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Original article:

ANALYSIS OF CAROTENOID ACCUMULATION AND EXPRESSION OF CAROTENOID BIOSYNTHESIS GENES IN DIFFERENT ORGANS OF CHINESE CABBAGE (*BRASSICA RAPA* SUBSP. *PEKINENSIS*)

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ABSTRACT

The relationship between carotenoid accumulation and expression of carotenoid biosynthesis genes was investigated in the flowers, stems, young leaves, old leaves, and roots of Chinese cabbage (*Brassica rapa* subsp. *pekinensis*). Quantitative real-time PCR analysis showed that the mRNA levels of *BrPSY*, *BrPDS*, *BrZDS*, *BrLCYB*, *BrLCYE*, *BrCHXB,* and *BrZEP* leading to the production of carotenoids were highest in the flowers or the leaves and lowest in the roots of Chinese cabbage. In contrast, the mRNA expression of *BrNCED,* a gene involved in abscisic acid (ABA) biosynthesis, was highest in the roots. High-performance liquid chromatography revealed that carotenoids, namely, lutein and *β*-carotene, were distributed predominantly in the flowers and leaves, with very little in the underground organ, the roots. Specifically, old leaves contained 120.3 μg/g lutein and 103.93 μg/g *β*-carotene, which is the most potent dietary precursor of vitamin A. Moreover, we found a relatively large amount of *cis* isomers of *β*-carotene, namely, 9-*cis β*-carotene and 13-*cis β*-carotene, in Chinese cabbage. These results provide insight into carotenoid biosynthetic mechanisms in Chinese cabbage and may be helpful in the metabolic engineering of carotenoid biosynthesis in plants.

Keywords: *Brassica rapa* subsp. *pekinensis*, Chinese cabbage, carotenoids, carotenoid biosynthesis genes, *β*-carotene

Abbreviations used: DEPC, diethylpyrocarbonate; HPLC, high-performance liquid chromatography; GGDP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, *ξ*-carotene desaturase; LCYB, lycopene *β*-cyclase; LCYE, lycopene ε-cyclase; CHXB, *β*-ring carotene hydroxylase; CHXE, *ε*-ring carotene hydroxylase; ZEP, zeaxanthin epoxidase; NCED, 9-*cis* epoxycarotenoid dioxygenase; ABA, abscisic acid; Y-leaves, young leaves; O-leaves, old leaves

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INTRODUCTION

Carotenoids are an important group of naturally occurring compounds derived from a terpenoid precursor. Carotenoids represent a diverse group of pigments that contribute to the red, orange, and yellow colors in plants (Cunningham and Gantt, 1998). Carotenoids also participate in a variety of critical processes in plants, such as light harvesting in photosynthetic membranes, as well as in protection of the photo-system from photo-oxidation (Havaux, 1998). Furthermore, carotenoids are precursors to aroma compounds, abscisic acid (ABA), and other derivatives involved in plant growth and development (Auldridge et al., 2006; Simkin et al., 2004). Carotenoids are important not only to the plant in which they are synthesized, but also to animals and humans. Carotenoids have long been considered as essential nutrients in human diets, primarily as a precursor of vitamin A (Giovannucci, 1999; Krinsky et al., 2003). Some studies suggest that high intake of carotenoids can reduce the risk of cancer, macular eye disease, and cardiovascular problems (Giovannucci, 1999; Mayne, 1996). Therefore, crops containing high levels of carotenoids have attracted the researchers since long. Recently, various transgenic approaches have been carried out to increase carotenoid levels in crops by overexpressing the genes involved in the biosynthetic pathway. Golden rice is the best known example for metabolic engineering of carotenoids in crops (Ye et al., 2000).

