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Regulatory control of high levels of carotenoid accumulation in potato tubers

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ABSTRACT

Potato (*Solanum tuberosum* L.) tubers contain a wide range of carotenoid contents. To decipher the key factors controlling carotenoid levels in tubers, four potato lines (Atlantic, Désirée, 91E22 and POR03) were examined by a combination of biochemical, molecular and genomics approaches. These lines contained incremental levels of carotenoids, which were found to be associated with enhanced capacity of carotenoid biosynthesis as evident from norflurazon treatment. Microarray analysis of high and low carotenoid lines (POR03 versus Atlantic) revealed 381 genes that showed significantly differential expression. The carotenoid metabolic pathway genes β-*carotene hydroxylase 2* (*BCH2*) and β-*carotene hydroxylase 1* (*BCH1*), along with *zeaxanthin epoxidase* (*ZEP*), and *carotenoid cleavage dioxygenase 1A* (*CCD1A*) were among the most highly differentially expressed genes. The transcript levels of *BCH2* and *BCH1* were lowest in Atlantic and highest in POR03, whereas those of *ZEP* and *CCD1A* were high in low carotenoid lines and low in high carotenoid lines. The high expression of *BCH2* in POR03 line was associated with enhanced response to sugars. Our results indicate that high levels of carotenoid accumulation in potato tubers were due to an increased metabolic flux into carotenoid biosynthetic pathway, as well as the differential expression of carotenoid metabolic genes.

Key-words: *BCH2* promoter; carotenoids; microarray; potato; sugar response.

INTRODUCTION

Carotenoids are indispensable for human nutrition and health. They provide precursors of vitamin A and offer protection against certain chronic diseases, such as cancer, heart diseases and age-related eye diseases (Fraser & Bramley 2004). Humans cannot make carotenoids from endogenous precursors and only obtain them from diet. Therefore, development of carotenoid-enriched food crops will maximize the nutritional and health benefits of carotenoids to humans. A comprehensive understanding of the regulation of carotenoid accumulation is critical in providing novel strategies for such endeavour.

Carotenoids accumulate in many flowers, fruits and vegetables, giving yellow, orange and red colours. Carotenoid accumulation in these tissues and organs is the net result of biosynthesis, degradation and stable storage of synthesized products (Lu & Li 2008; Cazzonelli & Pogson 2010). Multiple factors control the broad diversity of carotenoid composition and content in storage tissues. Regulation of the catalytic activity of carotenoid biosynthesis (Fig. 1) is generally recognized to be important in controlling the final levels of carotenoid accumulation. Phytoene synthase (PSY) is the rate-limiting step in the carotenoid biosynthetic pathway and manipulation of PSY expression in many plants has been demonstrated to enhance carotenoid synthesis by directing metabolic flux into the carotenoid biosynthetic pathway (Shewmaker *et al*. 1999; Fraser *et al*. 2002; Ducreux *et al*. 2005). Transcriptional regulation of carotenogenic gene expression has been shown to be important in controlling specific carotenoid accumulation in some cases (Ronen *et al*. 1999; Harjes *et al*. 2008) but not in others (Li *et al*. 2001). A family of carotenoid cleavage dioxygenases (CCDs) catabolizes enzymatic degradation of carotenoids. Expression of these genes inversely regulates carotenoid accumulation (Kato *et al*. 2006; Ohmiya *et al*. 2006; Campbell *et al*. 2010). In addition, regulation of stable storage in organelles represents another level of control. Induction of chromoplast formation and increase of chromoplast compartment size and number also impose a strong effect on final carotenoid levels in storage tissues (Liu *et al*. 2004; Lu *et al*. 2006; Galpaz *et al*. 2008).

The growing interest in having foods enriched with carotenoids have prompted the recent and significant progress in breeding and metabolic engineering of carotenoids in crops (Davuluri *et al*. 2005; Taylor & Ramsay 2005; Giuliano *et al*. 2008; Naqvi *et al*. 2010; Yan *et al*. 2010). Potato as the world's fourth largest crop generally contains low levels of tuber carotenoids, especially β-carotene, the most potent provitamin A carotenoid. The main carotenoids present in cultivated potato tubers include lutein, zeaxanthin and

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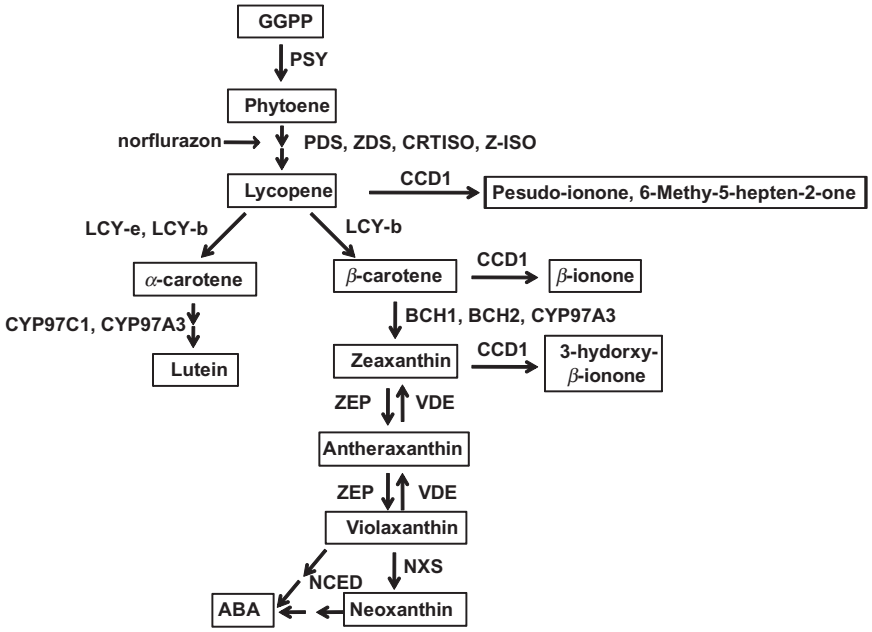

