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Overexpression of the *IbOr* gene from sweet potato (*Ipomea batatas* 'Hoang Long') in maize increases total carotenoid and β-carotene contents

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Abstract: Nutritional quality of most maize varieties is very low due to the lack of lysine and tryptophan and extremely low provitamin A carotenoids including β-carotene, α-carotene, and β-cryptoxanthin. In this study, we report the successful overexpression of the *IbOr* gene in H145 and H95 inbred maize lines under the control of maize seed-specific promoter globulin 1 (Glo1) for the purpose of improving β-carotene in maize. The results showed that the total carotenoid and β-carotene content of all analyzed transgenic maize plants were significantly higher than those of wild-type lines. For H145-IbOr transgenic maize, in the best line (H145-IbOr.10), the total carotenoid and β-carotene contents were increased up to 10.36- and 15.11-fold, respectively, compared to the wild type (H145-WT). In the case of H95-IbOr transgenic plants, 5.58-fold increase in total carotenoid and 7.63-fold increase in β-carotene were achieved in the H95-IbOr.6 line compared to nontransgenic plants (H95-WT). In all the transgenic plants derived from the wild-type maize line with less carotenoid content (H145-WT), the content of both total carotenoid and β-carotene was higher than in transgenic plants derived from the wild-type maize line having more carotenoid content (H95-WT). Our research is the first in successful overexpression of *IbOr* gene in maize.

Key words: β-Carotene, carotenoid content, *IbOr* gene, maize, sweet potato

1. Introduction

Maize is an important cereal in the global economy, which feeds one-third of the world's population and is the third largest food crop after wheat and rice. In Vietnam, maize is the second largest food crop after rice. Nutritional quality of most maize varieties is very low due to the lack of lysine and tryptophan and extremely low provitamin A carotenoids including α-carotene, β-carotene, and β-cryptoxanthin (Kurilich and Juvik, 1999). In order to improve the nutritional quality of maize, many efforts have been made to produce high quality maize (Sofi et al., 2009) and increased carotenoid content, particularly provitamin A carotenoids (Wurtzel et al., 2012).

Plant carotenoids are the main source of vitamin A for humans. Vitamin A deficiency is an important nutritional problem in many parts of the world. As estimated by the World Health Organization, vitamin A deficiency affects 250 million preschool children worldwide and leads to blindness for up to 500,000 children annually (http://www.who.int/nutrition/topics/vad/en/). In addition, carotenoids also have the potential to reduce cancer and cardiovascular diseases (Hadley et al., 2002). In livestock, the largest demand for carotenoids is in salmon and poultry farming (Tyczkowski and Hamilton, 1986).

Carotenoids in plants are synthesized in plastid membranes and are abundant in plastids (except proplastids) of flowers, fruits, and roots (Howitt and Pogson, 2006). Chromoplasts have a special mechanism for reserving large amounts of carotenoids by creating structures called carotenoid-lipoprotein structures that are located within the chromoplast. These structures are also called carotenoid isolation structures. Isolation structures serve as reservoirs for the isolation of carotenoids and inhibit the final products of carotenoid synthesis, which interfere with the formation of carotenoid in chromoplast membranes (Al Babili et al., 1999).

To improve the carotenoid content in crop plants, the increase of total carotenoid content should be regulated, including increasing synthesis, reducing decomposition, and optimizing the reserves. Therefore, to improve the carotenoid content, it is possible to interfere with the synthesis cycle by enhancing the expression of genes coding for key enzymes such as *PSY* (Burkhardt et al., 1997; Paine et al., 2005), *CrT1*, *PDS*, and *ZDS* (Romer et al., 2000) or inhibition of those genes involved in decomposition such as *LCY-e* or *CHY* (Diretto et al., 2007), and to create

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an optimal reserve body (Cookson et al., 2003; Li et al., 2012). Application of gene technology to express genes involved in carotenoid biosynthesis and accumulation is an important direction for improving carotenoid levels in diets. Transgenic maize lines with genes involved in biosynthesis pathways of intermediate keto-carotenoid, producing grains that vary from white and yellow to dark red, have been generated from white maize varieties (Aluru et al., 2008; Zhu et al., 2008).

