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Short sequence-paper

cDNAs for the synthesis of cyclic carotenoids in petals of *Gentiana lutea* and their regulation during flower development

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Abstract

cDNAs encoding lycopene ε-cyclase, lycopene β-cyclase, β-carotene hydroxylase and zeaxanthin epoxidase were isolated from a *Gentiana lutea* petal cDNA library. The function of all cDNAs was analyzed by complementation in *Escherichia coli*. Transcript levels during different stages of flower development of *G. lutea* were determined and compared to the carotenoid composition. Expression of all genes increased by a factor of up 2, with the exception of the lycopene ε-cyclase gene. The transcript amount of the latter was strongly decreased. These results indicate that during flower development, carotenoid formation is enhanced. Moreover, metabolites are shifted away from the biosynthetic branch to lutein and are channeled into β-carotene and derivatives.

Keywords: Cyclic carotenoid; *Gentiana lutea*; Flower development

Carotenoids are antioxidative pigments. Due to their lipophilic properties, carotenes and their oxygenated derivatives, the xanthophylls, are integral components of photosynthetic membranes and other membranes [1]. In higher plants, they are synthesized and located in the chromoplasts and chloroplasts. In the latter organelle, they participate in photosynthetic light-harvesting and photoprotection [2]. Chromoplasts are typically found in certain fruit or flowers where the carotenoids serve as coloring agents to attract pollinators and animals for seed dispersal [3]. In flower petals, the chromoplasts originate by conversion of fully developed chloroplasts [4]. During this chloroplast–chromoplast transition, the chlorophyll concentration decreases and the carotenoid content increases. Sometimes, the carotenoid composition of chromoplasts is changed. This formation of carotenoids is important to build up the internal plastid ultrastructure [5]. During flower development, the total carotenoid pathway is upregulated at the transcriptional level. In several species, enhanced expression of the phytoene synthase gene *psy* is a major factor. This has been demonstrated for petals from tomato [6,7], marigold [8] and *Gentiana lutea* [9]. In *G. lutea*, upregulation of *psy* is accompanied by increase of the *zds* transcript and a decrease of the transcript of geranylgeranyl pyrophosphate synthase. First indications were obtained for a quantitative change in the xanthophyll patterns of *G. lutea* during floral development. This could imply an intrapathway regulation, redirecting the biosynthesis into the α- and β-branch of the pathway.

For a detailed molecular analysis, cDNAs for lycopene ε-cyclase *lcy-e*, lycopene β-cyclase *lcy-b*, β-carotene hydroxylase *bhy* and zeaxanthin epoxidase *zep* were isolated from a *G. lutea* petal cDNA library [9]. Amplification by PCR using a *G. lutea* petal cDNA as template was performed with degenerated primers that corresponded to the strongly conserved amino acid sequence of individual carotenogenic enzymes. Sequences of the oligonucleotide primers were as follows: for *lcy-e*, 5′-ATGGTITTYATGGAYTAYMGIGA-3′ (forward) and 5′-AAICCYTGCCACATCCAYTTIGG-3′ (reverse); for *lcy-b*, 5′-GTITGGGTIGAYGARTTYGA-3′ (forward) and 5′-ARRAAICCRTGCCARTA-3′ (reverse); for *bhy*, 5′-ATGGCIGTIATGGCIGTITAYTA-3′ (forward)

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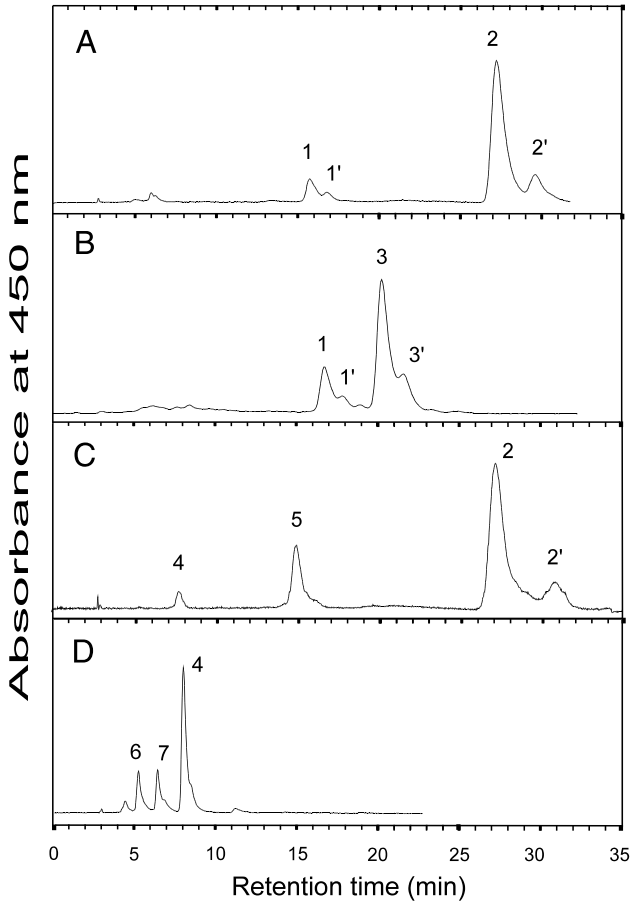