The carotenoid biosynthesis pathway has been described in detail in various plants, including tomato (Isaacson et al., 2002), tobacco (Busch et al., 2002), citrus (Kato et al., 2004), *Arabidopsis* (Park et al., 2002), and apricot (Marty et al., 2005). The first step in the pathway is the condensation of 2 geranylgeranyl diphosphate groups (GGDP) into phytoene, which is catalyzed by phytoene synthase (PSY) (Figure 1). Phytoene is transformed to lycopene by phytoene desaturase (PDS) and ξ-carotene desaturase (ZDS) through desaturation steps. The cyclization of lycopene is a key branch point in the pathway, yielding either α-carotene by catalysis with lycopene β-cyclase (LCYB) and lycopene ε-cyclase (LCYE), or β-carotene by catalysis with LCYB alone. α-Carotene is then converted into lutein and β-carotene is converted into zeaxanthin by hydroxylation, which are catalyzed by β-ring carotene hydroxylase (CHXB) and ε-ring carotene hydroxylase (CHXE), respectively. Zeaxanthin epoxidase (ZEP) catalyzes the epoxidation of zeaxanthin to produce violaxanthin. Violaxanthin is used to synthesize the plant hormone ABA through an oxidative cleavage catalyzed by 9-*cis* epoxycarotenoid dioxygenase (NCED). Natural carotenoids contain a mixture of different isomers (*cis* and *trans*) of the carotenoid molecules such as 9-*cis* β-carotene and 13-*cis* β-carotene; however, the biosynthetic genes are largely unknown (Khoo et al., 2011).

Chinese cabbage (*Brassica rapa* subsp. *pekinensis*) originated in China and is one of the most widely cultivated vegetables in Asia. Therefore, Chinese cabbage has been the subject of much research in a bid to evaluate its nutrient compounds (Artemyeva and Solovyeva, 2006; Krumbein et al., 2005). This study was undertaken to investigate carotenoid accumulation and the expression of genes related to carotenoid biosynthesis in the flowers, stems, young leaves, old leaves, and roots of Chinese cabbage in order to clarify the mechanism of carotenoid biosynthesis and the nutrient value of this plant.

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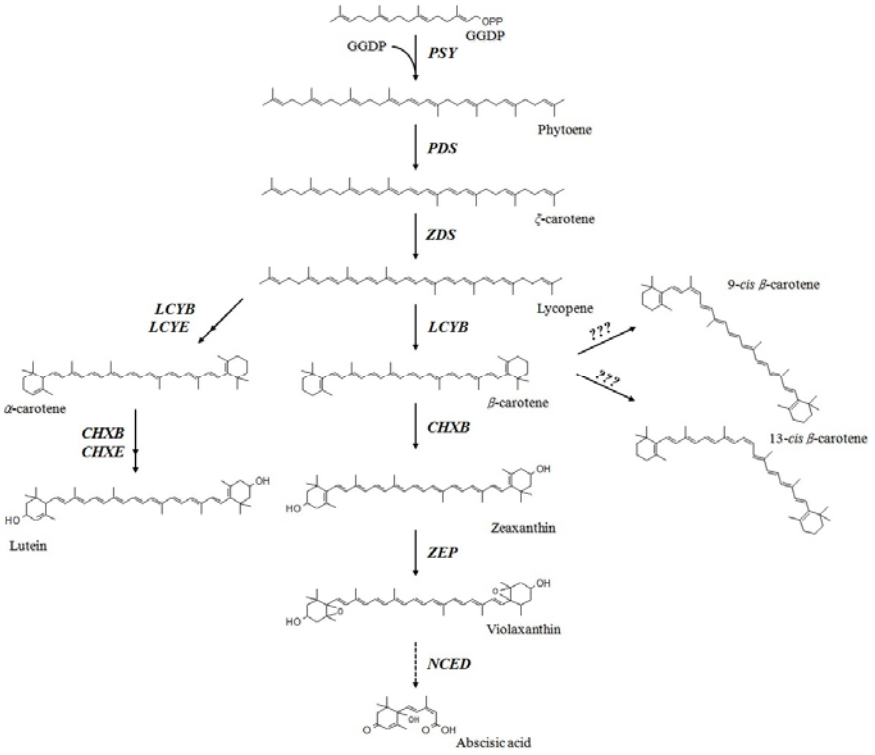

Figure 1: Carotenoid biosynthesis pathway in plants. GGDP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ξ-carotene desaturase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; CHXB, β-ring carotene hydroxylase; CHXE, ε-ring carotene hydroxylase; ZEP, zeaxanthin epoxidase; NCED, 9-*cis* epoxycarotenoid dioxygenase

MATERIALS AND METHODS

Plant materials

Chinese cabbage was grown in a greenhouse at the experimental farm of Chungnam National University (Daejeon, Korea). The flowers, stems, young leaves, old leaves, and roots were excised from mature plants. The samples were immediately frozen in liquid nitrogen, and then stored at -80 °C for RNA isolation, or freeze-dried for high-performance liquid chromatography (HPLC) analysis.