The *Or* gene (Li et al., 2001) plays a part in the accumulation of carotenoid and is involved in metabolism leading to differentiation of the nonpigmented plastids into chromoplasts, but does not interfere with carotenoid synthesis. Particularly, the *Or* gene plays the role of initiating the differentiation of nonpigmented plastids into chromoplasts in white or less pigmented tissues (Lu et al., 2006; Lopez et al., 2008), creating a metabolic sink to isolate and store carotenoids. The studies by Lu et al. (2006) and Lopez et al. (2008) have shown that the mutated *Brassica oleracea Or* gene (*BoORMUT*) showed its effect by triggering chromoplast differentiation and enhancing storage sink strength for carotenoid biosynthesis and accumulation. The overexpression of the wild-type *Or* gene also enhanced the carotenoid accumulation in sweet potato (Kim et al., 2013) and in calli of rice (Bai et al., 2014). The carotenoid accumulation driven by the *Or* gene associated with the formation of the metabolic sinks was also confirmed in transgenic potato (Zhou et al., 2008). The results obtained by Lu et al. (2006) showed that the *Or* gene codes for plastid protein containing a DnaJ Cys-rich domain that is associated with high carotenoid accumulation. In addition to the role in carotenoid accumulation, a recent study showed that the Or protein of *Arabidopsis thaliana* interacts directly with PSY protein and functions as a major regulator of active PSY protein abundance in mediating carotenoid biosynthesis (Zhou et al., 2015). The overexpression of the *Or* gene from *Ipomea batata*s (*IbOr*) in transgenic plants of potato, alfalfa, and sweet potato (Li et al., 2012; Goo et al., 2015; Park et al., 2015; Wang et al., 2015; Cho et al., 2016) has led to increased carotenoid accumulation without affecting carotenoid synthesis. A recent report on the role of the *Arabidopsis thaliana Or* gene (*AtOr*) in transgenic white maize and their hybrids (Berman et al., 2017) suggested the potential use of the *Or* gene to improve the carotenoid content of staple crops including maize.

In an attempt to regenerate transgenic maize plants with improved provitamin A carotenoid, we have cloned the *IbOr* gene from yellow-fleshed sweet potato cultivar Hoang Long and constructed a transformation vector carrying the gene. In this study, we report the successful overexpression of the gene in two inbred maize lines.

2. Materials and methods

2.1. Materials

Immature embryos of inbred maize lines H95 and H145 were obtained from the National Maize Research Institute, Vietnam Academy of Agriculture Science, Hanoi, Vietnam.

Plasmid pJET1.2 blunt/IbOr carrying the *IbOr* gene sequence (KX792094.1) from sweet potato cultivar Hoang Long (*Ipomea batatas* 'Hoang Long') and *Agrobacterium tumefaciens* strain C58 carrying transformation vector pCambia2300/Glo1/IbOr/Nos (Figure 1) were developed and provided by the Plant Cell Genetics Laboratory, Institute of Biotechnology, Vietnam Academy of Science and Technology, Hanoi, Vietnam. *E. coli* strain DH5α was supplied by Invitrogen (Life Technologies Holdings Pte. Ltd., Singapore).

The specific primers for amplification of the *IbOr* gene, IbOr-F: 5'-GCGGATCCATGGTATATT CAGGTAGAATC-3' with *Bam*HI recognition site and IbOr-R: 5'-GCGAGCTCTTAATCAAATGGGTCAA TTC-3' with *Sac*I recognition site, were designed using the Primer 3 program (NCBI, USA) and supplied by Macrogen Inc. (South Korea).

