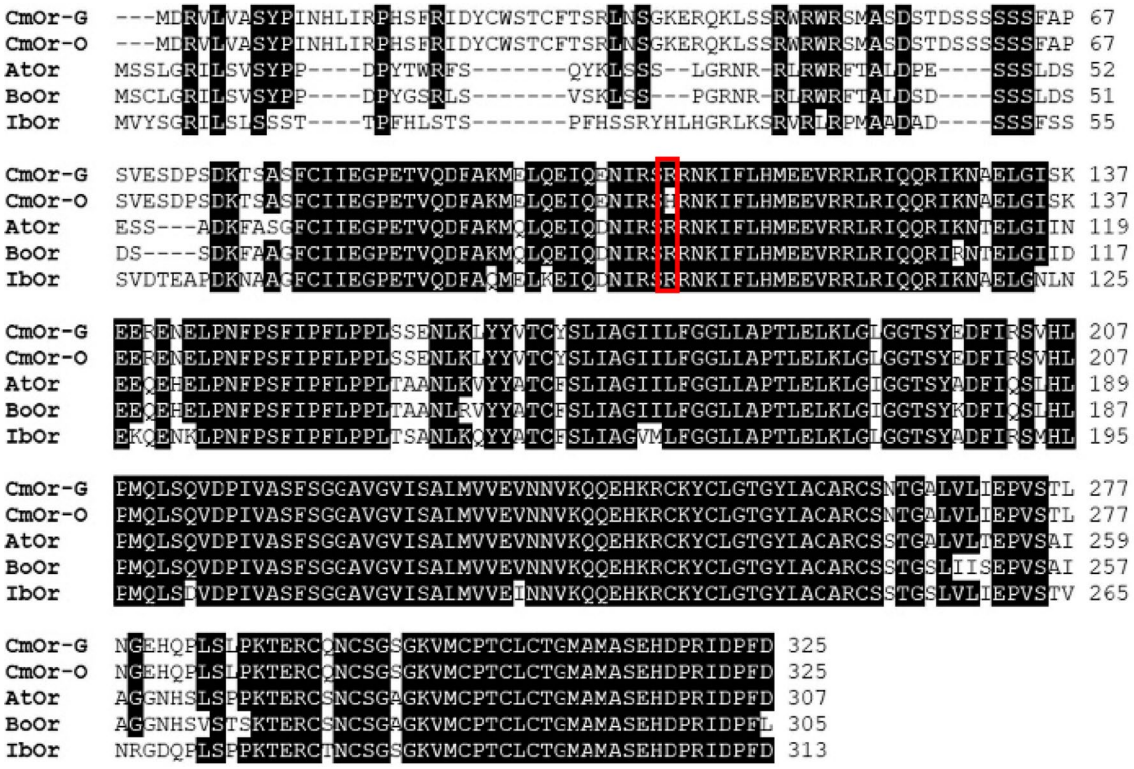

Fig. 1 Multiple sequence alignment of Or proteins. Alignment of full-length Or proteins, including CmOr-G (*Cucumis melo*, XP_008467325, green-fleshed fruit), CmOr-O (orange-fleshed fruit), AtOr (*Arabidopsis thaliana*, NP_200975), BoOr (*Brassica oleracea*, ABH07405), and IbOr (*Ipomoea batatas*, HQ828087). Red box indicates the site of the Arg to His mutation (color figure online)