Fig. 1. HPLC separation of carotenoids from *E. coli*/pACCRT-EBI$_{Eu}$/pUC-lcyb with an established lycopene background and expressed lycopene cyclase-β cDNA (A), *E. coli*/pACCRT-EBI$_{Eu}$/pUC-lcye, with an established lycopene background and expressed lycopene cyclase-ε cDNA (B), *E. coli*/pACCAR16ΔcrtX/pUC-bhy with an established β-carotene background and expressed β-carotene hydroxylase cDNA (C) and *E. coli*/pACCAR25ΔcrtX/pBluescript-zep with an established zeaxanthin background and expressed zeaxanthin epoxidase cDNA (D). Separation was carried out on a Nucleosil C18 column with acetonitrile/methanol/2-propanol (85:10:5).

and 5′-ACYTCYTCIARYTCYTTIGG-3′ (reverse) and for *zep*, 5′-AAYTGGTAYTGYAARTTYGA-3′ (forward) and 5′-TCYTCDATIGCCATRCAICC-3′ (reverse) (where Y = T/C, M = A/C, R = A/G, D = A/G/T and I = inosine).

With PCR-amplified fragments confirmed by nucleotide sequencing as probes, cDNAs of *lcy-e* (AB017368), *lcy-b* (AB017367), *bhy* (AB027187) and *zep* (AB017370) were isolated from the *G. lutea* petal cDNA library. Comparison of the amino acid sequences deduced from the gentian cDNAs with those from other higher plants such as tomato, tobacco, *Arabidopsis*, bell pepper and daffodil revealed identities between 60.7% and 79.8% among the respective proteins (data not shown).

The functions of these cDNAs were analyzed by complementation of *Escherichia coli* in which either a lycopene, β-carotene or zeaxanthin background was established [10]. For this purpose, plasmids pUC-lcye, pUC-lcyb, pUC-bhy and pBluescript-zep were constructed as in frame fusions for high-level functional expression of the corresponding enzymes. After growth for 2 days of the *E. coli* transformants at 28 °C, carotenoids were extracted from both cultures and separated and identified by HPLC using appropriate reference compounds and by their absorbance spectra, which were recorded on-line (see Ref. [9] for details). Fig. 1 shows the HPLC separations. In the case of a lycopene background, residual lycopene (all-E peak 1, Z-isomer peak 1′) was observed together with β-carotene (trace A, peaks 2, 2′) in the presence of the lycopene β-cyclase, or with monocyclic δ-carotene carrying one ε-ionone end group (trace B, peaks 3, 3′) upon expression of the lycopene ε-cyclase. In the culture that expressed the β-carotene hydroxylase gene, β-carotene was converted to the monohydroxy derivative β-cryptoxanthin (trace C, peak 5) and the dihydroxy carotenoid zeaxanthin (peak 4). In *E. coli*, synthesizing zeaxanthin (trace D, peak 4) formation of monoepoxy antheraxanthin (peak 7) and diepoxy violaxanthin (trace 6) was demonstrated in the presence of expressed