Figure 1. Carotenoid biosynthesis pathway in plants. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoids isomerase; Z-ISO, 15-*cis*-ζ-carotene isomerase; LCY-e, lycopene ε-cyclase; LCY-b, lycopene β-cyclase; BCH1, β-carotene hydroxylase 1; BCH2, β-carotene hydroxylase 2; CYP97C1, cytochrome P450-type carotene ε-hydroxylase; CYP97A3, cytochrome P450-type monooxygenase; ZEP, zeaxanthin epoxidase; VDE, violaxanthin deepoxidase; NXS, neoxanthin synthase; NCED, 9-*cis*-epoxycarotenoid dioxygenase; CCD1, carotenoid cleavage dioxygenase 1. Norflurazon inhibition site is indicated.

violaxanthin (Burgos *et al*. 2009). Over the past years, a large effort has taken place to alter carotenoid content and composition in this important food crop. 'Golden' potato tubers with enhanced β-carotene content have been generated through expression of a carotenoid mini-pathway, including *PSY*, a bacterial *phyotene desaturase* (*CrtI*) and *lyopene* β-*cyclase* (*LYC-b*) (Diretto *et al*. 2007, 2010). Tuber-specific overexpression of *PSY* leads to increased β-carotene and total carotenoid levels (Ducreux *et al*. 2005). Conversely, silencing of β-*carotene hydroxylase* (*BCH*), *zeaxanthin epoxidase* (*ZEP*) and *CCD4* results in increased β-carotene, zeaxanthin or violaxanthin content in tubers (Romer *et al*. 2002; Van Eck *et al*. 2007; Campbell *et al*. 2010). Enhancement of sink strength by expression of the cauliflower *Or* mutant gene increases β-carotene and total carotenoids in tubers (Lu *et al*. 2006; Lopez *et al*. 2008). Moreover, potato varieties with high carotenoid levels have been generated through traditional breeding (Brown *et al*. 2008; Kobayashi *et al*. 2008).

Genetic studies of the inheritance of carotenoids in potato tubers have identified the *Y* locus in chromosome 3 as the major regulatory allele, controlling yellow and white flesh colour of tubers (Bonierbale, Plaisted & Tanksley 1988). The *Y* gene has been associated with *BCH* and additional modifying genes are suggested to be involved in controlling total carotenoid levels (Brown *et al*. 2006). Indeed, *BCH* was found to be responsible for a major quantitative trait locus for flesh colour (Kloosterman *et al*. 2010). By association analysis of potato natural diversity, the yellow-fleshed tubers which accumulate high levels of zeaxanthin were found to be controlled by a combination of the dominant *BCH2* allele and homozygous recessive *ZEP* allele (Wolters *et al*. 2010). A recent study also reveals the association of low expression of *CCD4* with carotenoid content in yellow-fleshed tubers (Campbell *et al*. 2010).

To gain a better understanding of the control of carotenoid accumulation in potato tubers, we examined carotenogenesis in four potato cultivars (Atlantic, Désirée, 91E22 and POR03) that contain undetectable to high levels of carotenoids. Genome-wide identification of genes responsible for carotenoid accumulation was conducted by comparative microarray analysis of the expression profiles of Atlantic (negligible amount of carotenoids) and POR03 (high carotenoids) tubers. A number of genes associated with carotenoid metabolism were identified from approximately 12 000 spots and *BCH2* was found to be the most highly differentially expressed gene. To dissect the regulatory control of carotenoid biosynthetic gene expression in tubers, we isolated the promoters of *BCH2* from potato lines and examined the effect of sugars in activating *BCH2* expression.

MATERIALS AND METHODS

Plant materials

Potato tetraploids (*Solanum tuberosum* L.) Atlantic and Désirée, as well as diploids 91E22 (Brown *et al*. 2006) and POR03 were used in this study. POR03 (POR03PG6-3) was a selection from the cross EGA9403-4 × 91E22. EGA9403-4 was a diploid selection from true seeds obtained from the Polish Parental Line Breeding Department, IHAR, in Mlochow, Poland. These two tetraploid varieties and two diploid lines were selected, as most cultivated varieties are tetraploids with low carotenoids, and high carotenoid lines have only been observed among diploid lines. Moreover, a recent study shows that the ploidy change between diploid and tetraploid genotypes causes no statistically significant gene expression difference in the potato genome (Stupar *et al*. 2007). These potato lines were

grown in a greenhouse with a 14 h light/10 h dark photoperiod at 24 °C for over 3 months. Mature, freshly harvested tubers were used immediately for norflurazon (a phytoene desaturase inhibitor) inhibition studies or frozen in liquid nitrogen and stored at −80 °C for RNA extraction and high-performance liquid chromatography (HPLC) analysis.

Norflurazon treatment of potato tubers

Freshly harvested potato tubers were sterilized with 70% ethanol for 2 min and then 30% bleach for 15 min, followed by rinsing with sterile water for five times. After skins were removed, tubers were cut into thin slices and placed on the BCS medium (Li *et al*. 2006) containing 0.1 mM norflurazon. The treated tubers were maintained at 24 °C with a 16 h light/8 h dark photoperiod for up to 14 d in an incubator. Tuber tissue was harvested at 0, 3, 7 and 14 d post-treatment and stored at −80 °C. The experiments were repeated twice.

HPLC analysis of carotenoids in potato tubers

Carotenoids were extracted from potato tubers samples as described previously (Lopez *et al*. 2008), and analysed using a Waters HPLC system with a Spherisorb ODS2 (5 μm particle size) reversed-phase C18 column and a photodiode array detector (Waters, Milford, MA, USA). Individual carotenoids were identified and quantified as described previously (Li *et al*. 2001).

RNA extraction and microarray hybridization

At least five tubers from each potato line were cut into small pieces and pooled. Four grams of tuber samples from each line was used for RNA extraction. Total RNA was extracted by a hexadecyltrimethylammonium bromide (CTAB)/phenol method (Griffiths *et al*. 1999) and treated with RQ1 DNase (Promega, Madison, WI, USA) to completely remove genomic DNA. Fifty micrograms of total RNA was used to generate cDNA and labelled using the 3DNA Array Detection Array 50 Kit (Genisphere, Hatfield, PA, USA). Cy3 or Cy5-labelled cDNA probes were hybridized onto the tomato TOM2 long oligo arrays available through the Boyce Thompson Institute for Plant Research (http://ted.bti.cornell.edu/cgi-bin/TFGD/order/home.cgi). Microarray hybridization and data acquisition were carried out as reported previously (Moore *et al*. 2005). The hybridization was performed with four biological replicates.