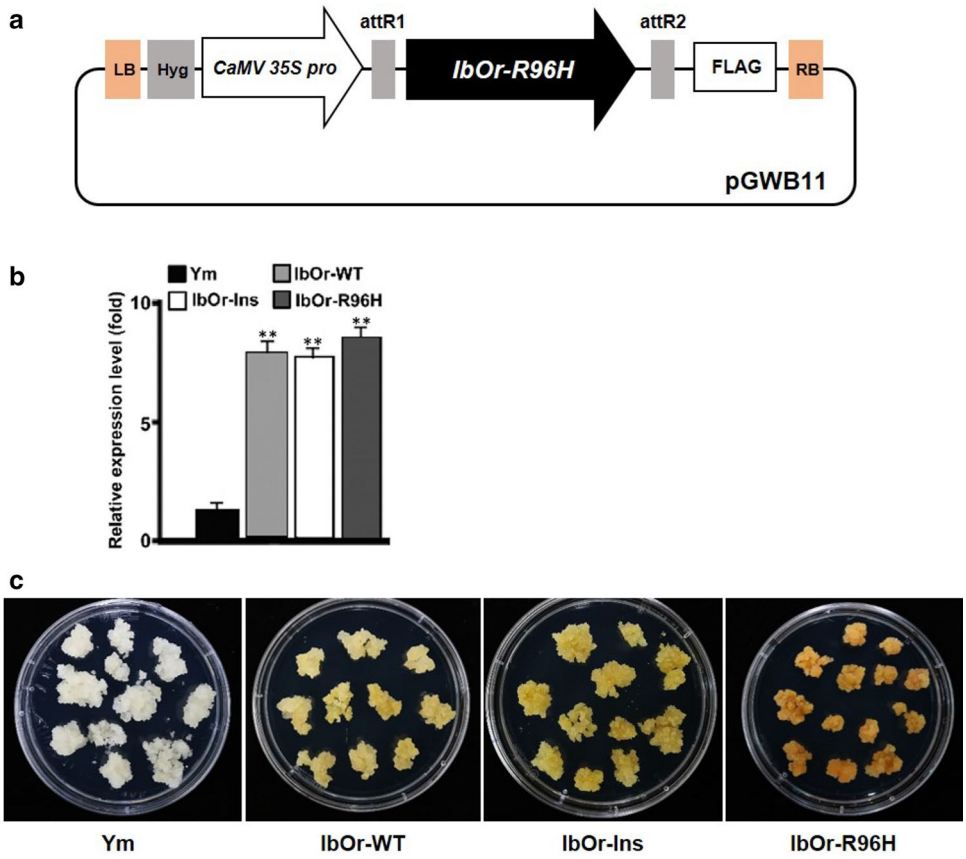

Fig. 2 Characterization of transgenic sweetpotato calli overexpressing IbOr-R96H. **a** Schematic diagram of the pGWB11/IbOr-R96H construct used for sweetpotato transformation. **b** Transcript levels of *IbOr* genes in the transgenic sweetpotato calli. Data are shown as mean ± SD of three biological repeats. Asterisks indicate significant differences compared with Ym calli, as determined by *t* tests ($P < 0.01$). **c** Phenotypes of transgenic sweetpotato calli overexpressing IbOr-WT, IbOr-Ins, or IbOr-R96H

IbOr-R96H promotes carotenoid accumulation in sweetpotato calli

To assess whether the color difference exhibited in transgenic calli overexpressing IbOr-R96H was correlated with carotenoid accumulation, carotenoid contents in these calli were quantified by HPLC. Consistent with previous data (Kim et al. 2013a) and color difference of the callus, the two IbOr-overexpressing transgenic calli (*IbOr-WT* or *IbOr-Ins*) displayed slightly higher levels of total carotenoids than those in the Ym callus. However, IbOr-R96H transgenic calli accumulated dramatically higher levels of total carotenoids (Fig. 3 and Supplemental Table S2). The total carotenoid content in IbOr-R96H transgenic calli was 23.8-fold higher than the level in the Ym callus, whereas the total carotenoid contents in IbOr-WT and IbOr-Ins transformed calli lines were 1.79- and 4.3-fold higher than those of the Ym callus, respectively. Interestingly, the β-carotene content in IbOr-R96H transgenic calli was 88.4-fold higher than the levels in the Ym callus. In addition, 9Z-β-carotene, 13Z-β-carotene, and β-cryptoxanthin contents were also higher in IbOr-R96H transgenic calli (Fig. 3). Similar to our previous results (Kim et al. 2013a), β-carotene contents in IbOr-WT and IbOr-Ins transgenic calli were 2.25 and 8.8-fold higher than those of the Ym calli. The α-carotene content in IbOr-R96H transgenic calli was also 7.6-fold higher than the levels in Ym calli. IbOr-R96H substantially increased the biosynthesis of β-carotene (Fig. 3 and Supplemental Table S2). In comparison with 16% β-carotene in the Ym calli, IbOr-WT and IbOr-Ins transgenic calli had β-carotene contents of 20% and 33%, and IbOr-R96H resulted in calli in which β-carotene accounted for approximately 60% of the total carotenoids (Fig. 3). The promotion of β-carotene biosynthesis by IbOr-R96H was consistent with results observed for $AtOr^{His}$ and $SbOr^{His}$ in transgenic *Arabidopsis* calli (Yuan et al. 2015). These results indicate that IbOr-R96H promotes carotenoid accumulation, similar to $AtOr^{His}$ and $SbOr^{His}$, and the effect of the His substitution in Or on carotenoid accumulation is conserved among plant species.

