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Down-regulation of β-carotene hydroxylase increases β-carotene and total carotenoids enhancing salt stress tolerance in transgenic cultured cells of sweetpotato

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ABSTRACT

Sweetpotato (*Ipomoea batatas* Lam.) is an important industrial crop and source of food that contains useful components, including antioxidants such as carotenoids. β-Carotene hydroxylase (*CHY-β*) is a key regulatory enzyme in the beta–beta-branch of carotenoid biosynthesis and it catalyzes hydroxylation into both β-carotene to β-cryptoxanthin and β-cryptoxanthin to zeaxanthin. To increase the β-carotene content of sweetpotato through the inhibition of further hydroxylation of β-carotene, the effects of silencing *CHY-β* in the carotenoid biosynthetic pathway were evaluated. A partial cDNA encoding *CHY-β* was cloned from the storage roots of orange-fleshed sweetpotato (cv. Shinhwangmi) to generate an RNA interference-*IbCHY-β* construct. This construct was introduced into cultured cells of white-fleshed sweetpotato (cv. Yulmi). Reverse transcription-polymerase chain reaction analysis confirmed the successful suppression of *IbCHY-β* gene expression in transgenic cultured cells. The expression level of phytoene synthase and lycopene β-cyclase increased, whereas the expression of other genes showed no detectable change. Down-regulation of *IbCHY-β* gene expression changed the composition and levels of carotenoids between non-transgenic (NT) and transgenic cells. In transgenic line #7, the total carotenoid content reached a maximum of 117 μg/g dry weight, of which β-carotene measured 34.43 μg/g dry weight. In addition, *IbCHY-β*-silenced calli showed elevated β-cryptoxanthin and zeaxanthin contents as well as high transcript level P450 gene. The 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity (DPPH) in transgenic cells was more than twice that in NT cells. RNA-*IbCHY-β* calli increased abscisic acid (ABA) content, which was accompanied by enhanced tolerance to salt stress. In addition, the production of reactive oxygen species measured by 3,3′-diaminobenzidine (DAB) staining was significantly decreased in transgenic cultured cells under salt stress. Taken together, the present results indicate that down-regulation of *IbCHY-β* increased β-carotene contents and total carotenoids in transgenic plant cells and enhanced their antioxidant capacity.

1. Introduction

Sweetpotato (*Ipomoea batatas* Lam.) is one of the most important food crops in the world. It provides β-carotene (8) (see Fig. 1), which is the primary carotenoid found in many crops such as carrot (*Daucus carota* ssp. *sativus* L.) and sweetpotato. White or pale yellow-fleshed sweetpotato contains very little β-carotene (8), whereas orange-fleshed sweetpotato contains large amounts. Consumption of orange-fleshed sweetpotato and processed foods containing it provides a sustainable and cost-effective source of provitamin A (Maoka et al., 2007). However, the biosynthesis and metabolism of carotenoids in sweetpotato are poorly understood.

Carotenoids represent a class of red, orange and yellow fat-soluble pigments that are widely distributed in plants, algae, fungi and cyanobacteria (Cunningham and Gantt, 1998). Carotenoids participate in light-harvesting processes and protect the photosynthetic machinery from photo-oxidative damage. Carotenoids are also essential nutrients and important health compounds (Fraser and Bramley, 2004) because animals and humans are unable to synthesize vitamin A. Sweetpotatoes, carrots and tomatoes are well-known carotenoid-rich foods. The World Health Organization estimates that vitamin A deficiency affects 250 million preschool children worldwide, 250,000–500,000 of which become blind every year with half dying within 12 months of losing their sight (Krinsky, 1998; Krinsky and Johnson, 2005).

Carotenoids are synthesized in higher plants by the conversion of geranylgeranyl pyrophosphate (*GGPP*) into phytoene (3) by phytoene synthase (*PSY*), followed by desaturation by phytoene

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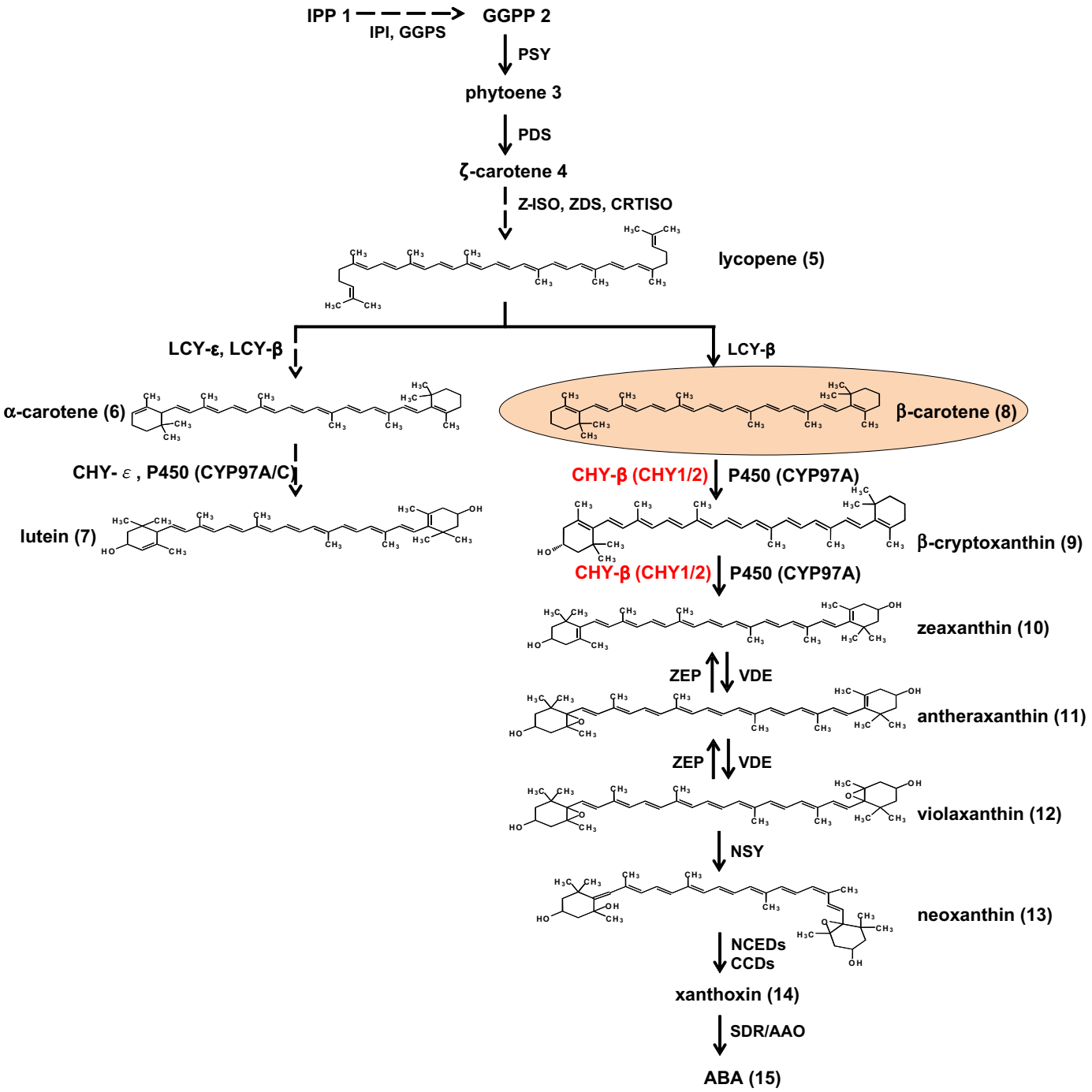