Expression analysis by real-time PCR

Total RNA was separately extracted from each organ of the Chinese cabbage plant by using Plant Total RNA Mini Kit (Geneaid, Taiwan). The quality and concentration of RNA were determined by agarose gel electrophoresis and spectrophotometry, respectively. The single-strand cDNA was synthesized using ReverTra Ace-α kit (Toyobo, Osaka, Japan), according to the manufacturer's protocol. A 25-fold dilution of the resulting cDNA was used for real-time PCR.

Based on the sequence information found in Genbank, we used Primer 3 (http://frodo.wi.mit.edu/primer3) to design primers to amplify the following carotenoid biosynthesis genes in Chinese cabbage: *BrPSY*, *BrPDS*, *BrZDS*, *BrLCYB*, *BrLCYE*, *BrCHXB*, *BrZEP,* and *BrNCED* (Table 1). *Actin* gene was used as a housekeeping gene. The melting curves and cycle thresholds for each real-time PCR primer pair was carefully examined before use. Real-time PCR was carried out in a 20-μL reaction volume containing 0.5 μM primer (each) and 1× SYBR Green Real-time PCR Master Mix (Toyobo). Real-time PCR reaction was repeated independently 3 times and analyzed by Bio-Rad CFX Manager 2.0 software (Bio-Rad Laboratories; Hercules, CA, USA). The PCR conditions were 94 °C for 5 min; 94 °C for 15 s, annealing temperature of 56 °C for 15 s, and 72 °C for 2 s for 40 cycles.

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Table 1: Primers used for real-time PCR

Name	Sequence (5' to 3')	Amplicon (base pair)	Accession number
BrPSY_RT F	GCTATCTACGTTTGGTGCAGAAGAA	189	FJ227935
BrPSY_RT R	AAATGGCTGAATATCGACAGGGTAT		
BrPDS_RT F	GAGCTCGAGGATGATGGTACTGTTA	175	FJ606826
BrPDS_RT R	TAACTGGCACACCAACTAGCTTCTC		
BrZDS_RT F	CCTTCTTGTCAAAGACCACACTCAT	160	FJ606827
BrZDS_RT R	AGCTAGTGAGTTCCTCAGCTTGTCA		
BrLCYB_RT F	AAGATATCCAAGAGAGGATGGTTGC	180	FJ606828
BrLCYB_RT R	CCACCATGTAACCTGTAGAAGGATG		
BrLCYE_RT F	ATGGATGAACAGTCTAAGCTCGTTG	185	FJ606829
BrLCYE_RT R	ACACCGTAGTTGTTTGTGAAAGGAA		
BrCHXB_RT F	CAGAGAAAACAAGCTCTCTGGACAC	185	GQ178285
BrCHXB_RT R	CATCTGCCAAGAGAATCGGTAGTAA		
BrZEP_RT F	AGACTTAAGCGCCATAAGAGGAGAA	185	FJ606830
BrZEP_RT R	ACTTGACATACCAAGTGCCAGAGAC		
BrNCED_RT F	CACATCCTCTGTTTTGTTCACGAC	171	AAV35466
BrNCED_RT R	AAGAGTTTGTTCCTGGAGTTGTTCC		
BrActin_RT F	TAGTGTTGTTGGTAGGCCAAGACAT	188	FJ969844
BrActin_RT R	GGAGCTCGTTGTAGAAAGTGTGATG		

