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Astaxanthin Production in Transgenic *Arabidopsis* with chyB Gene Encoding β-carotene Hydroxylase

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Oxycarotenoids, produced through the oxidation of carotenoids, play critical roles in plants. This reaction is mediated by a specific enzyme, β-carotene hydroxylase, which adds hydroxyl groups to the β-rings of carotenes. To investigate the effect of the β-carotene hydroxylase gene (*Chyb*) on oxycarotenoid biosynthesis, we generated transgenic *Arabidopsis* plants that over-expressed *Chyb* under the control of a 35S promoter. Their levels of zeaxanthin and neoxanthin were two- to three-fold greater relative to the WT, while that of violaxanthin, a final product in the xanthophyll pathway, was 1.3-fold higher than the control. In contrast, the amount of β-carotene declined as much as 2.4-fold, depending on the particular transgenic line. Interestingly, astaxanthin was produced in the transgenics, but not in the WT. These data suggest that, with the aid of unknown factors in the host, carotenoids could be converted into metabolites in the astaxanthin biosynthetic pathway. Microarray analysis was used to identify several genes that were consistently up-or down-regulated in transgenic chyB leaves compared with the controls. Here, we also discuss possible modifications in leaf carotenoids, and the importance of these data from a nutritional standpoint.

Keywords: *Arabidopsis*, astaxanthin, ß-carotene hydroxylase, microarray, oxycarotenoids, transgenic plant

Carotenoids are pigments with antioxidative properties (Krinsky, 1989) that are involved in photoprotection, light-harvesting, and membrane stabilization (Havaux, 1998; Niyogi, 1999). Their critical roles are reflected in the tight regulation of carotenoid biosynthesis throughout plant development and during specific physiological events associated with environmental stresses (Davison et al., 2002).

Rapid progress in the study of the carotenoid biosynthetic pathway in plants can be attributed to pioneering work on carotenogenesis in bacterial and plant systems. For example, this pathway has now been defined and the sequences of genes encoding enzymes involved in carotenoid biosynthesis have been determined in the photosynthetic bacterium *Rhodopseudomonas capsulate* (Marrs, 1981; Armstrong et al., 1994). Misawa et al. (1990) also have reported on the sequences and functions of carotenogenic genes in the bacterium *Erwinia uredovora*. These bacterial carotenogenic genes are indispensable tools for elucidating the plant carotenoid biosynthetic pathway (see Sandmann, 1994, 2001; Hirschberg et al., 1997; and Cunningham and Gantt, 1998). Briefly, lycopene is metabolized either twice by β-cyclase to produce β-carotene or once by β-cyclase and ε-cyclase (lut2) to produce β-carotene. The β-carotene rings are hydroxylated by ε-hydroxylase (lut1) and β-hydroxylase to make lutein. β-carotene is hydroxylated twice by β-hydroxylase to produce zeaxanthin. The hydroxyl groups are then added to the ends of each ring, resulting in xanthophylls that become more polar than β-carotene. Zeaxanthin is epoxidated twice to make violaxanthin, which is subsequently modified to produce neoxanthin.

The biological activities of carotenoid types are varied, and must be further characterized. Lycopene appears to have a protective effect against prostate cancer (Gann and Khachik, 2003), while both lutein and zeaxanthin seem to help guard against age-related macular degeneration (Krinsky et al., 2003). Research on vitamin A deficiency has demonstrated the importance of staple foods enhanced in carotenoids and having provitamin A activity (Ye et al., 2000). A number of genes encoding enzymes in the carotenoid biosynthetic pathway have now been cloned and characterized from both plant and microbial sources (Cunningham and Gantt, 1998; Hirschberg, 2001; Fraser and Bramley, 2004). However, the regulatory mechanism of this pathway is not very well understood, especially where it pertains to which carotenoids are accumulated and at what levels. Such information is of particular importance because plant storage organs are a major part of the human diet.