Fig. 1. Carotenoid biosynthesis pathway and related enzymes in plants. The pathway was slightly modified from the pathways by Spurgeon and Porter (1980), Giuliano et al. (2008) and Yamamizo et al. (2010). Abbreviations: *IPP*; isopentenyl diphosphate, *IPI*; isopentenyl diphosphate isomerase, *GGPS*; geranylgeranyl pyrophosphate synthase, *GGPP*; geranylgeranyl pyrophosphate, *PSY*; phytoene synthase, *PDS*; phytoene desaturase, *Z-ISO*; ζ-carotene isomerase, *ZDS*; ζ-carotene desaturase, *CRTISO*; carotenoid isomerase, *LCY-β*; lycopene β-cyclase, *LCY-ε*; lycopene ε-cyclase, *CHY-β*; β-carotene hydroxylase, P450; cytochrome P450 monooxygenases, *CHY-ε*; ε-ring hydroxylases, *VDE*; violaxanthin de-epoxidase, *ZEP*; zeaxanthin epoxidase, *NSY*; neoxanthin synthase, *NCEDs*; 9-cis-epoxycarotenoid dioxygenase, ABA; abscisic acid.

desaturase (*PDS*), 15-cis-ζ-carotene isomerase (*Z-ISO*), ζ-carotene desaturase (*ZDS*) and carotenoid isomerase (*CRTISO*) to form linear all-trans-lycopene (5). The cyclization of the linear carotenoid lycopene (5) catalyzed by lycopene β-cyclase (*LCY-β*) and/or lycopene ε-cyclase (*LCY-ε*) is a branch point in the pathway, leading to carotenoids with one ε- and one β-ring (α-carotene (6) and its derivatives; ε, β-carotenoids) or two β-rings (β-carotene (8) and its derivatives; β, β-carotenoids) (Cunningham and Gantt, 1998, 2001; Chen et al., 2010). Subsequently, α-carotene (6) and β-carotene (8) are modified by hydroxylation, epoxidation, or isomerization to express a variety of structural features. Beta-carotene hydroxylase (*CHY-β*) is a non-heme di-iron hydroxylase that functions in the conversion of β-carotene (8) and β-cryptoxanthin (9) to zeaxanthin (10). In *Arabidopsis*, four carotenoid hydroxylase enzymes have been reported: a pair of non-heme di-iron hydroxylases (*CHY1* and *CHY2*) and two heme-containing cytochrome P450 mono-oxygenases (*CYP97A* and *CYP97C*) (Tian et al., 2003; Kim and DellaPenna, 2006). *CYP97A* is β-carotene hydroxylase that

functions in the hydroxylation activity on the β-ring of α-carotene (6) and a minor activity on the β-rings of β-carotene (8). *CYP97C* is carotenoid ε-ring hydroxylase that functions in the conversion of α-carotene (6) to lutein (7) in *Arabidopsis* (Kim and DellaPenna, 2006). β-Xanthophyll is epoxidated–de-epoxidated by zeaxanthin epoxidase (*ZEP*) and violaxanthin de-epoxidase (*VDE*), giving rise to the xanthophyll cycle. Violaxanthin (12) is converted to neoxanthin (13) by neoxanthin synthase (*NSY*). The synthesized carotenoid end products can be catabolized to produce apocarotenoids. A family of carotenoid cleavage dioxygenases (*CCDs*) catalyzes the oxidative cleavage of carotenoids (Auldridge et al., 2006). The first *CCD* gene identified is 9-cis-epoxycarotenoid dioxygenase (*NCEDs*), which cleaves violaxanthin (12) and neoxanthin (13) to produce xanthoxin (14), the direct substrate for the synthesis of the phytohormone abscisic acid, ABA (15) (Spurgeon and Porter, 1980; Cunningham et al., 1996a,b; Cunningham and Gantt, 1998; Fraser and Bramley, 2004; Giuliano et al., 2008; Tanaka et al., 2008; Yamamizo et al., 2010) (Fig. 1).

Quantitative and qualitative manipulation of the carotenoid metabolic pathway in plants such as *Arabidopsis* (*Arabidopsis thaliana*), tomato (*Solanum lycopersicum* L.), pepper (*Capsicum* sp.), potato (*S. tuberosum* L.) and algae is now possible (Ducreux et al., 2005; Diretto et al., 2007a,b; Giuliano et al., 2008). The strategy commonly used to regulate carotenoid biosynthesis involves either the expression of a formerly silent enzyme or the modification of key pathway enzymes. For example, seed-specific overexpression of the *PSY* gene increases β-carotene (8), lutein (7), violaxanthin (12), and chlorophyll contents, which cause an increase in the ABA (15) content in *Arabidopsis* (Lindgren et al., 2003). During tomato fruit ripening, the expression of *PSY* and *PDS* increased while the expression of both *LCY-β* and *LCY-ε* (Bramley, 2002) decreased, leading to the accumulation of lycopene (5). In golden rice 2, ectopic expression of genes in a mini-carotenoid biosynthetic pathway containing a maize *PSY* and *Erwinia uredovora* carotene desaturase (*CrtI*) results in the accumulation of β-carotene (8) in rice endosperm up to 31 μg/g dry weight, a level that can fulfill daily vitamin A requirements (Paine et al., 2005). Metabolic engineering of carotenoids by tissue-specific silencing of *LCY-ε* and *CHY-β* enhances β-carotene (8) accumulation in potato tubers (Diretto et al., 2007a,b; Van Eck et al., 2007; Zhu et al., 2009), whereas silencing of *ZEP* (zeaxanthin (10) epoxidase) results in zeaxanthin enhancement in potato (Romer et al., 2002). Lu et al. (2006) reported that the mutated *Orange* (*Or*) gene is related to the accumulation of carotenoids in the chromoplast of the orange cauliflower mutant (Lu et al., 2006). The *Or* transgenic tubers accumulated phytoene (3), phytofluene and β-carotene (8), showing that chromoplast differentiation and regulation of chromoplast formation can have a profound effect on carotenoid accumulation in plants (Lu et al., 2006). Furthermore, β-carotene-enriched calli have been reported in other species. *Or* calli of cauliflower derived from *Or* mutant accumulated predominantly β-carotene (8) with small amount of lutein (7) (Lu et al., 2006). In potato tuber, tissue-specific expression of a bacterial CrtI and CrTY under control of the patatin promoter induced the accumulation of β-carotene (8) (Diretto et al., 2007a). Overexpression of algal β-carotene ketolase in carrot resulted in increased ketocarotenoid levels (Jayaraj et al., 2008).