Extraction and analysis of carotenoids

Carotenoids were extracted from Chinese cabbage samples (1 g) with 30 mL of ethanol containing 0.1 % ascorbic acid (w/v). This mixture was vortexed for 20 s, and then incubated in a water bath at 85 °C for 5 min. Subsequently, 120 µL of potassium hydroxide (80 % w/v) was added to saponify any potentially interfering oils. After vortexing and incubating at 85°C for 10 min, the samples were placed on ice, and 1.5 mL of cold deionized water and 0.05 mL of *β*-Apo-8'-carotenal (12.5 µg mL^{-1}), an internal standard, were added. Next, the carotenoids were extracted twice with 1.5 mL of hexane and centrifuged at 1200 *g* each time to separate the layers. Then, the extracts were freeze-dried under a stream of nitrogen gas and resuspended in 50:50 (v/v) dichloromethane/methanol. The extraction method used for carotenoid analysis was similar to that previously described (Howe and Tanumihardjo, 2006).

For HPLC analysis, the carotenoids were separated on an Agilent 1100 HPLC system with a C_{30} YMC column (250 × 4.6 mm, 3 m; Waters Corporation, Milford, MA) and detected with a photodiode array (PDA) detector at 450 nm. Solvent A consisted of methanol/water (92:8 v/v) with 10 mM ammonium acetate. Solvent B consisted of 100 % methyl *tert*-butyl ether (MTBE). The flow rate was maintained at 1 $mL \cdot min^{-1}$, and the samples were eluted

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with the following gradient: 0 min, 83 % A/17 % B; 23 min, 70 % A/ 30 % B; 29 min, 59 % A/41 % B; 35 min, 30 % A/70 % B; 40 min, 30 % A/70 % B; 44 min, 83 % A/17 % B; and 55 min, 83 % A/17 % B. Identification and peak assignment of carotenoids were primarily based on the comparison of their retention time and UV-visible spectrum data with that of standards and with guidelines previously presented (Fraser et al., 2000; Howe and Tanumihardjo, 2006).

RESULTS AND DISCUSSION

Expression of carotenoid biosynthesis genes in different organs of Chinese cabbage

The expression of carotenoid biosynthesis genes was investigated in the flowers, stems, young leaves, old leaves, and roots of Chinese cabbage by real-time PCR (Figure 2). Starting from the beginning of the carotenoid biosynthesis pathway, *BrPSY* was highly expressed in the flowers, young leaves, and old leaves; moderately expressed in the stems; and weakly expressed in the roots. Like *BrPSY*, transcription of *BrPDS*, *BrZDS,* and *BrLCYB* was abundant in the flowers and leaves, and low in the roots. Unlike lycopene *β*-cyclase (*BrLCYB*), lycopene *ε*-cyclase (*BrLCYE*) was expressed highly only in the flowers, with relatively lower expression in the other organs. *BrCHXB* transcription was abundant in the flowers, intermediate in the stems, and poor in the leaves and roots. Young leaves and old leaves exhibited high levels of *BrZEP* transcript, while the expression in the roots was the lowest. The highest expression of *BrNCED*, which is involved in ABA biosynthesis, was seen in the roots, where ABA may be produced in response to the environment (Fujita et al., 2006; Zhu, 2002).