2.2. Maize transformation and regeneration of transgenic plants

Maize transformation and regeneration of transgenic plants were performed as previously described by Nguyen et al. (2016): immature embryos at 10–13 days after pollination from the maize lines were used for transformation using

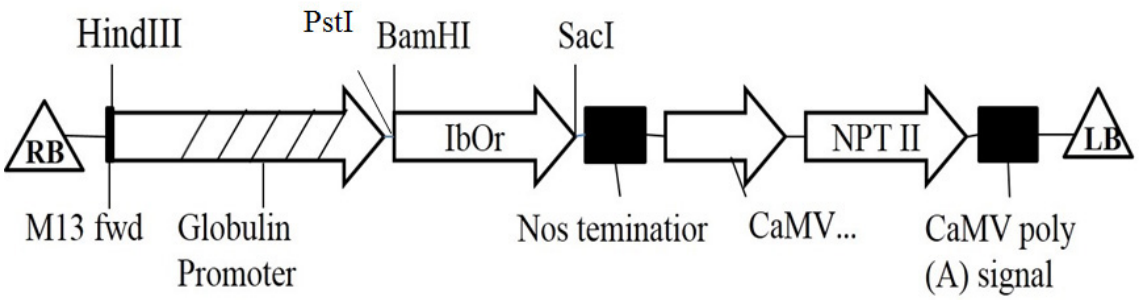

Figure 1. Schematic transformation vector pCambia2300/Glo1/IbOr/Nos.

Agrobacterium tumefaciens strain C58 harboring the pCambia2300/Glo1/IbOr/Nos construct. The treated embryos were cultured on modified N6 medium (basal N6 medium (Chu et al., 1975) supplemented with 2 mg/L 2,4-D, 10 mg/L $AgNO_3$, 2.3 g/L L-proline, 100 mg/L casein hydrolysate, 20 g/L sucrose, 10 g/L glucose, 100 μM acetosyringone, and 7.5 g/L agar; pH 5.8) for cocultivation for 3 days in the dark. After cocultivation, the embryos were transferred onto N6 medium supplemented with 2 mg/L 2,4-D, 10 mg/L $AgNO_3$, 2.3 g/L L-proline, 100 mg/L casein hydrolysate, 20 g/L sucrose, 10 g/L glucose, 500 mg/L cefotaxime, and 7.5 g/L agar (pH 5.8) with kanamycin (100 mg/L) for removal of leftover *Agrobacterium* and selection of calli. Selected transformed calli were transferred onto resting medium (medium without kanamycin) for 1 week and then onto regeneration medium (N6 medium plus 60 g/L sucrose, 0.2 mg/L BAP, and 7.5 g/L agar, pH 5.8) at 28 °C under a 10/14-h light/dark photoperiod. The regenerated shoots were transferred onto selection medium containing 100 mg/L kanamycin. Normal shoots were rooted on medium that consisted of ½ N6 medium plus 1.5 g/L NAA or IBA and 7.5 g/L agar at 28 °C, under a 10/14-h light/dark photoperiod. The complete plantlets were transplanted into thin plastic pots filled with a Tribat soil mixture (Sai Gon Biotechnology Company Ltd., Ho Chi Minh City), followed by planting in a safe greenhouse.

2.3. Molecular analyses of transgenic plants

2.3.1. PCR amplification of genomic DNA

We checked for the presence of the *IbOr* gene in transgenic maize plants by PCR of genomic DNA from T0 plants using specific primers. Reaction conditions were as follows: initial denaturing at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 50 °C for 50 s, and 72 °C for 90 s and a final extension at 72 °C for 8 min. The PCR reaction consisted of 10X PCR buffer: 2 μL; dNTP (1 mM): 2 μL; forward and reverse primers (50 ng/μL): 1 μL of each primer; Taq DNA polymerase (5 U/μL): 0.1 μL; DNA template (50 ng): 1 μL; and sterile distilled water: 12.9 L The PCR products were electrophoresed in 1% agarose gel and stained with EtBr for visualization under UV light.

2.3.2. Southern blotting

Genomic DNA samples from PCR-positive transgenic T1 maize plants with the *IbOr* gene were digested overnight at 37 °C with restriction enzyme *Bam*HI or *Sac*I. The plasmid DNA of pCambia2300/Glo1/IbOr/Nos was digested with *Bam*HI and *Sac*I to obtain the *IbOr* gene fragment (942 bp) for preparation of the probe. The probe was labeled with the Biotin DecaLabel DNA Labeling Kit (Thermo Scientific, USA). The digested genomic DNA from transgenic maize plants was separated on 1% agarose gel and transferred to Hybond-N nylon membrane (Magna Charge Nylon Company, USA). Hybridization was conducted at 42 °C. Hybridization and detection were performed according to the manufacturer's instructions using the Biotin Chromogenic Detection Kit (Thermo Scientific).