IbOr-R96H significantly alters carotenoid metabolic gene expression

We have previously shown that the expression levels of carotenogenesis-related genes are slightly increased in IbOr-WT and IbOr-Ins transgenic calli (Kim et al. 2013a). To determine whether the high accumulation of carotenoids in IbOr-R96H transgenic calli is correlated with altered carotenogenic gene expression, we investigated the transcript levels of carotenoid biosynthesis and degradation genes. We

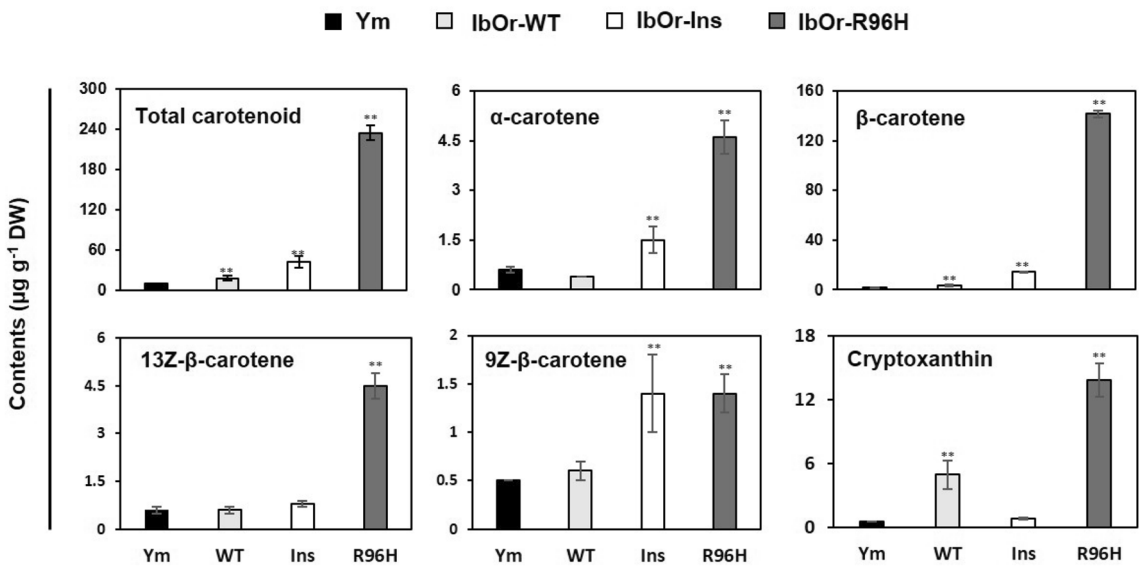

Fig. 3 Quantitative HPLC analysis of levels of total carotenoids and carotenoid compounds in transgenic sweetpotato calli expressing IbOr. Data are shown as mean ± SD of three biological repeats. Asterisks indicate significant differences compared with Ym calli, as determined by *t* tests ($P < 0.01$)

found that key genes of the carotenoid biosynthesis pathway were highly expressed in IbOr-R96H transgenic calli (Fig. 4). Expression levels of the upstream lycopene genes *geranylgeranyl pyrophosphate synthase* (*GGPS*), *PSY*, and *PDS* were higher in IbOr-Ins and IbOr-R96H transgenic calli than in Ym calli. *LCY-β*, *LCY-ε*, and *CHY-β* were also up-regulated in IbOr-Ins and IbOr-R96H transgenic calli (Fig. 4). Expression levels of *NCED*, *CCD1*, and CCD4,