Major carotenoids found in the diet are β-carotene (8), lycopene (5), and lutein (7) (Krinsky, 1998; Fraser and Bramley, 2004; Haugen and Bjornson, 2009). Their strong antioxidant activity both *in vivo* and *in vitro* has been demonstrated (El-Agamey et al., 2004; Johnson, 2009). The antioxidant activity of carotenoids involves the quenching of singlet oxygen as well as their rapid interaction with peroxyl radicals (ROO) as opposed to unsaturated acyl chains (Burton and Ingold, 1984; Krinsky and Yeum, 2003; El-Agamey et al., 2004). Stress tolerance, an important factor affecting plant yield, can be enhanced to increase food production. Carotenoid-derived products such as ABA can function as plant hormones or as signals of dehydration and salt stress. Furthermore, high carotenoid content increases resistance to abiotic stresses such as dehydration and salt by scavenging reactive oxygen species (ROS) or by acting synergistically with other antioxidants such as vitamins E and C. For example, in tomato, high salt concentrations increase vitamin C, lycopene (5) and β-carotene (8) content by up to 35% and enhance antioxidant capacity (Krauss et al., 2005). In romaine lettuce, a low salt-tolerant plant, long-term salt treatment leads to increased total carotenoid content without eliciting any change in color, and increases the content of lutein (7) and β-carotene (8) by up to 37% and 80%, respectively (Kim et al., 2008). Carotenoid biosynthesis of carrot is more active at high temperatures and under strong light conditions (Skrede et al., 1997; Rosenfeld et al., 1998). Actually, the carotenoid content is higher in spring and summer harvested carrots (Lim et al., 2009). These results show that carotenoids play critical roles in the stress tolerance of many plants, indicating their value as powerful antioxidants and important nutrients.

Metabolic engineering of carotenoids in sweetpotato was carried out using white-fleshed cv. Yulmi (Ym) callus. Because it produces violaxanthin (12) as the main carotenoid, which is down-stream of β-carotene (8), it is a good candidate to control the up-stream pathway. In addition, transgenic cells can be selected by their color variation from white to yellow, which indicates carotenoid accumulation. Furthermore, the generation of transformants in cultured cells is faster than in whole plants. The aims of this study were to induce the accumulation of β-carotene by inhibiting its hydroxylation through the down-regulation of *CHY-β* and to investigate the effects of altered carotenoid levels on antioxidant capacity under salt stress. The transcription of carotenoid biosynthetic genes was analyzed in *CHY-β*-silenced sweetpotato cultured cells. We also measured carotenoids and ABA (15) contents, and 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity in transgenic cultured cells.

2. Results and discussion

2.1. Cloning and transcription level of carotenoid biosynthesis-related genes in sweetpotato

Primer sequences for seven carotenoid biosynthetic genes of sweetpotato were designed using the sequences of *I. nill* which are available at TIGR Plant Transcript Assemblies (http://plantta.jcvi.org/): geranylgeranyl pyrophosphate synthase (GGPS; Plant TA Accession No. TA5597_35883), *PSY* (GenBank accession No. HQ828092), *ZDS* (GenBank accession No. HQ828088), *LCY-β* GenBank accession No. HQ828094, *LCY-β* GenBank accession No. HQ828093), *CHY-β* (GenBank accession No. HQ828095), and *ZEP* (GenBank accession No. HQ828089) were obtained from sweetpotato and sequenced, and these sequences were submitted to the NCBI GenBank database. The partial sequences of the various genes of interest were also next examined: specifically part of *GGPS* showed 95% and 76% sequence homology with those of *I. nil* (Plant TA Accession No. TA5597_35883) and tomato (GenBank accession No. AK325077.1), respectively, whereas *PSY* had 94% sequence homology with that of *Ipomoea* sp. Kenyan (GenBank accession No. AB499050.1). The partial sequence of *ZDS* displayed 92% sequence identity with that of *Ipomoea* sp. Kenyan (GenBank accession No. AB499052.1), whereas that of *LCY-β* showed 99%, 95%, and 83% sequence homology with those of *I. batatas* (GenBank accession No. GQ283003.1), *Ipomoea* sp. Kenyan (GenBank accession No AB499055.1) and pepper (GenBank accession No. GU085272.1), respectively. Additionally, the *LCY-β* path of

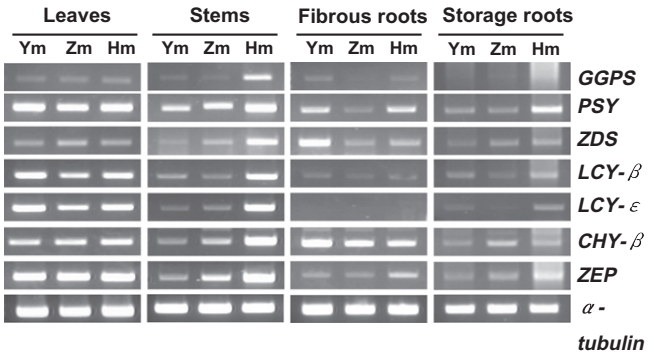

Fig. 2. Expression patterns of carotenoid biosynthesis-related genes in various tissues and cultivars of sweetpotato. Abbreviations: *GGPS*; geranylgeranyl pyrophosphate synthase, *PSY*; phytoene synthase, *ZDS*; ζ-carotene desaturase, *LCY-β*; lycopene β-cyclase, *LCY-ε*; lycopene ε-cyclase, *CHY-β*; β-carotene hydroxylase, *ZEP*; zeaxanthin epoxidase, Ym; Yulmi, Zm; Shinzami, Hm; Shinhwangmi.

sequence had 93% sequence homology with that of *Ipomoea* sp. Kenyan (GenBank accession no AB499054.1), and that of *CHY-β* had 98% sequence homology with that of *I. nil* (GenBank accession No. AB499058.1). The partial sequence of *ZEP* also showed 89% and 79% sequence homology with those of *I. nil* (Plant TA Accession No. TA6491_35883) and tomato (GenBank accession No. EU004202.1), respectively. Finally, the seven carotenoid biosynthesis-related genes were cloned and further used to detect their transcripts in plants and cultured cells of sweetpotato.