The amounts and types of carotenoids that can be accumulated have been modified in transgenic plants by manipulating the carotenoid biosynthetic pathway (Fraser and Bramley, 2004). In particular, overexpression of phytoene synthase affects carotenoid levels in storage organs, resulting in increases in total carotenoid content in carrot roots (Hauptmann et al., 1997), tomato fruit (Fraser et al., 2002), canola seed (Shewmaker et al., 1999), and *Arabidopsis* seed (Lindgren et al., 2003). In transgenic lines over-expressing phytoene synthase, β-carotene levels are substantially increased, while the effects on other carotenoids and isoprenoids are varied. For example, in tomato fruit, lycopene levels rise in parallel with an increase in β-carotene and total carotenoids. In canola, α-carotene and phytoene are enhanced as a result of overexpression of a bacterial phy-

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toene synthase. In *Arabidopsis* seeds, lutein levels are increased significantly by overexpression of an endogenous phytoene synthase. This range of effects may reflect the different origins of the gene that encodes this enzyme. Moreover, bacterial and plant proteins may vary in their ability to form protein–protein complexes, leading to different balances of products. Superimposed on this effect is a decrease in the flux control coefficient of phytoene synthase through overexpression. This causes other steps in the pathway to become rate-limiting (Fraser et al., 2002).

Davison et al. (2002) have shown that increasing the expression of the β-carotene hydroxylase enzyme in *Arabidopsis* gives rise to its higher content in the xanthophyll cycle and of zeaxanthin in the chloroplast membrane; notably, this manipulation leads to improved tolerance by plants under conditions of high light intensity and elevated temperatures. In our present study with transgenic *Arabidopsis*, we investigated the molecular and biochemical characterization of an introduced *chyB* gene, which encodes the β-carotene hydroxylase enzyme in the zeaxanthin biosynthetic pathway, causing a specific, two-fold increase in the size of the xanthophyll cycle pool. We also used microarray analysis of transcript profiles to study the global changes in gene expression that occurred as a result of this transgenic modification.

MATERIALS AND METHODS

Arabidopsis Plant Growth

Arabidopsis thaliana plants were grown in Arabaskets (Beta Tech, Gent, Belgium) containing sterilized Technic n. 1 DueEmme soil (The Netherlands), under conditions of 21 to 25°C and a 14-h photoperiod supplied at 150 µmol photons m^{-2} sec^{-1} (OSRAM L36 w/11-860 Lumilux PLUS Recyclable, Germany).

Preparation of Transformation Vector Containing *Arabidopsis* chyB cDNA

The chyB cDNA fragment AY117225 (Arabidopsis Biological Resource Center) was received as *Escherichia coli* DH10B strain with AY117225 cDNA in the amp-resistant pchyB1 cloning vector. cDNA was excised from that vector by *NotI–SalI* digestion, then blunt-ended with a Blunting Kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) and ligated into the pBI 101 transformation vector (Mitsuhara et al., 1996) that was previously digested with *SmaI* (Fig. 1). This vector contained a chimeric P35S promoter that confers a high level of expression by foreign genes. Correct orientation of the chyB cDNA in various *E. coli* colonies transformed with the pBI101::chyB vector was checked by PCR, using chyB1, chyB2, and CaMV primers (Table 1).

Arabidopsis Transformation

Agrobacterium tumefaciens strain LBA4404, containing the helper plasmid pGV3101 that confers resistance to gentamycin, was transformed via electroporation with our pBI101::chyB transformation vector, or with the empty pBI 101 vector as the control. It was grown overnight at 28°C on 500 mL of a Luria broth (LB) medium containing 50 µg mL^{-1}

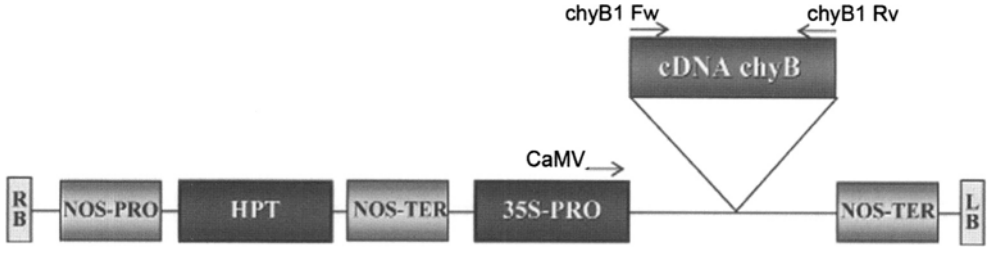

Figure 1. Construction of chyB overexpression vector for transforming plants of *Arabidopsis thaliana* var. Columbia. *SalI-NotI* fragment of *A. thaliana* chyB cDNA (AY117225 clone) was inserted into small pBI101 vector. Positions of primers used for confirming correct orientation of insert are indicated by arrows. Drawing is not to scale.