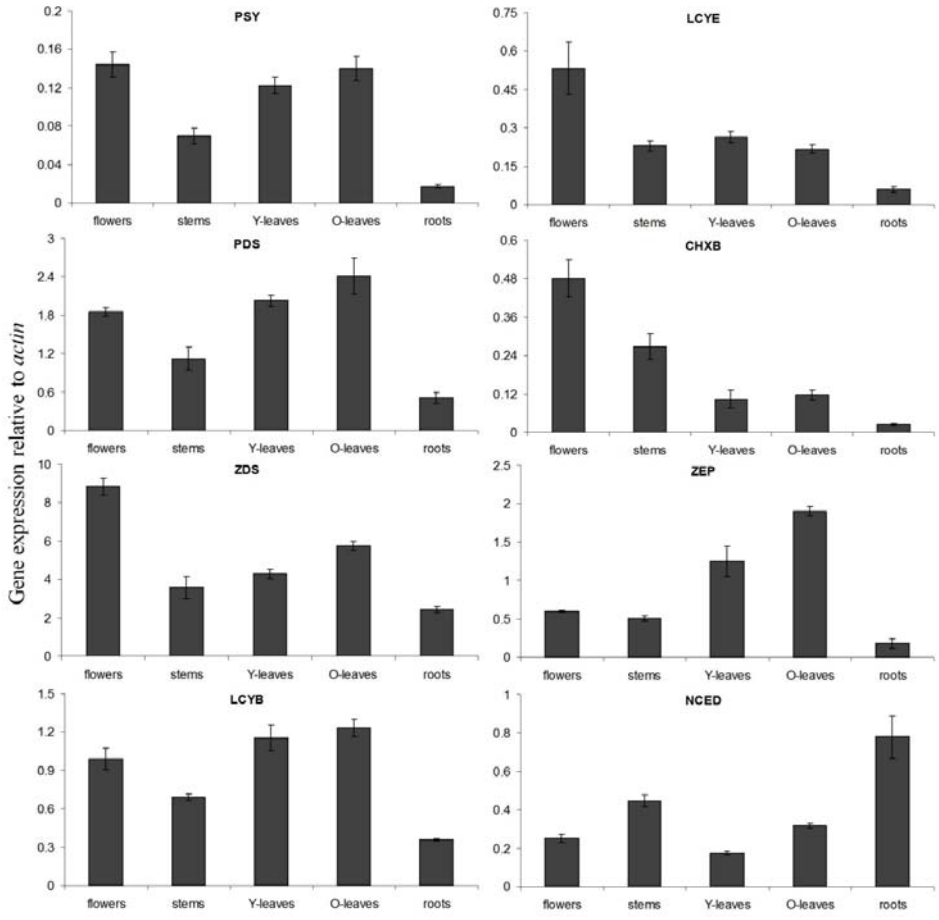

Figure 2: Expression of carotenoid biosynthesis genes in different organs of Chinese cabbage. The values and error bars represent the average and standard error from three independent reactions.

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Analysis of carotenoid accumulation in different organs of Chinese cabbage

The composition and content of carotenoids in the flowers, stems, young leaves, old leaves, and roots of Chinese cabbage were determined by HPLC (Table 2). Data indicate that carotenoids were found mostly in the flowers and leaves, and that the majority of carotenoids in Chinese cabbage were lutein and *β*-carotene, which are essential for photosynthesis. Lutein accumulation was the highest in the old leaves, where its concentration was 120.3 μg/g dry weight. The young leaves, flowers, and stems also contained a significant amount of lutein, viz., 101.87 μg/g, 72.44 μg/g, and 43.62 μg/g, respectively. This abundant accumulation of lutein may be responsible for the miniscule amount of its precursor, *α*-carotene, found in Chinese cabbage. Compared to *α*-carotene, *β*-carotene, which is the most potent dietary precursor of vitamin A, was synthesized in much higher amounts in Chinese cabbage. Specifically, *β*-carotene levels were highest in the old leaves and young leaves (103.93 μg/g and 87.13 μg/g, respectively), appreciable in the stems and flowers (31.95 μg/g and 24.99 μg/g, respectively), and lowest in the roots (0.16 μg/ g). High content of *β*-carotene may lead to the relatively high content of its *cis* isomers, namely, 9-*cis β*-carotene and 13-*cis β*-carotene, detected in Chinese cabbage. Chinese cabbage also contained a small amount of violaxanthin in the flowers (13.85 μg/g), old leaves (9.6 μg/g), and young leaves (8.21 μg/g), and only trace amounts of zeaxanthin were found.

In the present study, the expression of carotenoid biosynthesis genes was analyzed in different organs of Chinese cabbage. With the exception of *BrNCED,* which is involved in ABA biosynthesis, the genes were all expressed constitutively, with the highest level in the flowers or leaves, and the lowest, in the roots. This might explain the high accumulation of carotenoids in the flowers and leaves of Chinese cabbage. Carotenoids distribute mostly organs exposed to direct light (flowers, stems, and leaves), suggesting that light plays a role in carotenoid accumulation in Chinese cabbage. This is similar to previous studies in other plants in which the levels of carotenoids were very low in the underground organs (roots), but quite high in the flowers and leaves (Tuan et al., 2011a, b). There are very few plant species that synthesize and accumulate high levels of carotenoids in the roots such as carrot and sweet potatoes (Fuentes et al., 2012; Zhou et al., 2011). To date, some studies have shown that carotenoid biosynthesis in plants is highly regulated by light; however, the mechanisms are still unclear (Pizarro and Stange, 2009).