The expression profiles of carotenoid biosynthesis-related genes, *GGPS*, *PSY*, *ZDS*, *LCY-β*, *LCY-ε*, *CHY-β*, and *ZEP*, in different tissues of three cultivars of sweetpotato [white-fleshed: Yulmi (Ym), purple-fleshed: Shinzami (Zm), orange-fleshed: Shinhwangmi (Hm)] were investigated (Fig. 2). *GGPS* was very faintly detected in the leaves, fibrous roots, and storage roots of all three cultivars. However, it was strongly expressed in the stems of Hm, but not in the stems of other cultivars such as Ym and Zm. *PSY*, a key regulatory gene in carotenoid biosynthesis, was strongly expressed in the leaves and stems of the three cultivars, with higher expression in the storage roots of Hm. *ZDS* expression was consistently lower, except in stems of Hm and fibrous roots of Ym, where it was strongly expressed. The transcripts of *LCY-β*, *LCY-ε*, and *ZEP* were detected in leaves of all three cultivars and in stems of Hm, but were hardly detected in the fibrous roots or storage roots of the three cultivars. There was no clear difference among cultivars in the expression level of *CHY-β* in leaves, but its expression was elevated in stems of Hm and fibrous roots of Ym. Overall, the expression of carotenoid biosynthesis-related genes in the different tissues of the three cultivars was differentially regulated. The carotenoid contents of the storage roots of three sweetpotato cultivars were then measured. β-Carotene (8) was identified as a major carotenoid of storage roots of sweetpotato. Hm displayed the highest β-carotene (8) content with 282 μg/g dry wt, whereas the levels in Ym and Zm were 10 and 4 μg/g dry wt, respectively (Supplementary Table S1). These results suggest that there is no positive correlation between the expression levels of carotenoid biosynthesis-related genes and carotenoid accumulation. Clotault et al. (2008) also compared carotenoid contents and the expression levels of eight genes encoding carotenoid biosynthesis enzymes during the development of white, yellow, orange, and red carrot roots. All eight genes (*PSY1*, *PSY2*, *PDS*, *ZDS1*, *ZDS2*, *LCY-ε*, *LCY-β1* and *ZEP*) were expressed in the white cultivar even though it did not contain carotenoids. Therefore, these results can be considered hyperactivation of carotenoid catabolism in white cultivar.

2.2. Phenotype and RT-PCR analysis in RNAi-IbCHY-β transgenic Ym callus

Analysis of the carotenoid content and composition of the callus and storage roots of Ym established that β-carotene (8) is one of main carotenoids in storage roots. However, violaxanthin (12) accumulated to >50% of the total carotenoids in the callus. Thus, the Ym callus was selected for increased production of β-carotene (8) from violaxanthin (12) by an RNAi strategy involving down-regulation of β-carotene hydroxylation.

The RNAi-*IbCHY-β* vector was introduced into the Ym callus using *Agrobacterium*-mediated transformation and transgenic calli were selected on hygromycin B (HPT II) containing medium. The

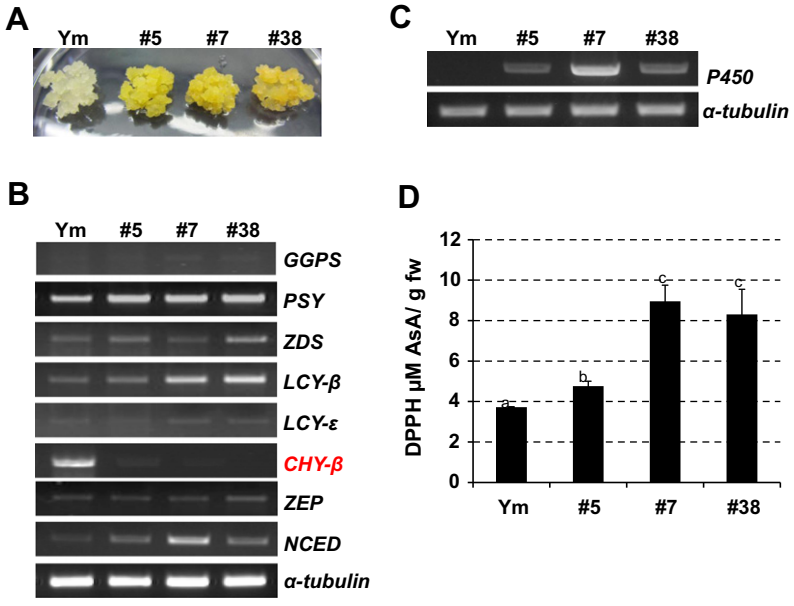

Fig. 3. Comparison of RNAi-*IbCHY-β* transgenic (#5, #7, #38) and non-transgenic sweetpotato calli. (A) Calli phenotypes of RNAi-*IbCHY-β* transgenic sweetpotato calli. (B) Transcript levels of carotenoid biosynthesis-related genes. (C) Transcript levels of the P450 gene. (D) DPPH radical scavenging activity. Ym, non-transgenic callus; #5, #7 and #38, transgenic callus line.

presence of the transgene was confirmed by PCR from genomic DNA using HPT II primers (data not shown). The resulting RNAi-*IbCHY-β* transgenic callus had a faint orange color, whereas the non-transgenic (NT) Ym callus had a faint yellow color (Fig. 3A). Down-regulation of *CHY-β* gene expression in transgenic callus was assessed by RT-PCR (Fig. 3B), which confirmed the successful development of RNAi-*IbCHY-β* transgenic sweetpotato cells. Finally, three transgenic lines (#5, #7, #38) showing significant down-regulation of *CHY-β* expression were selected for further study.

The transcript level of carotenoid biosynthesis-related genes in both the transgenic and NT Ym callus was compared (Fig. 3B, Supplemental Fig. S1A). *PSY* showed a higher expression level in transgenic cells than in NT cells. However, *ZDS*, *LCY-ε*, and *ZEP* expression in the transgenic callus were not changed. Interestingly, expression of *LCY-β* in the RNAi-*IbCHY-β* callus (lines #7 and #38) was slightly increased, consistent with previous observations of increased expression of *LCY-β* in *CHY-β*-silenced potato tubers (Diretto et al., 2007b; Giuliano et al., 2008). Because *LCY-β* converts both lycopene (5) to β-carotene (8) and δ-carotene to α-carotene (6), down-regulation of *CHY-β* caused β-carotene (8) accumulation, which may overflow into the α-carotene (6) pathway. Overall, the down-regulation of *CHY-β* in transgenic callus did not produce notable changes in the expression levels of other carotenoid biosynthesis genes, except for *PSY*, *LCY-β* and *NCED*. *NCED* showed a slightly higher expression level in transgenic cells than in NT cells suggesting the possibility of increased ABA content.

2.3. Carotenoid content in RNAi-IbCHY-β transgenic Ym callus

The carotenoid content of RNAi-*IbCHY-β* callus lines was analyzed by HPLC. In Ym calli, the total content of carotenoids was 6.50 μg/g dry wt, with violaxanthin (12) representing more than 51.8% (3.37 μg/g dry wt) followed by lutein (7) and β-carotene (8). Meanwhile, RNAi-*IbCHY-β* transgenic callus (lines #5, #7, #38) showed alterations in the composition and levels of carotenoids (Table 1). Silencing of *IbCHY-β* resulted in a marked increase (38-fold) in β-carotene (8), making it the major cellular carotenoid (ca. 30% of the total carotenoids in lines #7 and #38). With regard to xanthophylls, the content of β-carotene (8) down-stream intermediates zeaxanthin (10) and violaxanthin (12) increased 4.3–15-fold; however the proportions of both zeaxanthin (10) and violaxanthin (12) in total carotenoids were reduced from 7% to 2% and 51% to 26%, respectively. Also, in the two ε-β-branches, the α-carotene (6) content in transgenic cells was 3.6–5.2-fold higher in the transgenic lines. This was probably due to the elevated *LCY-β* expression level in transgenic cells (Fig. 3B, Supplemental Fig. S1A). The increased α-carotene (6) content is in agreement with the results of CHY1 and CHY2 silencing in potato (Diretto et al., 2007b). On the other hand, the lutein (7) levels differed according to the cell line and increased significantly in line #7 (8.6-fold) and moderately in line #5 (1.5-fold), but it decreased to an undetectable level in line #38. Interestingly, silencing of *IbCHY-β* led to a massive accumulation of β-cryptoxanthin (9) (up to 300-fold). This result is important because in citrus, corn and pea plants, it is a well-known antioxidant like lycopene (7) and β-carotene (8); it acts as a radical scavenger in certain biomolecules including lipids, proteins and nucleic acids (Johnson, 2009). The functions of β-cryptoxanthin (9) include pro-vitamin A activity and the stimulation of the repair of DNA oxidation damage and antioxidant activity in human cells (Lorenzo et al., 2009).