Table 1. Primers used in the cloning and analysis of gene expression

Primer	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size (bp)
chyB1	aaccgccgttacattcaaacc	acctacagggaaacgcttg	709
chyB2	ggtccaagaaacagcccatatgg	acatgttcgttcacgatggac	148
Actin	atggttgggatgggtcaaaaa	acggagctcgttgtagaaagt	220
T42644	gtggtccctcgctatcaaga	catcaagcgaggaaaaagga	214
AA586093	atggggcaatgaagatgaag	gaagaatccaaaggccatca	174
TC45368	gccggaagtggagcaaataa	atccgccaattgcttcttca	157
CK117631	cggtaagttcatgccgaaat	attaccttcgccgtcacaac	221
AY030867	cgcatatttccgcgtattct	tccaatttcacccgctttag	253
CB261784	agttcaaaggcgcaatgtct	caacaaccatggaacacataca	183
NM 115370	catcgatcctcttcgctctc	aggtgacacaccgttcttcc	154
AI099556	tgttgccggaggagttattc	atgtttgtttttcgggaacg	243
AK175255	tttcaaattcgggaaactcg	ccctccgacaacagacaaat	240
35S chyB	gcatcgacttcaaggaggac	acctacagggaaacgcttg	915

kanamycin (kan) and 25 μg mL^{-1} gentamycin. The bacterial cultures were centrifuged at 4000 *g* for 10min, and the pellets were re-suspended in sterilized infiltration buffer, to a final OD_{600} of 0.8. These bacteria were then used for transforming *Arabidopsis thaliana* var. 'Columbia' by the floral dip technique (Clough and Bent, 1998; Desfeux et al., 2000). T_1-transformed seeds were plated on MS (Murashige and Skoog, 1962) plates containing 1% plant agar (Duchefa, Amsterdam, The Netherlands) and 50 μg mL^{-1} kan. After they acquired their first true leaves, the T_1 kan-resistant plants were transferred to soil, and T_2 seeds were collected from single T_1 plants.

RT-PCR Analysis

Total RNA was extracted from *Arabidopsis* leaves with Trizol® reagent (Invitrogen, Carlsbad, CA, USA). RT-PCR amplification reactions were performed with an Access RT-PCR Kit (Promega Biosciences, San Luis, CA, USA), according to the manufacturer's instructions. Transcript levels for chyB were analyzed in transformed pBI101::chyB plants by amplifying the 709-bp chyB cDNA fragment with the primers listed in Table 1. After microarray analysis, mRNA expression profiles were evaluated via RT-PCR amplification of cDNA fragments for HKU07 (≈210 bp), HKU14 (≈170 bp), HKU22 (≈150 bp), HKU27 (≈220 bp), HKU31 (≈150 bp), and HKU56 (≈240 bp), using the primers shown in Table 1. Ein with actin (≈220 bp) served as the endogenous control.

Pigment Extraction and Saponification

Arabidopsis tissues (approx. 3 g) were cut and transferred to a round flask containing 25 mL of acetone and 25 mL of a methanolic solution of KOH (10% w/v) under nitrogen. This solution was used not only for hydrolysis of the esterified carotenoids, but also for shearing the petals to enhance pigment extraction. After 12 h, the pigments were transferred to diethyl ether in a separatory funnel and the extract was washed four times with distilled water to remove any trace of alkali. Finally, the ether extract was dried in a rotary evaporator set below 35°C.

High-Performance Liquid Chromatography Analysis

HPLC analyses were carried out on an Agilent 1100 system equipped with a quaternary pump, photodiode array detector, and column temperature control module (Agilent, Palo Alto, CA, USA). A 20 μL loop and C_{30} column (5 μm, 250×4.6 mm) (YMC, Wilmington, NC, USA) were used under conditions of 17°C and a flow rate of 1 mL min^{-1}. The diode array detector was set at 430, 450 and 486 nm. Methanol (MeOH), *tert*-butyl methyl ether (TBME), and water were used in the mobile phase, and the gradient elution was as described previously (Mouly et al., 1999, Meléndez-Martínez et al., 2005a, b): 0 min, 90% MeOH + 5% TBME + 5% water; 12 min, 95% MeOH + 5% TBME; 25 min, 89% MeOH + 11% TBME; 40 min, 75% MeOH + 25% TBME; 60 min, 50% MeOH + 50% TBME; and 62 min, 90% MeOH + 5% TBME + 5% water. The MeOH and TBME contained a small amount of butylated hydroxytoluene (BHT) (0.1%) and triethylamine (0.05%) to protect the carotenoids during chromatographic analysis (Hart and Scott, 1995).