Microarray data analysis

Spots with mean signal intensities less than local background intensities plus two standard deviations of the local background in both channels were regarded as empty spots and not included in the downstream statistical analysis. A print-tip Lowess normalization strategy was applied to normalize the ratio values for each array using the marray package (Yang *et al*. 2002) in Bioconductor (http://www.bioconductor.org/). Significances of gene expression changes between Atlantic and POR03 tubers were identified using the Patterns from Gene Expression package (PaGE) (Grant, Liu & Stoeckert 2005). Genes with a false discovery rate (FDR) (Benjamini & Hochberg 1995) less than 0.1 and a fold change not less than 1.5 were considered as differentially expressed genes. In addition, genes with fold changes no less than 1.5 in all four individual biological replicates were also considered as differentially expressed genes. Functional classification of differentially expressed genes was performed using the Plant MetGenMAP system (Joung *et al*. 2009). The entire array data set and the experimental descriptions were deposited into the Tomato Functional Genomics Database (Fei *et al*. 2011).

RNA gel blot and quantitative RT-PCR (qRT-PCR)

For Northern blot analysis, total RNA (20 μg) from potato tubers were resolved on 1.2% denaturing gels containing formaldehyde and blotted onto Hybond-N + membrane (GE Healthcare, Piscataway, NJ, USA) following the method as described previously (Li *et al*. 2001). Filters were probed overnight with P^{32}-labelled target gene fragments in ULTRAhyb hybridization buffer (Ambion, Austin, TX, USA). The templates were amplified from potato cDNA using gene-specific primers listed in Supporting Information Table S1, and labelled with random primers (Li *et al*. 2001). Following hybridization, filters were washed twice with 2 × saline-sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS) and 0.2 × SSC, 0.1% SDS at 42 °C, and then detected using STORM 860 PhosphorImager (GMI, Ramsey, MN, USA).

For qRT-PCR, the cDNA was synthesized using Superscript reverse transcriptase (Invitrogen, Carlsbad, CA, USA). qRT-PCR was conducted by using SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA). The cycling conditions involved denaturation at 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. The specificity of qRT-PCR was examined by the dissociation curve. The relative expression of *ZEP* and *CCD1A* was normalized to a potato *actin* gene (GenBank accession number GQ339765). Gene-specific primers used are listed in Supporting Information Table S1.

Determination of *BCH2* and *ZEP* allele composition

BCH2 allele composition of diploids 91E22 and POR03 and tetraploids Atlantic and Désirée was determined by sequencing PCR products obtained with primers CHY2ex4F and Beta-R822 (Wolters *et al*. 2010). *ZEP* allele composition was determined by sequencing PCR products obtained with primers AWZEP9 and AWZEP10 (Wolters *et al*. 2010).

Isolation of promoters of *BCH2* genes from potato

The promoter of *BCH2* from POR03 was isolated using GenomeWalker Universal Kit (Clontech, Mountain View, CA, USA). Based on the sequences of potato *BCH2*/*crtR-b2* cDNA (GenBank accession number: GU233534) and tomato *crtR-b2* cDNA (GenBank accession number: AK246159), two gene-specific primers B2R1and B2R2 were designed to amplify the promoter sequence. Genomic DNA was extracted from potato leaf tissues as described previously (Lopez *et al*. 2008). DNA (2.5 μg) was digested separately with *Dra*I, *Eco*RI, *Pvu*II and *Swa*I, and then ligated to GenomeWalker adaptors to create the libraries. The libraries were then used as templates in the nested PCR with adaptor primer 1 and B2R1 as primers in the first round of amplification, and nested adaptor primer 2 and B2R2 as primers in the second round of amplification. PCR products were cloned into pCR2.1 vector (Invitrogen) and sequenced. Based on the promoter sequence obtained from POR03, a new pair of primers NBPF1 and NBPR1 was designed to amplify the corresponding promoter regions from other varieties. The PCR products were cloned and verified by sequencing.

The promoter sequence from the *BCH2* allele 1 was determined by sequencing PCR products obtained with primer combinations NBPF2 + NBPR2 and UBPF1 + NBPR4, using DNA from monoploid genotype 7322 (Wolters *et al*. 2010) as template. The promoter sequence from *BCH2* allele 2 was determined by sequencing PCR products obtained with primer combinations NBPF2 + NBPR3 and UBPF1 + NBPR4, using DNA from monoploid genotype M5 (Wolters *et al*. 2010) as template. The search for *cis*-elements in the promoters was performed by PLACE (Higo *et al*. 1999).

Construction of Pro_{BCH2}:*GUS* (β-glucuronidase) plasmids and *Arabidopsis* transformation

To create the Pro_{BCH2}:*GUS* constructs, approximately 1.5 kb promoter sequence from Atlantic (allele 5) and POR03 (allele 3) were cloned into the pSG506 vector and then the chimeric genes were subcloned into pCAMBIA1300 vector as described previously (Chiu *et al*. 2010). The resulting $Pro_{BCH2\text{-}5}$:*GUS* and $Pro_{BCH2\text{-}3}$:*GUS* constructs were introduced into *Agrobacterium tumefaciens* GV3101 and transformed into *Arabidopsis thaliana* (ecotype Columbia) by the floral dip method.

Sugar treatment of *Arabidopsis* leaves

Four-week-old wild-type and Pro_{BCH2}:*GUS* transgenic *Arabidopsis* rosette leaves were detached and placed into H_2O, 6% glucose and 6% sucrose, respectively, and shaken for 24 h at room temperature in the dark (Zhou, Cooke & Li 2010). The treated rosette leaves were harvested and frozen immediately in liquid nitrogen.

Sugar determination in potato tubes

Dried potato tubers (0.02 g) were homogenized and extracted with boiling water for 15 min. After centrifugation at 13 000 *g* for 5 min, the supernatants were diluted 20-fold, and analysed by HPLC using a CarboPac PA1 4 × 250 mm analytical column and a CarboPac PA1 4 × 50 mm guard column (Dionex, Vienna, Austria) as described (Kaplan & Guy 2004).