Over the past decade, several studies have reported that metabolic engineering of carotenoids by tissue-specific silencing of *LCY-ε* and *CHY-β* enhanced β-carotene (8) accumulation in potato tubers (Diretto et al., 2007a,b). This potato showed increased contents of β-carotene (8), phytofluene, violaxanthin (12), neoxanthin (13) and lutein (7) except zeaxanthin (10). However, the *IbCHY-β* transgenic callus showed an increased zeaxanthin (10) content despite the fact that its fraction decreased from 7.3% to 2.9%. One explanation for the increased β-cryptoxanthin (9) and zeaxanthin (10) contents in transgenic cells could be the presence of a different hydroxylase that can add a hydroxyl group to the β-ring of carotenoids. The cytochrome P450 mono-oxygenase such as *CYP97A* in *Arabidopsis* is known to encode a β-carotene hydroxylase that is primarily responsible for hydroxylation of α and β-carotenes (6) and (8) (Kim and DellaPenna, 2006; Kim et al., 2009). There do not appear to be other genes related to β-carotene hydroxylation in the *Arabidopsis* genome; presumably homologues of a cytochrome P450 mono-oxygenase in transgenic cells is the candidate for β-carotene (8) hydroxylation responsible for increasing zeaxanthin (10) content. To support this hypothesis, we isolated cytochrome P450 hydroxylase in *I. batatas* (*IbP450*) (Supplemental Table S2). The transcription level of *IbP450* was higher in the *IbCHY-β* transgenic callus than in NT cells. This result indicated that both *IbCHY-β* and P450 might be functionally redundant for the β-ring hydroxylase (Fig. 3C) and that the *IbCHY-β* down-regulated transgenic callus has increased P450 expression to compensate for the loss of *CHY-β* activity. Thus, to completely down-regulate β-carotene hydroxylation, it is necessary to knock-out both β-carotene hydroxylase and the P450 mono-oxygenase together. Although β-carotene (8) is a very rare compound in Ym callus, it was clearly shown that Ym cells can accumulate β-carotene (8) to remarkable levels. This is the first direct evidence demonstrating metabolic engineering of carotenoid biosynthesis related genes in a sweetpotato culture cell system, which is further applicable to the production of valuable components including various antioxidants and carotenoids.

2.4. Antioxidant activity in RNAi-IbCHY-β transgenic Ym callus

Carotenoids from various sources are receiving increasing attention due to the desirable physiological functions associated with their antioxidant properties. To investigate antioxidant activity as a result of increased carotenoid contents in transgenic callus, the DPPH radical scavenging activity of *IbCHY-β* transgenic sweetpotato callus was assessed. *IbCHY-β* transgenic callus displayed 2.1–2.3-fold higher antioxidant activity than NT cells (Fig. 3D). This result correlated well with the carotenoid content of the cells, and provided evidence that higher carotenoid content is related to higher DPPH radical scavenging activity.

Table 1
Carotenoid content of non-transgenic and transgenic sweetpotato calli (μg/g dry wt).

Line	α-Carotene	Lutein	β-Carotene	β-Cryptoxanthin	Zeaxanthin	Violaxanthin	Total carotenoids
Ym	0.42 ± 0.04	1.23 ± 0.14	0.90 ± 0.02	0.10 ± 0.06	0.48 ± 0.07	3.37 ± 0.29	6.50 ± 0.69
#5	1.85 ± 0.73	1.85 ± 0.06	6.70 ± 0.81	9.03 ± 0.69	2.05 ± 0.12	49.74 ± 1.05	71.21 ± 3.46
#7	2.21 ± 0.28	10.58 ± 1.46	34.43 ± 2.22	29.70 ± 3.68	7.30 ± 2.02	33.63 ± 4.54	117.85 ± 24.83
#38	1.53 ± 0.03	ND	14.76 ± 0.42	12.63 ± 0.42	7.46 ± 0.12	13.45 ± 1.08	49.83 ± 8.46

Each carotenoid amount was measured by HPLC analysis with a minimum of five different calli. All content levels were expressed as the mean (μg/g dry wt) ± SD of two independent determinations. Ym, non-transgenic callus; #5, #7 and #38, transgenic callus line.

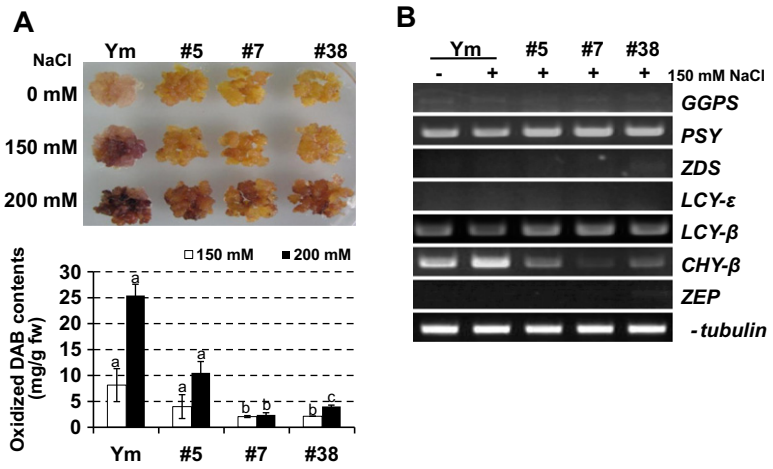

Fig. 4. Salt treatment of RNAi-*IbCHY-β* transgenic and non-transgenic sweetpotato calli. (A) H_2O_2 production after 24 h of treatment with 150 and 200 mM NaCl. Ym and RNAi-*IbCHY-β* transgenic sweetpotato calli were treated with a DAB-HCl solution and oxidized DAB content was compared. (B) Transcript levels of carotenoid biosynthesis-related genes after treatment with 150 mM NaCl. Ym, non-transgenic callus; #5, #7 and #38, transgenic callus line.