2.4. Carotenoid analysis

The total carotenoid extraction from the seeds of transgenic maize plants, 40 days after pollination, was carried out as described by Rivera and Canela (2012) with a small modification. Twenty to 25 seeds were ground to a fine powder using a mortar and pestle. Powder (100 mg) was then incubated with 15 mL of methanol containing 0.1 g/L butylated hydroxytoluene with continuous stirring for 20 min at 60 °C. Total carotenoids were quantified by measuring absorbance at 450 nm. HPLC analysis was carried out using the Hitachi Elite LaChrom HPLC system with Diode Array Detector L2450 (Hitachi High-Technologies, Japan). For HPLC separation, the solvent was evaporated under a stream of N_2 gas at 37 °C and redissolved in 50 μL of acetone. Twenty microliters of standard or sample were injected into a Zorbax Eclipse XDB-C18 (250 × 4.6 × 5) column (Agilent Technologies, USA) with a mobile phase of 20:80 (vol:vol) acetonitrile and MeOH. EZ Chrome Elite software (Agilent Technologies) was employed for data collecting and processing. The stock solutions of standard β-carotene (Sigma-Aldrich Fine Chemicals, USA) were prepared by dissolving 1 mg of standard in 10 mL of acetone. Working calibration solutions (0.1, 0.25, 0.5, 1, 2.5, 5, 10, 20, 30, 40, and 60 mg/mL) were then prepared by diluting stock solutions.

3. Results

3.1. Maize transformation and regeneration of transgenic plants

We used *Agrobacterium tumefaciens* C58 carrying the transformation vector pCambia2300/Glo1/IbOr/Nos for transformation of inbred maize lines H95 and H145. After infection of the immature embryos from the maize lines (30 and 40 min) and coculture (3 days), followed by callus production on medium containing cefotaxime (500 mg/L), the percentage of callus formation of the H95 and H145 was 72%–74% and 67%–69%, respectively (data not shown). On the selection medium containing kanamycin (100 mg/L), the selected calli varied from 15.35% to 16.19% and the regeneration frequency of the selected calli also varied depending on maize lines (Table 1). Finally, we obtained 65 and 51 transformed plants from the respective H95 and H145 wild-type maize lines.

3.2. Molecular analyses of transgenic maize plants

The results of PCR of genomic DNA from T0 transgenic plants (Figure 2) using specific primers (see Section 2) showed the presence of the *IbOr* gene in analyzed transformed maize plants. Out of the 116 T0 plants we obtained, a total of 74 positive plants bore the *IbOr* gene: 43 plants from H95 and 31 from H145. The transformation

Table 1. Percentage of selected calli and plant regeneration of the maize lines.

Maize lines	Selected calli (%)	Plant regeneration (%)	Number of regenerated normal T0 plants
H95	16.19	48.45	65
N618	15.35	52.49	51

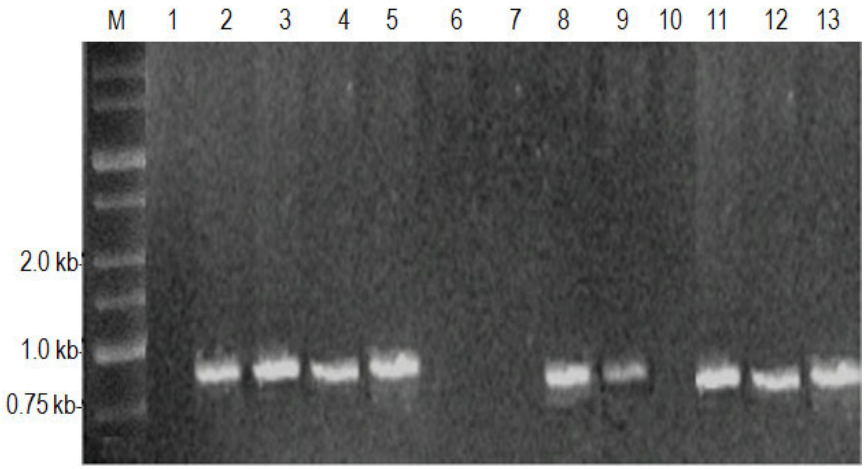

Figure 2. PCR analysis of some T0 plants with the *IbOr* gene: lane 1, control (nontransformed maize line); lanes 2, 3, 4, 5, 8, 9, 11, 12, and 13, transformed plants carrying the *IbOr* gene; lanes 6, 7, and 10, transformed plants with no *IbOr* gene.

frequencies ranged from 6.20% to 8.60% with an average of 7.40% (Table 2).