Determination of GUS activity

Quantitative analysis of GUS activity was measured with 4-methylumbelliferyl-D-glucuronide (4-MUG) as substrate as described previously (Chiu *et al*. 2010). Proteins were extracted from 4-week-old *Arabidopsis* rosette leaves in GUS extraction buffer (50 mM $NaHPO_4$ pH 7.0, 10 mM 2-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100) and their concentrations were determined by Bradford method (Bio-Rad, Hercules, CA, USA). Protein extracts (20 μL) were mixed with 180 μL 1 mM 4-MUG in extraction buffer, and incubated at 37 °C for 1 h. A 20 μL aliquot was transferred into 80 μL 0.2 M Na_2CO_3 and fluorescence was measured on a Dynex Fluorite 1000 fluorescence plate reader (Dynex Technologies, Chantilly, VA, USA) with excitation at 365 nm and emission at 450 nm. The standard curve was made by measuring 4-MU fluorescence. Four independent Pro_{BCH2}:*GUS* lines from each construct were used in the assay and the analysis was repeated three times.

RESULTS

Carotenoid accumulation in potato cultivars

Four potato lines (Atlantic, Désirée, 91E22 and POR03) were chosen to examine the regulation of carotenoid accumulation in tubers. Among them, Désirée had pink or red skin, and the rest yellow or light-brown skins. The four potato lines displayed different flesh colours. Atlantic was white, Désirée was light yellow and 91E22 and POR03 were orange-yellow and deep orange-yellow, respectively. HPLC analysis revealed that the four cultivars contained different levels of carotenoids. Atlantic contained undetectable amount of carotenoids, whereas POR03 had the highest amount of total carotenoids (Table 1). The increased levels of carotenoid accumulation in these four lines are consistent with the change of flesh colour from white to deep orange-yellow, indicating that carotenoids contribute significantly to tuber flesh colour.

The major carotenoids present in potato tubers are lutein, zeaxanthin and violaxanthin (Burgos *et al*. 2009). The composition of carotenoids varied among Désirée, 91E22 and POR03. The composition and relative levels of individual carotenoids are shown in Table 1. Lutein was the main carotenoid found in Désirée as shown in previous studies (Morris *et al*. 2004; Lopez *et al*. 2008). Zeaxanthin was the predominant carotenoid in 91E22 and POR03.

Table 1. Major carotenoids in freshly-harvested potato tubers

	Violaxanthin*	Lutein*	Zeaxanthin*	Total*
Atlantic	n.d.	n.d.	n.d.	n.d.
Désirée	n.d.	0.43 ± 0.05	n.d.	0.43 ± 0.05
91E22	0.15 ± 0.05	0.22 ± 0.02	4.85 ± 0.30	5.21 ± 0.37
POR03	0.55 ± 0.51	0.45 ± 0.25	9.13 ± 2.13	10.14 ± 2.90

*Carotenoid levels were expressed as μg g^{-1} fresh weight.
n.d., not detectable.

Increased carotenoid accumulation was associated with enhanced metabolic flux in potato tubers

Higher accumulation of carotenoids in plants could result from enhanced carotenogenic activity, reduced degradation and/or increased sink strength (Li *et al*. 2006; Li & Van Eck 2007). To decipher the mechanisms underlying carotenoid accumulation in potato cultivars, we compared the metabolic flux changes among the four cultivars following norflurazon treatment of the tubers. Norflurazon is known to be a pyridazinone herbicide, which specifically inhibits phytoene desaturase in the carotenoid biosynthetic pathway, resulting in the accumulation of phytoene (Breitenbach, Zhu & Sandmann 2001). The level of phytoene accumulation reflects the capacity of carotenoid biosynthesis in the tissue (Li *et al*. 2006). In all cultivars, phytoene was hardly detected in the freshly harvested tubers. In the presence of norflurazon, the phytoene level showed a general continuous increase during the 14 d of treatment, while the contents of the other carotenoids, i.e. lutein, violaxanthin and/or zeaxanthin, in these potato lines either remain constant or decrease. POR03, 91E22, Désirée and Atlantic accumulated phytoene at decreasing abundance, with the first three varieties containing about 7.4-, 4.2- and twofold more phytoene, respectively, than Atlantic at 7 d of treatment (Fig. 2). The results indicate that the increased levels of carotenoid accumulation in these four lines were associated with increased capacity of carotenoid biosynthesis.

Identification of differentially expressed genes by microarray analysis

To further explore the molecule basis of carotenoid accumulation in potato tubers, microarray analysis was employed to examine differences in gene expression between the carotenoid-undetectable cultivar Atlantic and the carotenoid-rich line POR03. Tomato and potato share significant gene sequence identities (Bonierbale *et al*. 1988), and tomato cDNA chips have been used for identification of differentially expressed genes from other related plant species (Ponce-Valadez *et al*. 2009). Thus, tomato TOM2 long oligonucleotide microarray slides were used to hybridize with Cy3- and Cy5-labelled cDNAs generated from total RNA of Atlantic and POR03 tubers. Approximately 12 120 spots were detected, among which 208 genes (1.72%) showed significantly higher expression (Supporting Information Table S2) and 173 (1.43%) showed significantly lower expression in POR03 than Atlantic (Supporting Information Table S3). These genes were involved in various processes, including metabolic process, response to stresses, biosynthetic and cellular process (Fig. 3). The major group of genes functions in metabolic process, suggesting that complex metabolic changes occur in POR03 tubers. Four carotenoid metabolic pathway genes were identified to be among the differentially expressed genes between POR03 and Atlantic. Among the highly up-regulated genes, *BCH2* (Y14810) and *BCH1* (SGN-U233360) exhibited 21.5- and 8.2-fold increase, respectively, in POR03 over Atlantic. Among the down-regulated genes in POR03, the expression of *ZEP* (Z83835) and *carotenoid cleavage dioxygenase 1A* (*CCD1A*; AY576001) (Simkin *et al*. 2004) in POR03 was markedly reduced, with 3.6- and 2.1-fold decrease, respectively, when compared to their expression in Atlantic.