2.5. Salt stress in RNAi IbCHY-β transgenic Ym callus

Recently, *SeLCY* (lycopene β-cyclase from *Salicornia europaea* L.) transgenic *Arabidopsis* plants have shown improved salt tolerance through an increase in carotenoid content, which impairs ROS and H_2O_2 production under salt stress (Chen et al., 2011). Additionally, several reports showed that salt treatment activated biosynthesis of carotenoids and enhanced ABA (15) biosynthesis in *Arabidopsis*, maize, and rice (Li et al., 2008, 2010; Lindgren et al., 2003).

To investigate whether increased carotenoid content affects osmotic stress under salt stress conditions, the antioxidant capacity was measured. H_2O_2 production in both the NT Ym and transgenic callus under salt stress was visually detected by adding 3,3,-diaminobenzidine (DAB)-HCl soln. to the cells. In cells treated with 150 mM NaCl, dark-brown cells were barely detectable in the transgenic callus, whereas more than 70% of the cells in the NT Ym callus were dark-brown. In 200 mM NaCl, more than 30% of the cells in the transgenic callus retained a yellow color, whereas most cells in the Ym callus were dark-brown. These results indicate that the amount of H_2O_2 in NT cells was much greater than in transgenic cells at both NaCl concentrations (Fig. 4A). To quantify H_2O_2 production, the level of oxidized DAB was calculated from the absorbance at 460 nm using a standard curve. In the cells treated with 150 mM NaCl, the oxidized DAB content of NT cells and transgenic cells was 7 and 2–4 mg/g fr. wt, respectively. After treatment with 200 mM NaCl, H_2O_2 production by the NT Ym callus was more than 5-fold higher than that of the transgenic callus (Fig. 4A). These results indicated that increased β-carotene (8) content attenuates the effects of salt stress by eliminating ROS.

To investigate changes in carotenoids under high salt conditions, the carotenoid contents of NT and transgenic cells were compared (Table 2). In NT Ym cells, total carotenoids and β-carotene (8) decreased 0.5- and 0.8-fold, respectively. Similarly, in RNAi-*IbCHY-β* transgenic cells, total carotenoids decreased 0.4–0.8-fold. Loss of carotenoids under salt stress has been reported in other plants where it was found to be related to photoinhibition or formation of ROS by lipid peroxidation (Aghaleh et al., 2009; Kato and Shimizu, 1985; Parida et al., 2002).

The expression patterns of seven carotenoid biosynthesis-related genes in salt-treated cells were also compared by RT-PCR (Fig. 4B, Supplemental Fig. S1B). There was no clear difference in the expression of *GGPS*, *PSY*, *ZDS*, *LCY-ε*, *LCY-β* and *ZEP* between NT and transgenic cells. Interestingly, the *CHY-β* transcript was detected in salt-treated transgenic callus, although its level was greatly reduced compared to that in NT cells. The representative PCR product was cloned and confirmed to be a *CHY-β* transcript. Because *CHY-β* expression increased as a consequence of salt

Table 2
Carotenoid content of salt-treated non-transgenic and transgenic sweetpotato calli (μg/g dry wt).

Line	Treatment	α-Carotene	Lutein	β-Carotene	β-Cryptoxanthin	Zeaxanthin	Violaxanthin	Total carotenoids
Ym	Control	0.42 ± 0.04	1.23 ± 0.14	0.90 ± 0.02	0.10 ± 0.06	0.48 ± 0.07	3.37 ± 0.29	6.50 ± 0.69
	NaCl	ND	0.98 ± 0.21	0.79 ± 0.21	ND	ND	1.36 ± 0.19	3.13 ± 0.71
		–	(0.79)[a]	(0.87)	–	–	(0.40)	(0.48)
#5	Control	1.85 ± 0.73	1.85 ± 0.06	6.70 ± 0.81	9.03 ± 0.69	2.05 ± 0.12	49.74 ± 1.05	71.21 ± 3.46
	NaCl	1.19 ± 0.05	ND	7.09 ± 0.56	4.04 ± 0.84	1.79 ± 0.24	21.67 ± 1.97	35.78 ± 7.47
		(0.64)	–	(1.15)	(0.44)	(0.87)	(0.43)	(0.50)
#7	Control	2.21 ± 0.28	10.58 ± 1.46	34.43 ± 2.22	29.70 ± 3.68	7.30 ± 2.02	33.63 ± 4.54	117.85 ± 24.83
	NaCl	1.29 ± 0.04	3.42 ± 0.45	17.87 ± 0.27	7.92 ± 1.19	0.79 ± 0.06	13.77 ± 2.10	45.06 ± 6.00
		(0.58)	(0.32)	(0.51)	(0.26)	(0.10)	(0.40)	(0.38)
#38	Control	1.53 ± 0.03	ND	14.76 ± 0.42	12.63 ± 0.42	7.46 ± 0.12	13.45 ± 1.08	49.83 ± 8.46
	NaCl	1.36 ± 0.09	ND	18.30 ± 2.66	9.09 ± 0.10 (0.71)	ND	13.57 ± 0.47	42.32 ± 4.69
		(0.88)	–	(1.23)	(0.71)	–	(1.00)	(0.84)

Each carotenoid amount was measured by HPLC analysis with a minimum of five different calli. All content levels were expressed as the mean (μg/g dry wt) ± SD of two independent determinations. Ym, non-transgenic callus; #5, #7 and #38, transgenic callus line.

[a] Fold variation (NaCl/control).

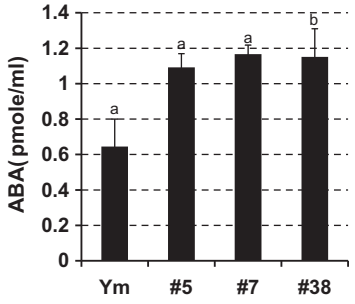

Fig. 5. ABA contents in RNAi-*IbCHY-β* transgenic (#5, #7, #38) and non-transgenic calli. ABA concentration in non-transgenic Ym and RNAi-*IbCHY-β* calli grown on MS1D. Data represent the means of three biological replicates each containing multiple calli. Ym, non-transgenic callus; #5, #7 and #38, transgenic callus line.

treatment in NT cells, *CHY-β* expression in transgenic cells might be also induced by salt treatment exceeding the down-regulation level controlled by the CaMV 35S promoter.

2.6. ABA contents in transgenic sweetpotato cell lines

Based on the result showing that the expression level of *NCED* was higher in transgenic callus than in NT Ym callus (Fig. 3B, Supplemental Fig. S1A) and that transgenic cells showed the greatest tolerance to the presence of NaCl, we compared ABA (15) contents in both transgenic and NT calli. ABA (15) contents were 0.64 and 1.20 pmol/ml in NT and transgenic calli, respectively (Fig. 5). This result also explains why RNAi-*IbCHY-β* calli showed salt stress tolerance. Because ABA (15) is a well known abiotic stress signal in plants (Li et al., 2010; Lindgren et al., 2003; Yang and Guo, 2007), it is possible to develop abiotic stress tolerance transgenic plants through the metabolic engineering of carotenoid biosynthesis.

Overall, the salt stress tolerance of the transgenic callus can be attributed to the synergistic effects of the β-carotene (8) antioxidant activity and increased ABA (15) biosynthesis, which is involved in multiple stress responses.