cDNA Microarray Analysis

Total RNA was extracted from the leaves with TRIZOL reagent (Life Technologies, U.S.A.) according to the manufacturer's instructions. These tissues were purified by passing them through an RNeasy column (Qiagen). After checking its quality by gel electrophoresis (Agilent Bioanalyzer, Agilent Technologies), 40 μg of total RNA was reverse-transcribed with the oligo dT12-18 primer and aminoallyl-dUTP. The synthesized cDNA was then dye-labeled (NHS-ester Cy3 or Cy5; Amersham Biosciences, USA) according to the method described by Hughes et al. (2001). The labeled cDNA was applied to the DNA microarray (*Arabidopsis* microarray; Agilent Technologies) and hybridized at 60°C for 17 h. After washing, the microarray was scanned on a ScanArray 5000 (GSI Lumonics) and the image was analyzed using QuantArray software (GSI Lumonics). Signal intensity for each spot was calibrated by subtracting the intensity of the negative control. In analyzing these data, genes with expression levels >500 were taken into account, i.e., genes were considered to be up-regulated in the transgenics if they had a signal intensity ratio >2.0 (transgenic:WT) or down-regulated if that ratio was <0.5.

RESULTS

Analysis of *A. thaliana* 35S::chyB Lines

In all, 30 plants of *Arabidopsis thaliana* ecotype 'Columbia' (Col) were transformed with *Agrobacterium*, then grown and allowed to self-fertilize before their T_1 seeds were collected. Ninety-one T_1 plants with resistance to kanamycin (k^r trait) were analyzed by PCR for integrity of the inserted transgene (Fig. 2), using primers that annealed to the 35S and chyB sequences. Presence of the intact transgene was confirmed in 20 T_1 lines. Their T_2 seeds were collected and tested for segregation of the k^r trait. In eight T_2 lines, the segregation ratio of k^r:sensitivity to kan (k^s) was 3:1, thereby confirming a single insertion site (Table 2). From each of these, 20 T_3 seed families were tested for segregation of the k^r trait. These T_3 lines, homozygous for one insertion site, were isolated and propagated for further analysis. Among the eight homozygous 35S::chyB lines, those of 2/4, 7/3, 9/8, and 13/2 showed increased mRNA accumulations relative to the WT 'Columbia' (Fig. 3).

Changes in Carotenoid Contents from *A. thaliana* 35S::chyB Line

To determine the xanthophyll profiles in transgenic plants, carotenoid extractions and HPLC analysis were conducted

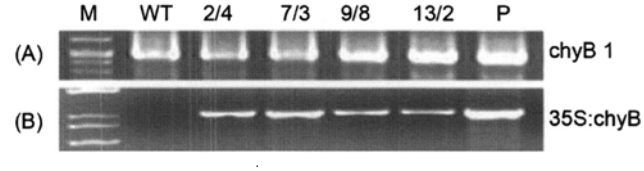

Figure 2. PCR analysis for integrity of inserted transgene. Primer sequences are listed in Table 1.

Table 2. Segregation analysis of *Arabidopsis thaliana* transformed T2 seedlings

T_2 lines	Seedlings			X^2 - values (k^r:k^s=3:1)
	Total	k^r	k^s	
2/4	180	131	49	0.47
3/5	169	131	38	0.51
7/3	177	131	46	0.12
9/8	88	64	24	0.24
11/7	308	225	83	0.62
13/2	141	105	36	0.04
14/5	122	91	31	0.01
17/2	187	146	41	1.02
18/1	227	180	47	2.34

Segregation of k^r trait in T_2 35s:chyB seedlings. For each T_2 line, the total number of seedlings tested and the number of seedlings showing either k^r or k^s trait have been reported. The hypothesis of a segregation ratio k^r:k^s=3:1 has been accepted in all these T_2 lines with the X^2-test (X^2-value in the last column) with 0.05 significance level.