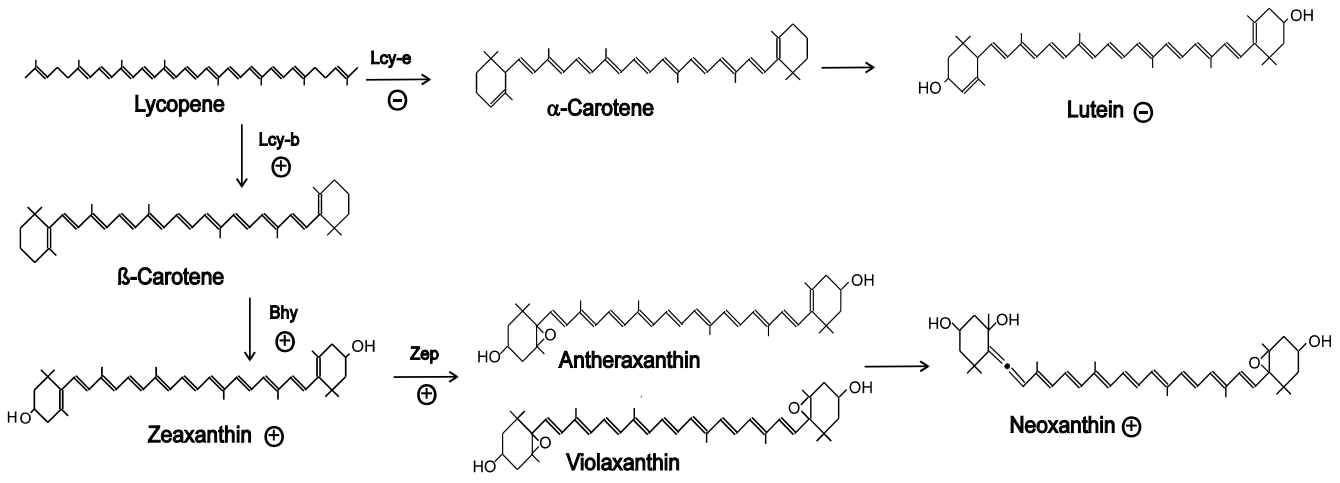

Fig. 2. Formation of cyclic carotenoids and their conversion into lutein and neoxanthin in *G. lutea*. Transcriptional upregulation of a reaction and increased proportional carotenoid levels during flower development are indicated by ⊕; downregulation and decreased levels are indicated by ⊖.

Table 1
Carotenoid composition during different stages (S1 to S5) of flower development[a] (A) and changes of expression levels of lycopene ε-cyclase, *lcy-e*; lycopene β-cyclase *lcy-b*; β-carotene hydroxylase, *bhy*; and zeaxanthin epoxidase (*zep*) (B)

	Stages of flower development				
	S1	S2	S3	S4	S5
(A)					
Total carotenoids (μg/g dw)	450	502	569	676	766
Percent distribution[b] of					
Neoxanthin	2.8	2.6	1.4	1.1	6.1
Violaxanthin	21.6	20.6	13.2	9.9	18.5
Antheraxanthin	10.8	11.0	9.8	9.0	13.1
Lutein	43.4	41.8	37.0	35.1	30.2
Zeaxanthin	2.5	3.8	10.7	7.9	11.1
β-cryptoxanthin	1.7	1.5	3.2	3.6	3.8
β-carotene	13.6	14.4	15.9	25.2	13.2
(B)					
Transcript levels (%)[c]					
lcy-e	100	83	104	16	43
lcy-b	100	88	98	101	190
bhy	100	127	140	127	193
zep	100	124	150	165	182

[a] The description of the individual stages of flower development is given in the text.
[b] Carotenoids were separated by HPLC on a polymeric Vydac 218TP54 C18 column with methanol as eluent. Differences to the sum of 100 are due to traces of α-carotene, β-cryptoxanthin and β-carotene epoxide. These values are means of three determinations using flowers from individual plants. The variation of the carotenoid values was within 10% of the absolute amounts.
[c] The transcript values are related to 25 S rRNA and are expressed in percent of the transcript amounts of stage 1.

zeaxanthin epoxidase. Structures and pathways of the carotenoids are shown in Fig. 2.