3. Conclusion

To increase β-carotene (8) content in sweetpotato, cultured cells were generated containing a silenced *IbCHY-β* gene. These cells had increased β-carotene (8) and total carotenoid contents (a maximum of 117 μg/g dry wt). In addition, *IbCHY-β* cells had shown elevated levels of zeaxanthin (10) and other xanthophylls (such as β-cryptoxanthin (9)) contents accompanied by strong upregulation of P450 gene, which encodes a β-carotene hydroxylase. Transgenic cell lines also showed enhanced antioxidant capacity such as elevated DPPH radical scavenging activity. RNAi-*IbCHY-β* transgenic calli accumulated more ABA content. As a consequence of all that, transgenic calli exhibited enhanced tolerance to salt stress. The present study is the first to report significant carotenoid accumulation and increased antioxidant activity following the down-regulation of *IbCHY-β* gene expression in a plant cell culture system. It is suggested that enhanced levels of carotenoids contribute to salt stress tolerance in transgenic cells.

4. Experimental

4.1. Plant materials

Three cultivars [*I. batatas* cv. Yulmi (Ym), Shinzami (Zm), Shinhwangmi (Hm)] of sweetpotato were used in this study: Ym, Zm, and Hm denote white-, purple-, and orange-fleshed sweetpotatoes, respectively. Sweetpotato plants were grown for 50 d in a growth chamber at 25 ± 1 °C. The maximum irradiance was approximately 10,500 μmol m^{-2} s^{-1} and the mean day length was 16 h. To induce callus formation in sweetpotato, small pieces of the storage roots of Ym were surface-sterilized with EtOH-H_2O (70:30, v/v) for 30 s, and then with 1% NaOCl for 20 min. After rinsing three times with sterile H_2O, callus induction was initiated by placing small pieces directly onto plates of MS1D medium containing MS salts supplemented with 30 g/l sucrose, 1 mg/l 2,4-D, 100 mg/l myo-inositol, 4 mg/l thiamin-HCl (pH 5.8), and 3 g/l gelrite at 25 °C in the dark. Callus proliferation was carried out on fresh MS1D plates and calli were maintained in media for later use.

4.2. Construction of RNAi vector

To clone a partial *IbCHY-β* cDNA from sweetpotato, primers for *CHY-β* were synthesized based on the sequence of the *I. nill CHY-β* gene (GenBank accession No. AB499058) from the NCBI database (http://www.ncbi.nlm.nih.gov/). A partial *IbCHY-β* fragment was amplified from the cDNA of storage roots of sweetpotato (cv. Hm) by RT-PCR using Advantage 2 pfu DNA polymerase mix (Clontech, Tokyo, Japan) with a *CHY-β* primer set (5′-GTTTACTGTTTAGTCCTTTAAGTCG-3′, 5′-AACATCTCAGTATATGGAACTTCTC-3′). The PCR product was then cloned into the pGEM-TEasy vector (Promega, Madison, WI, USA) and sequenced. For construction of the RNAi-*IbCHY-β* vector, *IbCHY-β* primers were designed from the sequence of a partial fragment of *IbCHY-β* containing restriction enzyme sites [(*Bam*HI and *Xho*I underlined) 5′-GGATCCAAGTTCCGTATACTGAGATGTTCG-3′, 5′-CTCGAGCACTCTCCTAAAATAAGGCACGTC-3′]. PCR was performed in 20 μl of reaction solution containing 1 μg of cDNA template and 0.1 μM of *CHY-β* primers with an initial denaturation step at 95 °C for 2 min followed by 30 cycles of denaturation at 95 °C for 20 s, annealing at 58 °C for 30 s, polymerization at 72 °C for 30 s, and a final extension at 72 °C for 10 min. The PCR product was cloned into a pGEM-TEasy vector, and the resulting pGEM::*IbCHY-β* vector was digested with *Bam*HI and *Xho*I restriction enzymes and ligated into the pENTR11 vector, which had already been digested by the same enzymes. The pENTR11-*IbCHY-β* clones were integrated by site-specific recombination using the LR Clonase enzyme mix (Invitrogen, Carlsbad, CA, USA) into the plant RNAi expression vector pH7GWIWG2(I) containing the cauliflower mosaic virus (CaMV) 35S promoter. The resulting expression construct was used for transformation into *Escherichia coli* DH5α strains. Transformants were selected on LB agar plates containing 50 mg/l spectinomycin/streptomycin.

4.3. Transformation into sweetpotato callus

The EHA105 strain of *Agrobacterium tumefaciens* was transformed with the pH7GWIWG2(I)-*IbCHY-β* construct by the freeze–thaw method (Hofgen and Willmitzer, 1988). Transformed *A. tumefaciens* was grown overnight on a shaker at 200 rpm containing 200 μM acetosyringone (AS) and 250 mg/l spectinomycin/streptomycin in YEP liquid media at a temperature of 28 °C. Transformed *A. tumefaciens* and calli (Ym) were mixed in co-culture medium (MS1D + 100 μM AS), gently shaken for 30 min, and then sonicated for 1 min. Calli were then transferred to sterile filter paper and blotted dry. The calli were placed on MS1D plates containing 100 μM AS and cultivated in the dark at 28 °C for 4 days, after which they were rinsed five times with sterile H_2O, transferred to sterile filter paper and blotted dry. The calli were transferred to 1DCH (MS1D + cefotaxime 400 mg/l + 5 mg/l hygromycin) medium, and then subcultured on fresh medium at 3-week intervals for selection.

Table 3
Primer sequences of carotenoid biosynthesis-related genes of sweetpotato.

Target gene	Sequence	Direction	Amplicon size (bp)
GGPS	AGTAGGTGTGTTGTATCAAGTTGT	FOR	308
	AACAGGTAAGAGCATATAGTGTAGC	REV	
PSY	TATTTACCTCAAGAGAATTAGCTC	FOR	399
	TCAGCTTCTTCAGTACAGTATTACA	REV	
ZDS	GGTGTTATACAAAACACGATTACAT	FOR	302
	AAAGGAAAAGAGAAGAGAAGAACTA	REV	
LCY-ε	GAAAATTGTACGTATATATCGACTTC	FOR	365
	TAGTTATTTGTGAAAGGAAGATCAG	REV	
LCY-β	TAGATATGAAGGATATTCAGGAAAG	FOR	358
	AGTAGAATATCCATACCAAAACACA	REV	
CHY-β	GTTACTGTTTAGTCCTTTAAGTCG	FOR	334
	AACATCTCAGTATATGGAACTTCTC	REV	
ZEP	GTAGTAAACATGGTACTTGGATCAC	FOR	355
	CATTTCTAGTGATTTCTTGTTCTG	REV	
NCED	GGGAAGATCCCGGAGTGTAT	FOR	381
	GTGGTACGGCAAATCGTCTT	REV	
P450	CAGCCAGGGAAATGGTGAGT	FOR	606
	CAGCAAGTGTGCAAAAGGAG	REV	
α-Tubulin	CAACTACCAGCCACCAACTGT	FOR	220
	CAAGATCCTCACGAGCTTCAC	REV	