The Southern analyses (Figure 3) performed on PCR-positive T1 plants showed that the *IbOr* gene was integrated into the genome of transformed maize lines. The copy number of the transgene was one to four. The analyzed transgenic maize plants that carried the *IbOr* transgene were taken for further analyses of carotenoids.

3.3. Carotenoid analysis of transgenic maize lines

According to published studies, the *Or* gene from cauliflower (*Brassica oleracea* var. *botrytis*), the sweet potato *Or* gene (*IbOr* gene), and the *Arabidopsis thaliana Or* gene (*AtOr*) are involved in the development of chromoplasts and promote the accumulation of large amounts of β-carotene (Lu et al., 2006; Lopez et al., 2008; Kim et al., 2013; Bai et al., 2014). We analyzed the total carotenoid content and β-carotene in the produced T1 transgenic maize lines. The seeds (40 days after pollination) from four transgenic H95-IbOr lines and five transgenic H145-IbOr lines were analyzed. Total carotenoid and β-carotene contents of all transgenic maize lines were higher than those of wild-type (non-transgenic) plants (Table 3). The total carotenoid and β-carotene contents in the best H145-IbOr transgenic maize (H145-IbOr.10) were respectively 10.36- and 15.11-fold higher than those in the wild type (H145-WT). Furthermore, the total carotenoids and content of β-carotene in the H95-IbOr.6 transgenic plants were 5.83- and 7.63-fold higher than in the wild type (H95-

Table 2. Transformation frequencies of the maize lines.

Maize lines	Number of treated embryos	Number of T0 plants tested	Number of PCR-positive plants	Percentage of PCR-positive plants (%)	Transformation efficiency* (%)
H95	500	65	43	66.15	8.60
N618	500	63	31	49.20	6.20
Total	1000	116	74	63.79	7.40

*Transformation efficiency is the percentage (%) of positive T0 plants obtained after PCR analysis per the number of embryos treated with *A. tumefaciens* carrying the transgene.

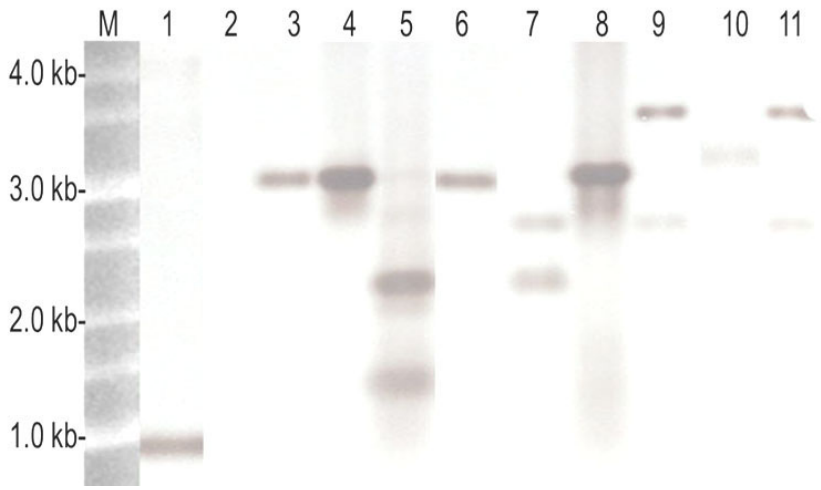

Figure 3. Southern analysis of selected T1 transgenic plants. Genomic DNA was digested by *Sac*I enzyme and probed with *IbOr* gene fragment: lane M, DNA ladder; lane 1, positive control (PCR amplification of *IbOr* gene); lane 2, nontransformed plants; lanes 3–11, transgenic plants.