Expression of the differentially expressed carotenoid genes was correlated with carotenoid levels in the potato lines

To validate the microarray results and to find out whether the expression of the four differentially expressed

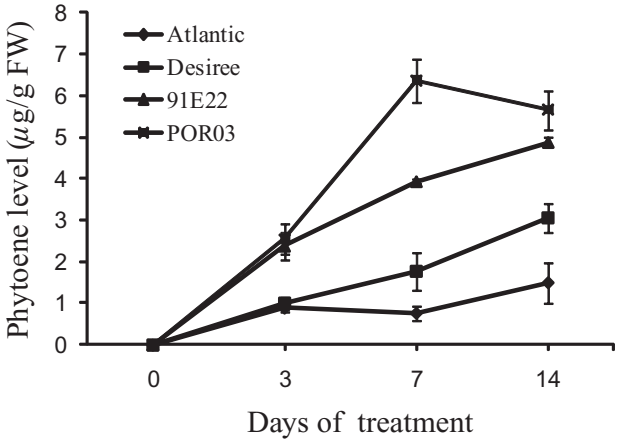

Figure 2. Phytoene synthesis in freshly harvested tuber slices treated with norflurazon. Fresh potato tubers were sterilized and tuber slices were placed on BCS medium containing 0.1 mM norflurazon for up to 14 d. Tuber slices were collected at the indicated time points. Phytoene levels were measured by high-performance liquid chromatography. Error bars indicate ±SD ($n = 4$).

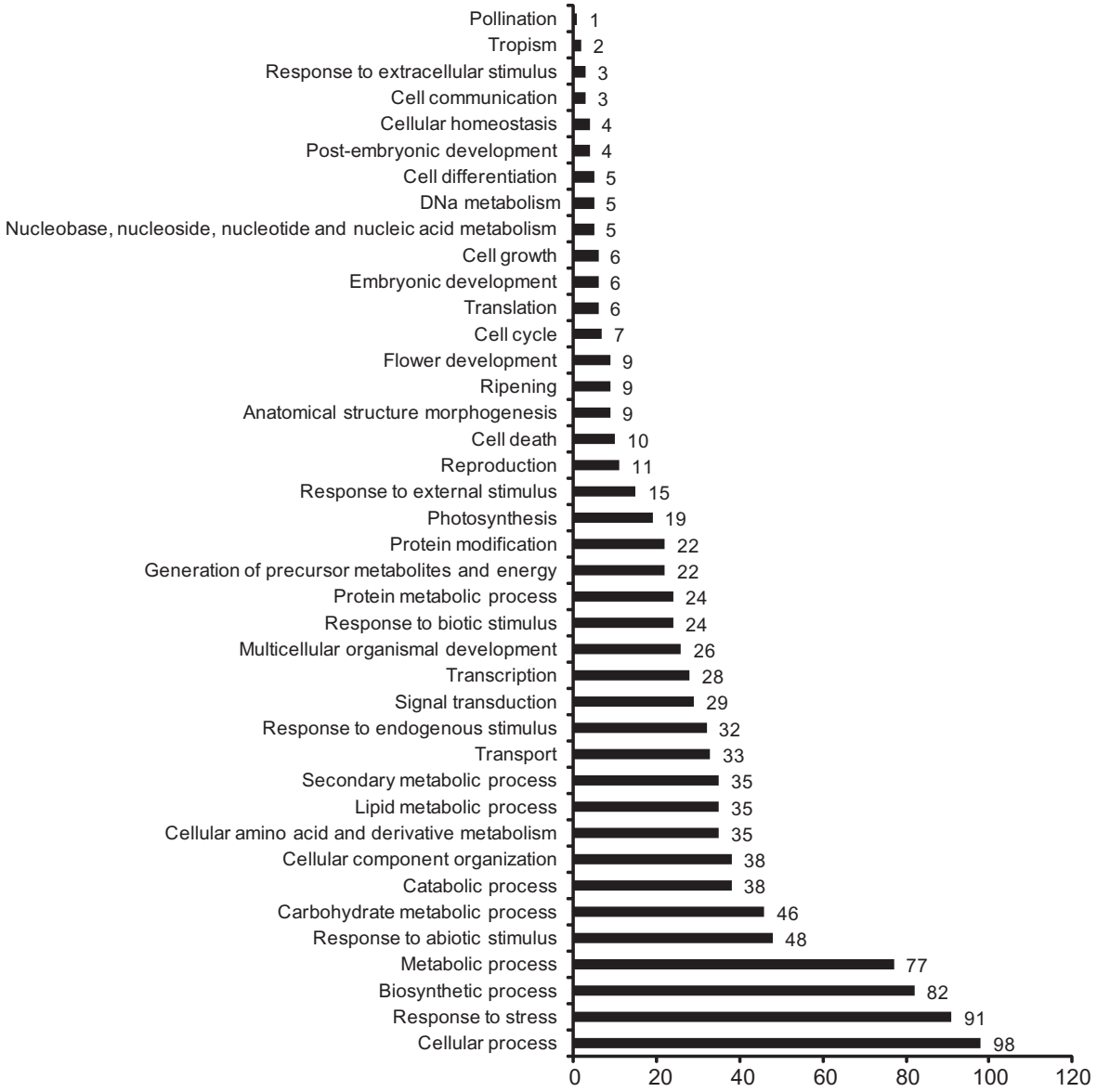

Figure 3. Functional categories of genes differentially expressed in POR03 and Atlantic tubers.

carotenoid genes was correlated with carotenoid content in the tubers, the transcript levels of *BCH2*, *BCH1*, *ZEP* and *CCD1A* were examined by RNA gel blot or qRT-PCR analysis in the four potato lines. We observed that *BCH2* was expressed higher in carotenoid-rich lines POR03 and 91E22 than in low carotenoid cultivars Désirée and Atlantic, implicating that the enhanced expression of potato *BCH2* could account for higher accumulation of carotenoids in these potato varieties. In addition, *BCH1* showed weak expression in these four lines, but it clearly was expressed higher in POR03 than the other varieties (Fig. 4a). Expression of *ZEP* and *CCD1A* was analysed by qRT-PCR. The results showed that the relative expression levels of *ZEP* and *CCD1A* was higher in low carotenoid-containing cultivars (Atlantic and Désirée) and lower in carotenoid-rich cultivars (91E22 and POR03), showing a negative association with carotenoid accumulation in potato tubers (Fig. 4b).