Table 2: Carotenoid composition and content in different organs of Chinese cabbage (μg·g^{-1} dry weight). Results are expressed as the mean (± standard error) (n = 3).

Carotenoids	Flowers	Stems	Young leaves	Old leaves	Roots
α-Carotene	0.61 (0.03)	0.28 (0.02)	0.57 (0.07)	0.57 (0.05)	N.D.
Lutein	72.44 (6.58)	43.62 (5.72)	101.87 (8.53)	120.3 (12.1)	0.48 (0.09)
β-Carotene	24.99 (1.18)	31.95 (3.35)	87.13 (1.16)	103.93 (2.78)	0.16 (0.05)
9-*cis β*-Carotene	5.84 (0.28)	6.2 (0.55)	18.15 (1.1)	18.1 (0.99)	0.06 (0.01)
13-*cis β*-Carotene	3.55 (0.23)	3.18 (0.44)	8.65 (0.52)	9.5 (0.75)	N.D.
Zeaxanthin	0.95 (0.14)	0.49 (0.1)	1.14 (0.1)	1.95 (0.15)	N.D.
Violaxanthin	13.85 (1.28)	3.57 (0.5)	8.21 (0.72)	9.6 (0.86)	0.09 (0.00)

N.D. = not detected

The leaves of Chinese cabbage contain significant amounts of lutein and *β*-carotene, compounds that may reduce the risk of stroke, heart disease, and cancer (Kritchevsky, 1999; Mayne, 1996). Epidemiological studies suggest that a diet rich in carotenoids is associated with reduced risk of heart disease and cancer (Melendez-Martinez et al., 2004). However, treating smokers with synthetic all-*trans* *β*-carotene showed no reduction in the incidence of lung cancer, and failed to affect cancer and cardiovascular disease (Hennekens et al., 1996; The Alpha-Tocopherol Beta Carotene Cancer Prevention Study Group, 1994). Furthermore, a 9-*cis* *β*-carotene-rich diet was shown to inhibit atherogenesis and fatty liver formation in mice (Harari et al., 2008). We found that Chinese cabbage has relatively high amounts of 9-*cis* *β*-carotene and 13-*cis* *β*-carotene; however, the biosynthetic mechanism of these *cis* isomers of *β*-carotene is still unknown in plants.

It has been well-documented that carotenoids are indispensable for human nutrition and health (Giovannucci, 1999; Krinsky et al., 2003; Mayne, 1996). Humans can not make carotenoids from endogenous precursors and only obtain them from diet. However, many daily food crops contain only trace to low amounts of carotenoids. Recently, trials in metabolic engineering of carotenoids in crops have been successful (Li and Van Eck, 2007). In "Golden Rice 2", the expression of maize *PSY1* in combination with the bacterial *crtI* gene result in the accumulation of *β*-carotene in rice endosperm up to 37 μg g^{-1} dry weight (Paine et al., 2005). A similar approach has been employed in successfully producing ''golden'' potato tubers containing an increase of *β*-carotene to a level of 47 μg g^{-1} dry weight (Diretto et al., 2007). Furthermore, overexpression of the bacterial *crtB* in a seed-specific manner led to 50-fold increase in total carotenoids in the oilseeds of canola (Shewmaker et al., 1999). On the other hand, the contents of *β*-carotene, zeaxanthin, violaxanthin, and lutein were enhanced by reducing the expression of lycopene epsilon-cyclase using RNAi in *Brassica napus* seeds (Yu et al., 2008). In this study, the relationship between carotenoid accumulation and expression of carotenoid biosynthesis genes in different organs may broaden our knowledge of the molecular mechanisms involved in carotenoid biosynthesis in Chinese cabbage. Understanding the regulation of carotenoid accumulation will facilitate metabolic engineering of carotenoid biosynthesis in Chinese cabbage, a popular vegetable in Asia.

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