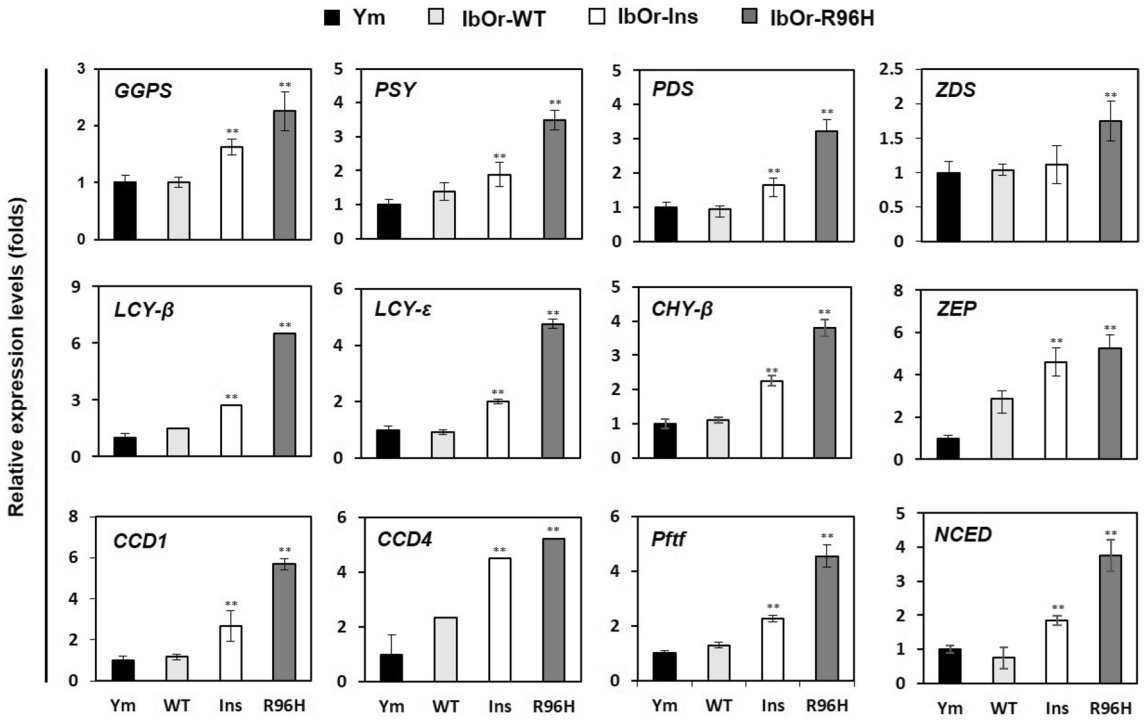

Fig. 4 Expression patterns of genes involved in the carotenoid biosynthetic pathway (biosynthesis and degradation) in transgenic sweetpotato calli expressing *IbOr* variants. Data are shown as mean ± SD of three biological repeats. Asterisks indicate significant differences compared with Ym calli, as determined by *t* tests ($P < 0.01$)

which is involved in carotenoid cleavage, were significantly higher in IbOr-R96H transgenic calli than in Ym calli. The transcript level of *Pftf* gene, which is involved in the differentiation from chloroplast to chromoplasts, was also increased in IbOr-R96H transgenic calli. These results indicated that increased levels of carotenoids accumulation in IbOr-R96H transgenic calli likely resulted from higher expression of carotenoid biosynthetic gene.

IbOr-R96H transgenic calli exhibit an enhanced tolerance to salt and heat stress

IbOr-WT or IbOr-Ins transgenic calli show increased tolerance to salt stress, and transgenic sweetpotato plants overexpressing *IbOr-Ins* display improved resistance to heat and oxidative stresses (Kim et al. 2013a; Park et al. 2016). Thus, we speculate that IbOr-R96H transgenic calli also exhibit increased salt tolerance. To evaluate salt stress resistance, Ym and transgenic calli were treated with 150 mM NaCl for 24 h. As shown in Fig. 5a, we did not observe significant cell death in response to salt stress in Ym, IbOr-WT, IbO-Ins, or IbOr-R96H transgenic calli. We next measured the level of salt-induced oxidative stress in calli by DAB staining, which results in a dark-brown color when oxidized by H_2O_2. Compared with Ym calli, which exhibited a dark-brown color, IbOr-R96H transgenic calli showed reduced DAB staining intensities after salt stress treatment. These results indicated that IbOr-R96H expression confers an enhanced resistance to salt stress in transgenic calli (Fig. 5a). MDA is an important indicator of cell membrane damage under oxidative stress conditions (Hodges et al. 1999). After salt stress treatment, MDA contents were higher in Ym calli than in IbOr-WT, IbOr-Ins, and IbOr-R96H transgenic calli (Fig. 5b). These results suggest that the degree of cell membrane damage was greater in Ym calli than in transgenic calli overexpressing IbOr variants under salt stress.