4.4. RNA isolation and RT-PCR analysis

Non-transgenic (NT) and transgenic callus tissues were ground in N_2 with a mortar and pestle, and total RNA was extracted with an Easy-Spin™ total RNA extraction kit (iNtRON, Daejeon, Korea) according to the manufacturer's instructions. First-strand cDNA synthesis was performed in a 20 μl reaction mixture containing 1 μg of total RNA, 0.5 μg oligo dT, 40 μM of each of the four dNTPs, 2 μl 10× buffer, 200 units of M-MLV reverse transcriptase, and 20 units of RNase inhibitor (MBI-Fermentas, St. Leon-Rot, Germany). The reaction mixture was incubated at 42 °C for 60 min and stopped by heating at 65 °C for 10 min. PCR amplification reactions were incubated initially for 3 min at 94 °C, followed by 30 cycles at 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s using each of the gene-specific primers. To determine the expression patterns of genes, reverse transcription PCR using gene-specific primers was performed using the *α-tubulin* gene (GenBank accession No. DV037573) transcript level as an internal standard. A 10 μl aliquot of each RT-PCR product was analyzed on a 1.5% (w/v) agarose gel to visualize amplified cDNAs. The primers used in this study are shown in Table 3.

4.5. Quantitative analysis

The densitometry data corresponding to the band intensities of different sets of experiments was generated by analyzing the gel images on the Image J program (Version 1.33, USA http://rsb.info.nih.gov/ij/).

4.6. Carotenoid analysis

Carotenoids were extracted from calli and analyzed by HPLC, according to the method of Lim et al. (2009). All extraction procedures were conducted under subdued light to avoid degradation and loss of the pigments. Lyophilized callus (100 mg) was homogenized using a pre-chilled mortar and a pestle with acetone (3 ml, 0.01% butylated hydroxytoluene, BHT), sea sand, Na_2SO_4, and $NaHCO_3$. The sample was ultrasonicated twice with acetone (15 ml, 0.01% BHT) for 10 min. The extract was then centrifuged at 3075×*g*, at 4 °C for 5 min, after which the supernatant was concentrated *in vacuo*, and dissolved in CH_2Cl_2:acetone (1:1, 200 μl). The sample solution was filtered through a 0.45 μm membrane filter (Whatman, PTFE, 13 mm) and then subjected to HPLC analysis using an Agilent 1100 HPLC system (Hewlett–Packard, Palo Alto, CA, USA) that consisted of a temperature controlled autosampler, column oven, and a binary pump. A standard or sample soln. (20 μl) was injected directly onto a YMC C_{30} carotenoid column (3 μM, 4.6 × 250 mm) with solvent A [MeOH– *tert*-butylmethyl ether (MTBE)–H_2O (81:15:4)] and solvent B [MeOH–MTBE–H_2O (6:90:4)] using a step gradient elution of 100% solvent A for the first 15 min, then 100% solvent A to 100% solvent B over the next 35 min. A conditioning phase (50–60 min) served to return the column to its initial state. The flow rate was 0.7 ml/min and the column temperature was 22 °C. The eluent was detected at 450 nm using an UV–Visible detector. Chemstation software (Hewlett–Packard) was used to operate the HPLC-DAD system. Carotenoids were quantified using an external calibration method. Briefly each standard (1 mg) was dissolved in CH_2Cl_2 (10 ml containing 0.01% BHT). Working calibration solns. (50, 20, 10, 5.0, 2.5, 1.0, 0.50, 0.25, 0.10, and 0.025 μg/ml) were then prepared by diluting stock solns. of the external standards. Standards of α-carotene (6), β-carotene (8), β-cryptoxanthin (9), lutein (7), violaxanthin (12), and zeaxanthin (10) were purchased from CaroteNature (Lupsingen, Switzerland). Under these chromatographic conditions, standard carotenoids produced peaks at t_R (min) values of 11.5 for violaxanthin (10), 23.3 for lutein (9), 26.6 for zeaxanthin (10), 33.5 for β-cryptoxanthin (9), 37.3 for α-carotene (6), and 39.2 for β-carotene (8).

Carotenoid contents were measured by HPLC analysis with a minimum of five different calli. All content levels were expressed as the mean (the average content in g dry weight) ± SD (the standard deviation) of two independent determinations.

4.7. ABA (15) extraction and measurement

The ABA (15) extraction from cells was performed as previously described (Artsaenko et al., 1995) with some modifications. RNAi-*IbCHY-β* and NT Ym 3-week-old calli grown in MS1D medium were harvested, frozen in N_2, and ground to a powder. ABA (15) was extracted by suspending powdered sample (400 mg) with acetone-H_2O (4 ml, 80:20, v/v) overnight at 4 °C in the dark. The suspension was centrifuged at 2000*g* for 10 min, and the supernatant was transferred to a clean tube. The supernatant was diluted 1:100 with TBS (trizma base 3.03 g/l, NaCl 5.84 g/l, magnesium chloride hexahydrate 0.20 g/l, NaN_3 0.20 g/l). ABA (15) concentrations were then determined with a Phytodetek ABA enzyme immunoassay test kit (Agdia Inc., Elkhart, USA). Absorbance was detected with the i-Mark Microplate Reader (BIO-RAD, CA, USA) at 405 nm. Two independent experiments were performed, and the same results were obtained.

4.8. Antioxidant activity and DAB staining assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity of the cells was measured as previously described (Pieroni et al., 2002) with some modifications. Transgenic and NT calli (ca. 200 mg) were ground in MeOH (1 ml). Samples were centrifuged at 15000×*g* for 5 min. and the supernatant was collected for analysis. A volume of each extract (100 μl) was added to 0.5 mM DPPH in MeOH (750 μl). The mixture was shaken vigorously and left in the dark at room temperature for 30 min. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm against a blank (MeOH). L-Ascorbic acid (AsA, 0.015–0.125 mM) was used as the standard for the calibration curve, and DPPH radical scavenging activity was expressed as the mole AsA equivalent per gram of tested sample.

To measure cellular H_2O_2 contents, each callus was placed in a 1 mg/ml solution of 3,3′-diaminobenzidine (DAB)-HCl (pH 3.8) for 5 h at 25 °C under continuous light (Chadwick et al., 1995). DAB

was oxidized by H_2O_2 to a dark brown color. Oxidized DABs were visualized in cells as dark-polymerization products resulting from the reaction of DAB with H_2O_2. For quantification of H_2O_2 production, the DAB solution was separated from the callus and used for measuring absorbance at 460 nm. Oxidized DAB content was calculated from the standard curve of DAB.

4.9. NaCl treatments

Transgenic and NT callus were treated with MSID liquid medium containing 150 and 200 mM NaCl for 24 h and then harvested.

4.10. Statistical analysis

Data were analyzed by one way analysis of variance (ANOVA). The subsequent multiple comparisons were examined based on the least significant difference (LSD) test. All statistical analysis was performed using the Statistical Package for the Social Sciences (SPSS 12) and statistical significance was set at $P < 0.05$ and 0.01.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2011.11.003.

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