BCH2 and *ZEP* allele composition

In the diploid genotypes 91E22 and POR03, each gene may contain two different alleles, while in the tetraploid genotypes Atlantic and Désirée, up to four different alleles for each gene may be present. The alleles for *BCH2* and *ZEP* are distinguished by specific single-nucleotide polymorphisms (SNPs) as shown in Supporting Information Tables S1 and S3 from a previous publication (Wolters *et al.* 2010). The allelic composition for *BCH2* and *ZEP* genes in these four potato lines was determined by amplification and sequencing of gene regions and analysis of allele-specific SNPs as described by Wolters *et al.* (2010).

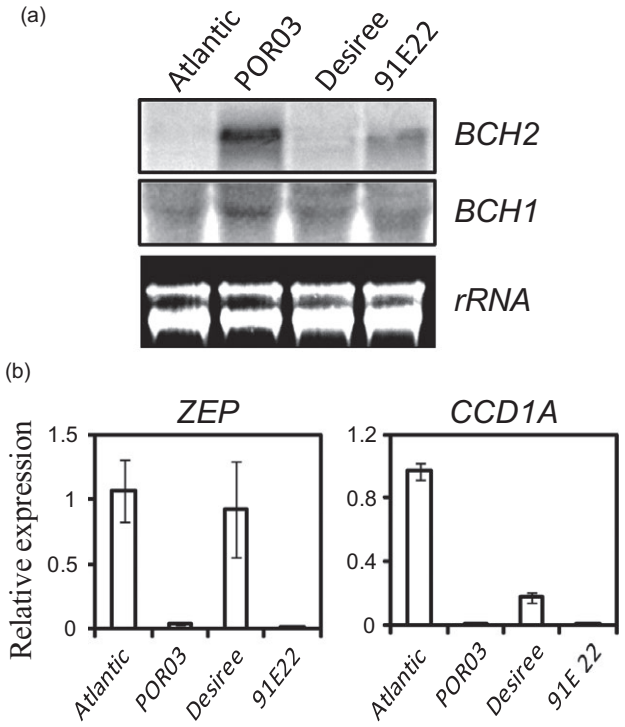

Figure 4. Transcript levels of differentially expressed carotenoid genes in tubers of four potato lines. (a) Northern blot analysis of *BCH2* and *BCH1* expression in tubers. Total RNA was extracted from pooled tubers and 20 μg of RNA was resolved on a 1.2% denaturing gel. rRNA was used for equal loading control. (b) qRT-PCR analysis of *ZEP* and *CCD1A* expression. Total RNA (5 μg) was used in reverse transcription. The experiments were repeated with three biological repeats.

Both diploid genotypes 91E22 and POR03 contained *BCH2* alleles 1 and 3. Allele 3 is the dominant allele that has been shown to be associated with a high level of zeaxanthin accumulation in potato tuber (Wolters *et al*. 2010). Cultivar Désirée contained one *BCH2* allele 3 together with one allele 1 and two alleles 2. Therefore, its *BCH2* allelic composition was 1,2,2,3. Cultivar Atlantic did not contain the dominant allele 3, but instead, one allele 1 and three alleles 5 (allelic composition 1,5,5,5). Alleles 1, 2 and 5 are recessive alleles (Wolters *et al*. 2010).

Both diploid genotypes 91E22 and POR03 were homozygous for recessive *ZEP* allele 1, which has a reduced level of expression in all diploid genotypes with orange tuber flesh (Wolters *et al*. 2010). The tetraploids Atlantic and Désirée did not contain *ZEP* allele 1.

Structural analysis of promoters of *BCH2* genes

BCH2 was expressed highly in the carotenoid-rich lines, indicating its important role in controlling carotenoid accumulation in tubers. To find potential *cis*-elements that control *BCH2* expression in potato tubers, we isolated and compared the promoter sequences of different alleles of *BCH2* present in these four potato lines, which include the dominant allele 3 and the recessive alleles 1, 2 and 5. The *BCH2* upstream promoter sequence of 1648 bp (allele 5) and 1493 bp (allele 3) from Atlantic and POR03, respectively, was cloned through genome DNA walking. The *BCH2* promoter sequences from allele 1 (1501 bp) and allele 2 (1713 bp) were obtained from monoploid genotypes 7322 and M5, respectively. Alignment of the four promoter sequences revealed 82 SNPs and 18 Indels, of which three were relatively large (Indel 7, 434 bp; Indel 10, 210 bp; Indel 14, 383 bp) (Supporting Information Fig. S1). Analysis of the regulatory regions of *BCH2* by PLACE (http://www.dna.affrc.go.jp/ PLACE/signalscan.html) (Higo *et al*. 1999) revealed several variable putative *cis*-element sequences (Supporting Information Fig. S1). Most noticeably, the promoter region of *BCH2* from dominant allele 3 contains three unique *cis*-elements. One is recognized by MybSt1 (a potato MYB homolog), the second is recognized by AtMYC2 transcription factor and the third is WBOXHVISO1. WBOXHVISO1 has previously been reported to be involved in sugar signalling (Sun *et al*. 2003; Hammargren *et al*. 2008). That whether these motifs play a role in activating *BCH2* expression of dominant allele 3 remains to be determined.