Table 3. Content of carotenoids profiles of 35S::chyB line and wild type Columbia

Component	RT(min)	Contents (nmol/g_{DW})	
		Wild type	35S::chyB lines
Neoxanthin	9.92	40.3	138.1
Violaxanthin	12.00	217.6	246.9
Astaxanthin	18.21	0	19.1
Lutein	19.40	1628.9	1456.4
zeaxanthin	26.76	79.2	172.2
β-carotene	28.92	514.6	207.2
cis-β-carotene	29.63	104.3	34.9
Total		2584.9	2274.8

with the homologous transgenic line (Table 3). There, the amount of carotenoids was increased by 2- to 3-fold; by 1.8- to 2.1-fold for zeaxanthin and violaxanthin; and by 3.4-fold for neoxanthin, the final product in the xanthophyll pathway. In contrast, the level of β-carotene decreased by up to 2.4-fold relative to the WT. β-carotene, an initial product in the xanthophyll pathway, is synthesized to β-cryptoxanthin and zeaxanthin by β-carotene hydroxylase. Therefore, the amount of β-carotene could decline due to the expression of *chyB*. In addition, we detected higher levels of astaxanthin in the transgenics, but not in the WT plants. However, no difference in lutein amounts was seen between genotypes.

Microarray Analysis of Transgenic Line

To demonstrate the effects of transformation at the transcript level, we took a microarray approach. Expression patterns in three biological replicates of chyB-expressing leaves (Lines chyB 2/2, chyB 7/3, and chyB 13/2) were compared with empty-vector control lines. Following data-processing, several genes were shown to be consistently expressed, and were significantly up-regulated or down-regulated relative to the controls (Table 4). Our filtering strategy selected changes based on low *P* values (<0.05), combined with the fold-change in expression, for signals that were significantly greater than the background noise. The results of these microarray experiments were confirmed by semi-quantitative RT-PCR for several of the most significant changes identified here (Fig. 4). Expression patterns for those genes also were determined in the developing chyB transgenic lines and compared with the controls.

Some of the genes up-regulated in the transgenic plants included HKU 22 (encoding fibrillin), HKU 27 (mitogen-activated protein kinase), and HKU 31 (chalcone flavanone isomerase), while expression levels appeared either unchanged (HKU 07) or only moderately changed, i.e., HKU 14 and HKU 56 (Fig. 4). Finally, expression levels for some genes decreased in the transgenic plants relative to the controls.

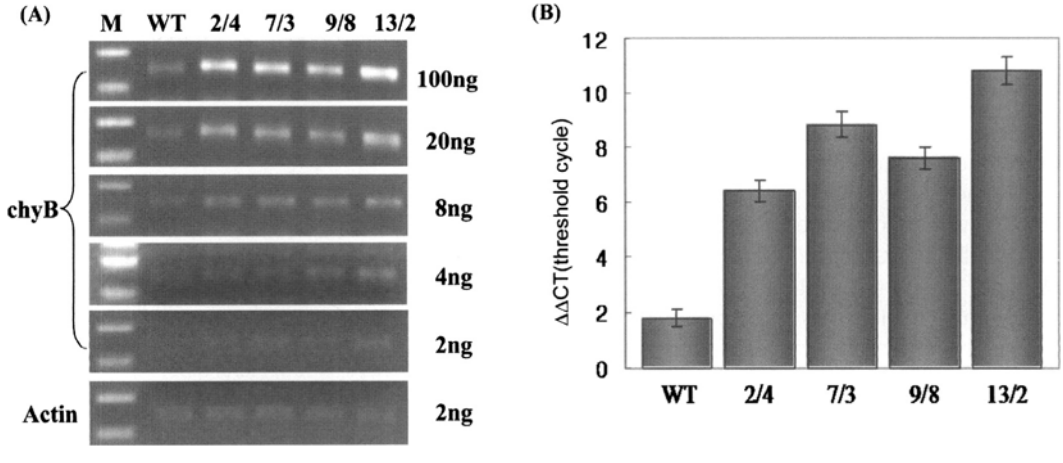

Figure 3. Expression of chyB in *A. thaliana* 35S::chyB lines.
A, RT-PCR amplification of chyB transcript in wild-type 'Columbia' line and T3 lines homozygous for single insertion event of transgene. Amplification of actin served as control for equal loading of RNA in each sample. **B**, Quantitative RT-PCR analysis. CT values were calculated using actin expression level as control. Error bars show standard error of mean for 3 replicate measurements. Primer sequences are listed in Table 1.