Transgenic sweetpotato plants overexpressing *IbOr-Ins* show enhanced tolerance to heat stress and this can be attributed to the chaperone activity of IbOr, involved in protecting the photosynthetic apparatus and carotenoid biosynthesis pathway (Park et al. 2016 and Kang et al. 2017a, b). Thus, we examined whether the IbOr transgenic calli also exhibited increased tolerance to heat stress due to IbOr overexpression. As shown in Fig. 6a, visible callus damage was lower in IbOr transgenic calli than in Ym calli. MDA contents also were higher in Ym calli than in IbOr-WT, IbOr-Ins, and IbOr-R96H transgenic calli after heat stress treatment (Fig. 6b). We did not observe dramatic differences among IbOr-WT, IbOr-Ins, and IbOr-R96H transgenic calli in response to salt or heat stresses.

Discussion

A SNP in *Or* is associated with a difference in carotenoid accumulation between orange- and green/white-fleshed melon fruits (Tzuri et al. 2015). The overexpression of *AtOr* gene with a mutation in the site corresponding to the CmOr SNP (altering Arg to His) results in high total carotenoid and β-carotene contents (Yuan et al. 2015). In this study, we successfully generated transgenic sweetpotato calli overexpressing IbOr-R96H. Interestingly, the overexpression of IbOr-R96H turned white-fleshed into orange-fleshed transgenic sweetpotato calli (Fig. 2). In addition, the IbOr-R96H-overexpressing calli showed dramatically elevated total carotenoid (23.8-fold) and β-carotene levels

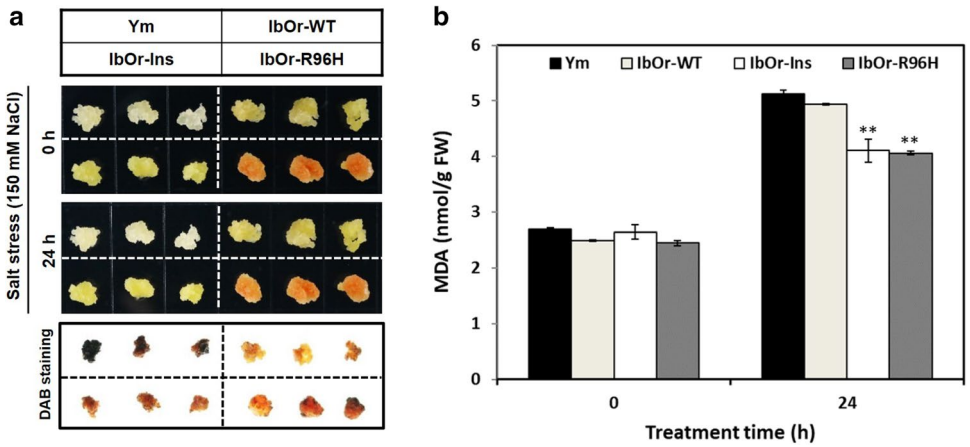

Fig. 5 Tolerance of transgenic sweetpotato calli expressing IbOr to salt-induced oxidative stress. **a** Schematic details (upper). Visible damage in sweetpotato calli after treatment with 150 mM NaCl for 24 h (middle). Detection of ROS accumulation in sweetpotato calli by DAB staining to visualize H_2O_2 (lower). **b** MDA contents in transgenic calli incubated at 150 mM NaCl for 24 h. Asterisks indicate significant differences compared with Ym calli, as determined by *t* tests ($P < 0.05$)