Activity of the *BCH2* promoters was stimulated by sugars

WBOXHVISO1 box was found in *Arabidopsis nucleoside diphosphate kinase* (*NDPK3a*) and barley *iso1* promoters and was shown to mediate sugar signalling and to be induced by sucrose and glucose (Sun *et al*. 2003; Hammargren *et al*. 2008). Potato tubers are starch-rich organs but also contain reducing sugars and sucrose. To examine the effect of sugars on induction of the *BCH2* expression, the promoter sequences from recessive allele 5 (from Atlantic) and dominant allele 3 (from POR03) were fused to GUS reporter gene to produce $Pro_{BCH2\text{-}5}$*:GUS* and $Pro_{BCH2\text{-}3}$*:GUS* constructs, respectively, and transformed into *Arabidopsis*. Transgenic *Arabidopsis* rosette leaves expressing *ProBCH2:GUS* constructs were treated with 6% sucrose and glucose. No significant difference ($P > 0.05$) in GUS activity between $Pro_{BCH2\text{-}5}$*:GUS* and $Pro_{BCH2\text{-}3}$*:GUS* was found in the detached rosette leaves treated with water (Fig. 5a). However, following treatment with 6% glucose or sucrose, an increase of GUS activity could be detected in transgenic leaves expressing both constructs. The GUS activity driven by *BCH2* promoter from allele 3 in POR03 was significantly higher than that from allele 5 in Atlantic when the transgenic leaves were treated with glucose and sucrose. The results suggest that the higher expression of *BCH2* in POR03 tubers may be associated with an enhanced response of the *BCH2* promoter to sugars (Fig. 5a).

Sugar levels in potato tubers

In order to examine whether *BCH2* expression was correlated with soluble sugars in potato tubers, the levels of reducing sugars of glucose, fructose and maltose and

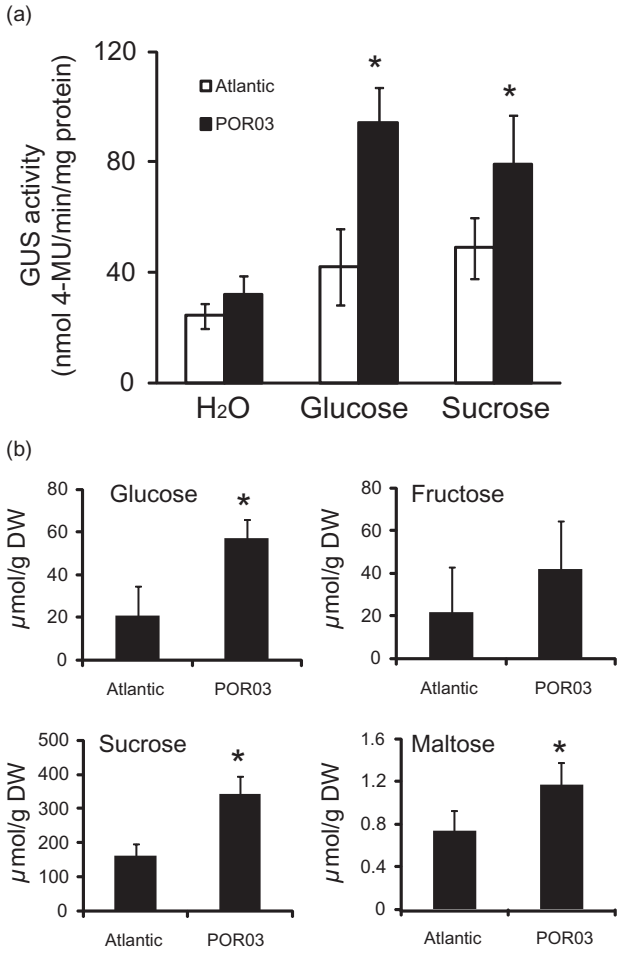

Figure 5. Analysis of promoter activity of *BCH2* in transgenic *Arabidopsis* plants and sugar levels in potato lines Atlantic and POR03. (a) GUS activity driven by *BCH2* promoters of allele 3 in POR03 and allele 5 in Atlantic after treatment with 6% sucrose and glucose for 24 h. GUS activity was measured using 4-MUG as substrate. The analysis was repeated three times. (b) Sugar levels in potato tubers. Error bars indicate +SD of three repeat measurements. Values marked by asterisks indicate a statistically significant difference ($P < 0.05$) ($n = 3$).

disaccharide sucrose were analysed in Atlantic and POR03. POR03 contained significantly ($P < 0.05$) more glucose than Atlantic (Fig. 5b). There was no significant difference in fructose level between Atlantic and POR03 ($P > 0.05$). Sucrose was the major soluble sugar detected in tubers (Fig. 5b). Sucrose content was doubled in POR3 compared with Atlantic. Maltose level in tubers was less than the other three soluble sugars, and POR3 contained significantly more maltose than Atlantic (Fig. 5b). Collectively, the results showed that the higher expression of *BCH2* in POR03 than in Atlantic could result from its response to higher endogenous tuber glucose or sucrose levels.

DISCUSSION

Plants have evolved sophisticated mechanisms to control carbon flux into and through the carotenoid biosynthetic pathway. Evaluation of the accumulation of metabolites in the presence of pathway inhibitors, such as norflurazon, provides a convenient way to monitor carotenoid biosynthesis from overall carotenoid metabolism (Li *et al*. 2006). By measuring phytoene levels in potato tubers treated with norflurazon, we found that the increased carotenoid accumulation in potato tubers was associated with enhanced capacity of carotenoid biosynthesis. PSY as the first committed enzyme in carotenoid biosynthetic pathway is generally recognized to be the key in directing metabolic flux into the carotenoid biosynthetic pathway (Cazzonelli & Pogson 2010). Alteration of its expression has profound effects on carotenoid accumulation in plants (Shewmaker *et al*. 1999; Paine *et al*. 2005). The upstream pathway enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS) has also been shown to influence carotenoid biosynthesis (Enfissi *et al*. 2005). Previous studies in transgenic potato tubers show that alteration of some carotenoid gene expression plays an important role in carotenoid biosynthesis (Ducreux *et al*. 2005; Diretto *et al*. 2006). We compared the expression of PSY and DXS and found similar levels of expression among these four potato lines (data not shown), indicating that other unknown mechanisms likely regulate carotenoid metabolic flux in potato tubers.