Table 3. The carotenoid content in transgenic maize lines.

Maize lines	Total carotenoids		β-carotene	
	(μg/g DW)	Increased (fold)	(μg/g DW)	Increased (fold)
H95-WT	11.90	1.00	6.10	1.00
H95-IbOr.1	20.90	1.76	12.36	2.03
H95-IbOr.4	36.22	3.04	24.82	4.07
H95-IbOr.6	66.45	5.58	46.54	7.63
H95-IbOr.10	48.32	4.06	28.53	4.68
H145-W	2.67	1.00	1.28	1.00
H145-IbOr.1	14.96	5.61	10.44	8.15
H145-IbOr.4	19.51	7.32	11.80	9.21
H145-IbOr.5	23.13	8.68	10.23	7.98
H145-IbOr.10	27.60	10.36	19.35	15.11
H145-IbOr.15	24.12	9.05	17.63	13.76

WT). The increase of total carotenoids and β-carotene led to the difference in color of the ears and grains: the ears and grains of transgenic plants are more yellow than those of the wild type (Figure 4). Interestingly, the total carotenoid and β-carotene contents in all transgenic plants derived from the wild-type maize (H145-WT) with less carotenoid content were higher than those of transgenic plants from wild-type maize with more carotenoid content (H95-WT).

4. Discussion

Orange gene (*Or gene*) was shown to be involved in carotenoid accumulation by promoting chromoplast differentiation and creating a metabolic sink (Li et al., 2001). This function was confirmed by overexpression of cauliflower *Or gene* in transgenic potato (Lopez et al., 2008; Zhou et al., 2008; Li et al., 2012) and by transfer of the *Arabidopsis Or gene* (*AtOr*) into rice zygotic embryos (Bai et al., 2014) or by expression of *AtOr* in transgenic

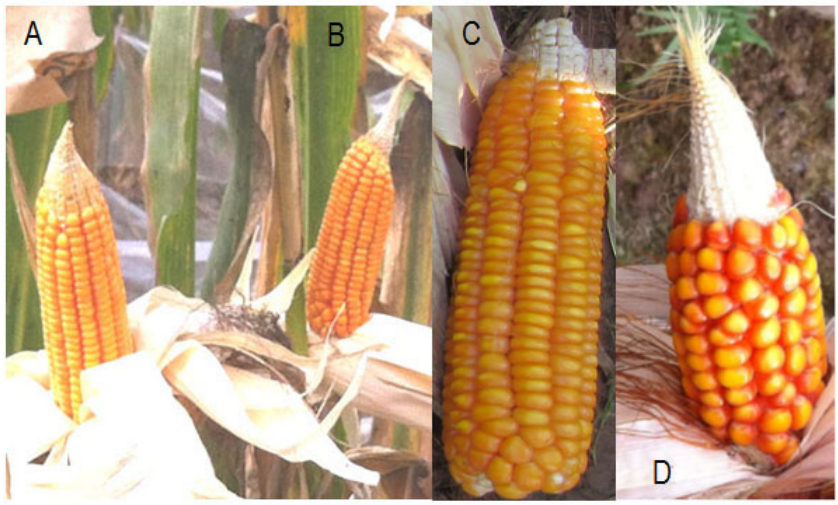

Figure 4. Morphology of the ears and grains of wild-type nontransgenic and transgenic maize plants: A) H145-WT; B) H145-IbOr; C) H95-WT; D) H95-IbOr.