Table 4. Clones that are consistently expressed at higher or lower levels in 35S::chyB line the empty-vector controls

Clones	Gene ID	Fold ±expression	Description
HKU07	T42644	11.20	Hypothetical protein DKFZp566N1047.1-human (fragment)
HKU14	AA586093	5.03	Arabidopsis thaliana cDNA clone 66G9XP 3-, mRNA sequence
HKU22	TC45368	4.71	Fibrillin homologue CDSP34 precursor chloroplast.
HKU27	CK117631	3.70	Arabidopsis thaliana cDNA clone mitogen-activated protein kinase
HKU29	AY080867	3.77	Arabidopsis thaliana At1g36280 mRNA sequence
HKU30	CB261784	4.02	Arabidopsis thaliana cDNA clone MPIZp767N2115Q 5-PRIME, mRNA sequence
HKU31	NM_115370	4.13	Chalcone-flavanone isomerase / chalcone isomerase (CHI), identical to SP|P41088
HKU51	AK117363	3.25	Arabidopsis thaliana At3g11020 mRNA for putative DREB2B transcription factor
HKU56	AI099556	3.24	Arabidopsis thaliana cDNA clone 156A23XP 3-, mRNA sequence
HKU59	AK175255	3.25	Arabidopsis thaliana mRNA for putative dirigent protein,

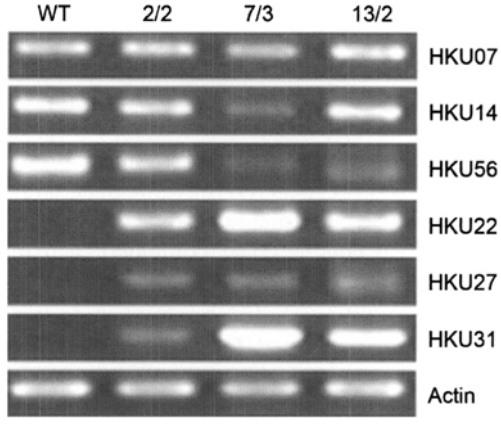

Figure 4. Semi-quantitative RT-PCR analysis of expression levels for candidate genes identified by microarray analysis in developing leaves from 35S::chyB line and wild-type control. Primer sequences are listed in Table 1.

DISCUSSION

There is growing interest worldwide to manipulate carotenoid biosynthesis in plants. All carotenoid species that contain a β-ring can be converted to retinol and, thus, are precursors of vitamin A. Here, we also investigated the effects of *chyB* overexpression on other transcripts in *Arabidopsis* in order to identify functions that might regulate this biosynthesis.

Based on our results from the HPLC analysis of leaves, the amounts of xanthophyll-cycle carotenoids -- violaxanthin and zeaxanthin – were significantly increased relative to the WT, while β-carotene levels decreased (Table 3). Davison et al., (2002) have demonstrated that the pool of these particular carotenoids, as well as tolerance to heat stress, can be increased dramatically in *Arabidopsis* transformed with the β-hydroxylase gene, a report consistent with our data. Furthermore, astaxanthin, which has not previously been detected in the xanthophyll cycle, was also found to be synthesized here. Two different enzymes, β-hydroxylase and C-4-oxygenase, are generally necessary for its synthesis from β-carotene. Astaxanthin synthesis also occurs in *E. coli* with the C4-oxygenase of *Haematococcus* (Lotan and Hirschberg, 1995; Breitenbach et al., 1996), in transgenic tobacco with the *Haematococcus CrtO* gene (Mann et al., 2000), and in transgenic *Synechococcus* with the *Cyanobacteria CrtO* gene (Harker and Hirschberg, 1997). This synthesis in transgenic organisms might result from hydroxylation and ketolation reactions with C4-oxygenase or β-hydroxylase, which are present in both the host and the transgene in those organisms. However, our report is the first to demonstrate the presence of C4-oxygenase in *Arabidopsis*. Here, the biosynthesis of astaxanthin was possibly generated due to the activation of C4-oxygenase by the accumulation of metabolites, which was prompted by the overexpression of β-hydroxylase. Alternatively, the *chyB* gene may have the same function as that for C4-oxygenase. In fact, a hydroxylase gene has shown ketolase activity in *Haematococcus* (Lotan and Hirschberg, 1995; Breitenbach et al., 1996). Changes in its transcript levels have now been analyzed in transgenic plants and in empty-vector control lines using the 37,000 gene microarrays available from an oligo chip of *Arabidopsis* (Agilent). One of these up-regulated cDNAs encodes fibrillin, a protein involved in carotenoid storage (Rey et al., 2000). Although not previously reported in *Arabidopsis*, its up-regulation by enhanced carotenogenesis is entirely consistent with its known role. Nevertheless, functions of the other up-regulated cDNAs are not as well-characterized. HKU 27 does contain a conserved protein kinase domain, suggesting that it has a regulatory role in carotenogenesis.

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