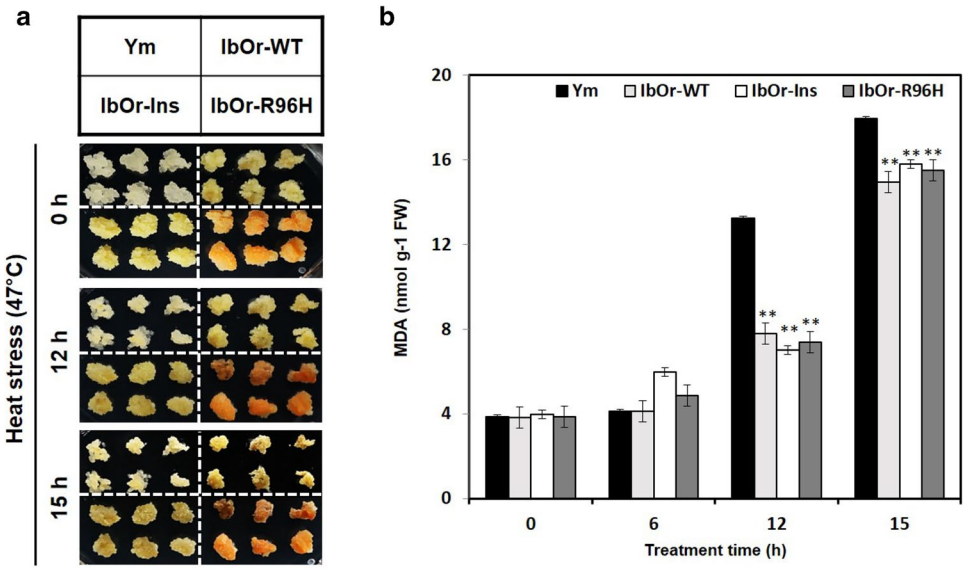

Fig. 6 Analysis of heat stress (47 °C) tolerance in transgenic sweetpotato calli expressing IbOr. **a** Schematic details (upper). Visible damage in calli after high-temperature stress (47 °C) treatment for 15 h (lower). **b** MDA contents in transgenic calli incubated at 47 °C for 15 h. Asterisks indicate significant differences compared with Ym calli, as determined by *t* tests ($P < 0.05$)

(88.4-fold) compared to those in Ym calli. In particular, the total carotenoid and β-carotene levels in IbOr-R96H-overexpressing transgenic calli were approximately 13.3- and 39.3-fold higher, respectively, than those in IbOr-WT transgenic calli (Supplemental Table S1). However, the total carotenoid content in the $AtOr^{His}$-overexpressing lines is up to fourfold higher than that in the AtOr-overexpressing lines (Yuan et al. 2015). IbOr-R96H increased the β-carotene ratio to 60%, whereas $AtOr^{His}$ resulted in calli with β-carotene accounting for 50% of the total carotenoids (Fig. 3). These results suggest that the manipulation of IbOr-R96H is a useful strategy to improve nutritional quality by increasing the total carotenoid and β-carotene contents.

Carotenoids belong to the isoprenoid family, including C40 tetraterpenoids and lipid-soluble antioxidants in chloroplasts. They have nutritional value as precursors of vitamin A, with protective effects against cancer, cardiovascular diseases, and eye diseases (DellaPenna and Pogson 2006). To address chronic diseases and malnutrition in developing countries, plant biotechnology for sustainable agriculture is required. In plants, metabolic engineering of the carotenoid biosynthetic pathway has been used to develop carotenoid-enriched crops. The first generation of Golden Rice expressing *PSY* from daffodil and the bacterial phytoene desaturase (*crtI*) from *Erwinia uredorva* accumulated 1.6 μg g^{-1} total carotenoids (Ye et al. 2000). A second generation of Golden Rice in which maize PSY was introduced has 23-fold higher levels of total carotenoids (37 μg g^{-1}) compared to those in the first Golden Rice (Paine et al. 2005). Interestingly, the overexpression of IbOr-R96H in sweetpotato calli increased the accumulation of total carotenoids up to 234.1 μg g^{-1} DW including 141.5 μg g^{-1} DW β-carotene. The IbOr-R96H transgenic sweetpotato calli have 146-fold higher levels of total carotenoids than those in the first-generation Golden Rice. Thus, *IbOr* gene is a novel and powerful target for plant breeding to increase carotenoid contents. Although comparison of carotenoid contents in sweetpotato callus and Golden Rice is investigated in completely different tissue, it will be confirmed by measurement of carotenoid content in the edible tissue such as storage root of IbOr-R96H overexpressing sweetpotato plant.