Floral development can be followed through five different stages [9], from hard buds less than 1.5 cm long (S1), to still closed buds of around 2 cm (S2), to open buds with yellow petals (S3), to opening flowers (S4) and to fully open and developed flowers (S5). During this maturation, the carotenoid content increases from 450 to 766 μg/g dry weight (Table 1A). A transient decrease was observed for neoxanthin, but at S5 the value was slightly higher than at S1. Violaxanthin and antheraxanthin decreased from S2 onwards but were almost back to the initial values at S5. Zeaxanthin as well as β-cryptoxanthin start from a low level and reached a four- or two-fold increase at S5. The distribution of β-carotene was rather constant except for S4 where it was increased. All these carotenoids belong to the β-branch of the biosynthetic pathway and among them neoxanthin and zeaxanthin exhibit the highest proportional increase during flower development. In contrast to these carotenoids, lutein is derived from α-carotene. The lutein pattern steadily decreases from 43.4% in S1 to 30.2% in S5. In parallel, expression of the lycopene ε-cyclase gene *lcy-e* and lycopene β-cyclase *lcy-b* involved in the formation of α- and β-carotene and expression of *bhy* and *zep* for conversion of β-carotene was determined for the five developmental stages (Table 1B). The Northern analysis was carried out as described [9], from 20 μg of total RNA in each case. As a control, 25 S rRNA was stained with ethidium bromide. All transcript values for individual genes were normalized with respect to the corresponding value of the 25 S rRNA. Expression values were given in relation to the expression levels of the S1 stage. They are means of three individual experiments with variations of less than 10%. In comparison to S1, transcript levels of all genes related to β-carotene formation and metabolism increased by a factor of about 2. However, the *lcy-e* transcript decreased to 43% from S1 to S5. Both antagonistic effects are responsible for higher relative zeaxanthin and neoxanthin concentrations at the expense of lutein. These changes of transcripts modulate the relative abundance of carotenoids from the α- or β-biosynthetic branch. They are rather moderate compared to the upregulation of rate-limiting phytoene synthase in the range of 10-fold, which leads to enhanced total carotenoid biosynthesis in various flowers during anthesis [6,9]. Since zeaxanthin is highly photoprotective [11] and its enhanced production in plastids [12,13] or nonplastidic membranes [14] increased tolerance against solar radiation, accumulation of higher concentrations of zeaxanthin in the fully developed flower can be regarded as a protective strategy to cope with photooxidation in light-exposed *G. lutea* petals.

In Fig. 2, the results on the interpathway regulation of carotenoid biosynthesis during chloroplast to chromoplast transition related to flower development in *G. lutea* are illustrated. In developed flowers, downregulation of chlorophyll biosynthesis is accompanied by upregulation of carotenoid biosynthesis. The pathway to lycopene is strongly enhanced mainly by increased expression of the phytoene synthase gene [9]. Especially the increased transcript levels of lycopene β-cyclase and decreased levels of lycopene ε-cyclase correspond very well with a diversion of the metabolic flow away from α-carotene and its hydroxylation product lutein and into the β-branch leading to a higher proportion of zeaxanthin.

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References

[1] D. Siefermann-Harms, Physiol. Plant. 69 (1987) 561–568.
[2] K.K. Niyogi, Annu. Rev. Plant Physiol. Plant Mol. Biol. 50 (1999) 333–359.
[3] G.E. Bartley, P.A. Scolnik, Plant Cell 7 (1995) 1027–1038.

[4] M.R. Marano, E.C. Serra, E.G. Orellano, N. Carrillo, Plant Sci. 94 (1993) 1–17.
[5] O. Emter, H. Falk, P. Sitte, Protoplasma 157 (1990) 128–135.
[6] P.D. Fraser, M. Truesdale, C.R. Bird, W. Schuch, P.M. Bramley, Plant Physiol. 105 (1993) 405–413.
[7] G. Giuliano, G.E. Bartley, P.A. Scolnik, Plant Cell 5 (1993) 379–387.
[8] C.P. Moehs, L. Tian, K.W. Osteryoung, D. DellaPenna, Plant Mol. Biol. 45 (2001) 281–293.
[9] C. Zhu, S. Yamamura, H. Koiwa, M. Nishihara, G. Sandmann, Plant Mol. Biol. 48 (2002) 277–285.
[10] N. Misawa, M. Nakagawa, K. Kobayashi, S. Yamano, K. Nakamura, K. Harashima, J. Bacteriol. 172 (1990) 6704–6712.
[11] A.A. Woodall, S.W.-M. Lee, R.J. Weesie, M.J. Jackson, G. Britton, Biochim. Biophys. Acta 1336 (1997) 33–42.
[12] T. Götz, G. Sandmann, S. Römer, Plant Mol. Biol. 50 (2002) 127–140.
[13] P.A. Davison, C.N. Hunter, P. Horton, Nature 48 (2002) 203–206.
[14] T. Götz, U. Windhövel, P. Böger, G. Sandmann, Plant Physiol. 120 (1999) 599–604.