Towards further understanding of the regulatory control of carotenoid accumulation in potato tubers, we conducted a genome-wide gene expression analysis between the white flesh cultivar Atlantic and the deep orange-yellow flesh line POR03. Genome-wide analysis identified four carotenoid metabolic pathway genes, i.e. *BCH2*, *BCH1*, *ZEP* and *CCD1A,* which showed differential expression. BCH2 and BCH1 are non-heme di-iron enzymes. The biochemical function of BCH is to convert β-carotene to zeaxanthin. Two copies of *BCH* are known in potato (Diretto *et al*. 2007), as well as in *Arabidopsis* (Sun, Gantt & Cunningham 1996; Tian & DellaPenna 2001) and tomato (Galpaz *et al*. 2006). Here, we showed that *BCH2* was the most highly differentially expressed gene and its transcript levels were tightly associated with carotenoid content in these four potato lines, consistent with previous studies obtained by association mapping and a microarray analysis (Brown *et al*. 2006; Kloosterman *et al*. 2010; Wolters *et al*. 2010). The results suggest that BCH2 functions as a major factor in directing the flux through the β-branch of the pathway to lead zeaxanthin synthesis in tubers. Unlike tomato *BCH1*, which is believed to be mainly involved in carotenoid biosynthesis in photosynthesis tissue (Galpaz *et al*. 2006), a correlated expression of *BCH1* is also observed among potato cultivars, indicating that it could play a role, even if less important than *BCH2*, in zeaxanthin biosynthesis in tubers.

ZEP is responsible for the conversion of zeaxanthin to violaxanthin. We found that *ZEP* transcript level was drastically reduced in POR03 and 91E22 compared with Atlantic and Désirée. Reduction of *ZEP* expression is reported to cause to a dramatic increase of zeaxanthin content, as well as the total tuber carotenoid level in transgenic potato lines (Romer *et al*. 2002). Similarly, low transcript levels are

observed in high carotenoid lines from examination of a range of potato germplasm (Morris *et al*. 2004). It is apparent that tuber-specific expression of *ZEP* adversely affects tuber carotenoid content.

Zeaxanthin was the major component of carotenoids accumulated in tubers of the diploid genotypes POR03 and 91E22. The enhanced expression of *BCH2* and the reduced expression of *ZEP* were consistent with the recent report showing that the presence of a dominant *BCH2* allele 3 and a homozygous recessive *ZEP* allele 1 is responsible for zeaxanthin accumulation in orange-yellow flesh tubers (Wolters *et al*. 2010). Eight *BCH2* alleles have been identified from potato and the presence of a single allele 3 is sufficient for its yellow flesh colour (Wolters *et al*. 2010). We examined the *BCH2* and *ZEP* allele compositions of the four potato lines. While the *BCH2* allele composition for white flesh cultivar Atlantic was 1, 5, 5, 5, Désirée had alleles 1, 2, 2, 3, and the diploid lines contained alleles 1 and 3. Although one allele 3 was present in POR03, 91E22 and Désirée, the different levels of carotenoid accumulation among them confirm the role of additional genes in controlling total carotenoid accumulation in potato tubers as suggested (Brown *et al*. 2006). Five *ZEP* alleles have been identified in potato and a recessive homozygous allele 1 is found to be associated with orange-yellow flesh phenotype (Wolters *et al*. 2010). The high carotenoid lines POR03 and 91E22 were homozygous for *ZEP* allele 1 with low transcript level. The low expression reduces the rate for further metabolizing zeaxanthin to other xanthophylls. In contrast, Désirée did not contain allele 1 and showed much higher transcription of *ZEP* than POR03 and 91E22, which offers an explanation for lower-level carotenoid accumulation in Désirée in comparison with the high carotenoid lines.

A family of CCDs catalyses enzymatic turnover of carotenoids for the production of apocarotenoids, such as β-ionone, pseudoionone and geranylacetone (Auldridge, McCarty & Klee 2006). In *Arabidopsis*, there are nine members of CCDs that cleave multiple carotenoid substrates into apocarotenoids (Tan *et al*. 2003). CCDs help maintain the homeostasis of carotenoids in plants (Ohmiya *et al*. 2006). *CCD1A*, a homolog of tomato *CCD1A* (Simkin *et al*. 2004), was identified here to be differentially expressed between Atlantic and POR03, and its transcript level was found to be dramatically reduced in high carotenoid lines. A dramatic reduced expression of *CCD1A* in high carotenoid varieties suggests a reduced carotenoid turnover. A recent study of another member of the *CCD* gene family, *CCD4*, in potato shows that down-regulation of *CCD4* by RNAi results in two- to fivefold increased carotenoids in potato tubers (Campbell *et al*. 2010). It appears that CCD activity affects final carotenoid accumulation in potato tubers, but the role of individual member varies depending on varieties.

Sugars mainly sucrose have been shown to induce expression of some tuber-specific genes, such as *patatin*, *sucrose synthase Sus4-16* and *StCDPK1* in other tissues where they are not normally expressed (Grierson *et al*. 1994; Fu, Kim & Park 1995; Raices *et al*. 2003). Sucrose promotes conversion of chloroplasts into chromoplasts for carotenoid accumulation in citrus peel segments (Huff 1983). Sucrose availability affects *PSY1* expression and lycopene accumulation in tomato fruits (Télef *et al*. 2006). A number of carotenoid biosynthetic genes are transcriptionally activated by glucose in cyanobacterium *Synechocystis* (Ryu *et al*. 2004). Here, we found that *BCH2* expression was activated in leaf tissue by exogenous application of glucose and sucrose, indicating that free sugars in tubers could play a role in promoting *BCH2* expression. Indeed, POR03 tubers contained more glucose and sucrose than Atlantic. This provides an explanation for high expression of *BCH2* in POR03 tubers. Further analysis of the sugar-responsive *cis*-element in the *BCH2* promoter will help dissect the local network regulating carotenogenesis in potato tubers.

Taken together, our results showed that the high carotenoid accumulation in potato tubers is due to a combination of increased metabolic flux into the carotenoid biosynthetic pathway and the transcriptional control of a number of carotenoid metabolic genes. The synergistic effect of elevated expression of *BCH2* and *BCH1* as well as suppressed expression of *ZEP* and *CCD1A* is responsible for zeaxanthin accumulation in the yellow-fleshed varieties.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Structure analysis of the *BCH2* promoters from Atlantic, Désirée, 91E22, and POR03.

Table S1. Primers used for amplification of probes for northern blot analysis, genome walking, and qRT-PCR analysis of genes differentially expressed based on microarray data.

Table S2. Top 100 up-regulated genes in microarray analysis of potato tubers from Altantic and POR03.

Table S3. Top 100 down-regulated genes in microarray analysis of potato tubers from Altantic and POR03.

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