Or genes related to the carotenoids overaccumulation have been studied in sweetpotato, *Arabidopsis*, cauliflower, melon, potato, and sorghum. In particular, IbOr isolated from an orange-fleshed sweetpotato function in carotenoid accumulation and tolerance to abiotic stresses. The overexpression of *IbOr* in sweetpotato calli, sweetpotato storage roots, alfalfa, and potato tubers increased carotenoid contents and abiotic stress tolerance (Kim et al. 2013a, b; Goo et al. 2015; Park et al. 2015; Wang et al. 2015; Cho et al. 2016; Park et al. 2016). In the carotenoid biosynthetic pathway, IbOr directly interacts with IbPSY and stabilizes IbPSY via chaperone activity under heat and oxidative stresses (Park et al. 2016). Moreover, overexpression of *IbOr* in *Arabidopsis* plants exhibit increased tolerance to heat and oxidative stress (Park et al. 2016). Here, we demonstrated that transgenic sweetpotato calli overexpressing *IbOr-WT*, *IbOr-Ins*, and *IbOr-R96H* display salt (150 mM NaCl) and heat (47 °C) stress tolerance (Figs. 5, 6). Transgenic IbOr calli exhibited less damage and lower MDA contents than those of Ym calli. However, there were no significant differences among IbOr transgenic callus lines, unexpectedly. We previously reported that IbOr regulates process of photosynthesis by protecting degradation of IbPsbP protein under heat stress (Kang et al. 2017a). These results suggest that IbOr protein can protect plants from abiotic stresses such as heat, salt, and oxidative stress not only by controlling carotenoid

metabolic pathway but also by directly stabilizing photosynthesis. Although IbOr-R96H transgenic calli showed the dramatic accumulation of carotenoids, the lack of a difference in stress tolerance among transgenic calli overexpressing *IbOr* variants is explained by the inability of non-green tissues to photosynthesize, such as calli.

NCED enzymes synthesize ABA by cleavaging the *cis*-isomers of violaxanthin and neoxanthin (Nambara and Marionpoll 2005). *NCED* transcript levels increased in IbOr-R96H transgenic calli (Fig. 4). The increased resistance to salt and heat stress in IbOr-R96H transgenic calli might be involved in the ABA-signaling pathway. Expression levels of *CCD1* and *CCD4* genes coding carotenoid cleavage enzyme are negatively related with carotenoid accumulation in various plants, including *Arabidopsis* seed (Gonzalez-Jorge et al. 2013), potato (Campbell and Taylor 2010), strawberry (García-Limones et al. 2008), and chrysanthemum flowers (Ohmiya et al. 2006). Despite increased levels of carotenoid accumulation in sweetpotato plants overexpressing *IbOr*, these transgenic sweetpotato also showed high transcript levels of *CCD1* and *CCD4* genes (Park et al. 2015). The down-regulation of *LCY-ε* also induces *CCD1* and *CCD4* expression (Ke et al. 2019). Moreover, expression levels of *CCD1* and *CCD4* are not always accompanied with carotenoid accumulation levels in morning glory (Yamamizo et al. 2010). We showed that the up-regulation of IbOr-R96H in sweetpotato calli increased not only total carotenoid levels but also *CCD1* and *CCD4* expression levels (Figs. 3, 4). These results indicate that the rate of carotenoid accumulation exceeds the rate of degradation in IbOr-R96H transgenic calli. However, the precise mechanism regulating carotenoid metabolism pathway remains to be elucidated in plant.

For further analyses of IbOr-R96H regulatory mechanisms, transgenic sweetpotato plants overexpressing IbOr-R96H are under investigation. We anticipate that IbOr-R96H transgenic sweetpotato plants will exhibit enhanced production of carotenoids and environmental stress tolerance including high temperature, salt, and drought stresses. The rational regulation of IbOr-R96H will contribute to the generation of efficient industrial plants to cope with food and nutrition security as well as climate change by enabling sustainable agriculture on global marginal lands.

Acknowledgements This work was supported by grants from the Systems & Synthetic Agrobiotech Center (PJ01318401 and PJ01318402), the Biogreen 21 Project for the Next Generation, Rural Development Administration, Korea, the National Natural Science Foundation of China (31700335), and the KRIBB initiative program.

Author contribution statement SEK, HSK, and SSK contributed to the research design. SEK, HSK, ZW, QK, CJL, SUP, and YHL performed site-directed mutagenesis, transformation, qRT-PCR, and stress-tolerant assays. WSP and MJA performed HPLC for the measurement of carotenoid contents. SEK, HSK and SSK primarily wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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