maize plants with limited carotenoid pools (Berman et al., 2017). Kim et al. (2013) reported that the sweet potato *Or gene* (*IbOr*) promotes accumulation of carotenoids by inducing expression of carotenoid biosynthesis genes in transgenic sweet potato calli. Zhou et al. (2015) identified the interaction between *Arabidopsis* OR proteins and phytoene synthase (PSY) in plastids. This interaction has no effect on *PSY* gene expression, but positively mediates PSY protein level and carotenoid content. In another study, the overexpression of the *AtOr* gene increased the accumulation of carotenoids by upregulating a series of endogenous carotenogenic genes such as maize phytoene synthase 1 (*ZmPSY1*) and the *Pantoea ananatis* phytoene desaturase gene *(PaCRTI*) (Bai et al., 2016). Park et al. (2016) showed that the *IbOr* transgene in transgenic sweet potato and *Arabidopsis* functions as a holdase chaperone, enhancing tolerance to heat as well as oxidative stress. This protects the stability of IbPSY protein and leads to carotenoid accumulation. The different expression manners of the *Or* gene have led to varied phenomena: changing both the content and the composition of carotenoids (Lopez et al., 2008), promoting the accumulation of β-carotene and other carotenoids (Lopez et al., 2008; Li et al., 2012), and increasing the total carotenoid content without affecting the composition of carotenoids (Goo et al., 2015; Berman et al., 2017). In our work, the overexpression of the *IbOr* gene from yellow-fleshed sweet potato cultivar Hoang Long in transgenic maize plants resulted in the increase of both total carotenoids (1.76–10.36-fold) and β-carotene (2.03–15.11-fold).

The function of the *Or* gene is related to the differentiation of chromoplasts and accumulation of carotenoids in most of the plants investigated so far; however, the functional relation between the *Or* transgene and endogenous carotenoid biosynthesis genes as well as levels of carotenoids in wild-type plants is still not fully understood. Several reports showed that the overexpression of the *Or* gene can affect the endogenous carotenoid synthesis genes (Bai et al., 2014, 2016; Goo et al., 2015), while other studies showed that the overexpression of the *Or* gene stimulates accumulation of carotenoids without an effect on endogenous carotenoid synthesis genes (Yuan et al., 2015; Zhou et al., 2015). Recently, Berman et al. (2017) showed that the overexpression of the *Or* gene depends on the level and composition of carotenoid of the wild-type plants. It was reported that the *AtOr* gene can enhance carotenoid levels by promoting the formation of carotenoid-sequestering structures when the carotenoid pool is limited, but has no further effect when carotenoids are already abundant (Berman et al., 2017). Our results showed that in all transgenic plants derived from wild-type maize with less carotenoid content (H145-WT), the content of both total carotenoids and β-carotene was higher than in transgenic plants from wild-type maize with more carotenoid content (H95-WT).

The *IbOr* gene was first isolated from storage roots of sweet potato (*Ipomoea batatas* (L.) Lam. 'Sinhwangmi'), and its function in transgenic sweet potato calli was studied (Kim et al., 2013). Later on, Park et al. (2015) overexpressed the *IbOr* gene with insertion of seven amino acids between 131 and 142 of the wild-type IbOr protein (*IbOr-ins*) under the control of the cauliflower mosaic virus (CaMV) 35S promoter in an anthocyanin-rich sweet potato cultivar and Wang et al. (2015) transferred the *IbOr* gene to alfalfa under the control of an oxidative stress-inducible peroxidase (SWPA2) promoter via *Agrobacterium*

tumefaciens-mediated transformation and generated transgenic alfalfa plants with high carotenoid content that exhibited enhanced tolerance to drought stress. Our successful transfer of the *IbOr* gene under the control of maize globulin 1 seed-specific promoter and regeneration of transgenic maize plants with enhanced total carotenoid and β-carotene content is the first work to overexpress the *IbOr* gene in maize. In previous research, the genes for biosynthesis of carotenoids such as bacterial genes *crtB* (for phytoene synthase) and *crtI* (for the four desaturation steps of the carotenoid pathway catalyzed by phytoene desaturase and z-carotene desaturase) (Aluru et al., 2008) or a combination of 5 carotenoid synthesis genes (*Zmpsy1* (*Zea mays* phytoene synthase 1), *PacrtI* (*Pantoea ananatis* phytoene desaturase), *Gllycb* (*Gentiana lutea* lycopene β-cyclase), *Glbch* (*G. lutea* β-carotene hydroxylase), and *ParacrtW (Paracoccus* β-carotene ketolase)), have been used to produce transgenic maize plants with enhanced carotenoid content including β-carotene. Our research is the first in successful overexpression of the *IbOr* gene in maize. Our results show the potential application of the *IbOr* gene for biofortification of maize with β-carotene.

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