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ORIGINAL ARTICLE

Characterization of a β-carotene hydroxylase of *Adonis aestivalis* and its expression in *Arabidopsis thaliana*

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Abstract Carotenoids are plant secondary metabolites that comprise two main groups: carotenes and xanthophylls. The latter group includes zeaxanthin which is synthesized by β-carotene hydroxylase catalyzing the hydroxylation of the β-rings of β-carotene molecules. To develop tools to alter carotenoid biosynthesis in plants, we isolated a cDNA clone encoding a candidate β-carotene hydroxylase, *CrtH*1, from the flower petals of *Adonis aestivalis*. CrtH1 protein has homology to β-carotene hydroxylases from other organisms, and possesses the four histidine motifs conserved in this family of enzymes. Sequence analysis predicted the presence of a putative plastid transit peptide at the amino terminus and four transmembrane helical regions. Southern-blot analysis showed *CrtH*1 to be encoded by a multicopy gene family with at least three members in *A. aestivalis*. Analysis of *CrtH*1 transcript abundance by Northern blotting indicates it is highly expressed in flower petals, roots and stems, with relatively low expression in leaves and developing seeds. CrtH1 was able to catalyze the formation of zeaxanthin and its intermediate precursor β-cryptoxanthin from β-carotene in functional assays conducted in *E. coli*. Expression of *CrtH*1 in *Arabidopsis thaliana* wild type and a mutant deficient for endogenous β-carotene hydroxylases enhanced the biosynthesis of violaxanthin in the seeds.

The sequence of the β-carotene hydroxylase, CrtH1, has been submitted to Gene Bank, accession #EF120636.

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Abbreviations

BHT	Butylated hydroxytoluene
DEPC	Diethylpyrocarbonate
IPTG	Isopropyl-β-D-thiogalactopyranoside
ORF	Open-reading frame
TAG	Triacylglyceride

Introduction

Carotenoids are ubiquitous secondary metabolites and pigments responsible for some organ coloration in plants and microorganisms (Ausich 1997). Carotenoids are used as colorants in the food industry (Fraser 1997) and as essential supplements in livestock and fish feed formulations (Johnson and Schroeder 1996). The importance of carotenoids in human health is well documented and includes roles as antioxidants with potential anti-cancer therapeutic properties (Mayne 1996).

Carotenoids are synthesized in all photosynthetic plants, algae and cyanobacteria, and act as important components of the photosynthetic machinery, where they act as photoprotecting antioxidants and regulate membrane fluidity (Umeno et al. 2005). Many non-photosynthetic bacteria (*Erwinia herbicola, Thermus aquaticus*) and fungi (*Neurospora crassa*) also synthesize carotenoids. In plants, carotenoids are synthesized and localized together with chlorophyll in chloroplasts, or alone in the chromoplasts of flowers, fruits and other tissues (Giuliano et al. 2000; Römer et al. 2002). Based on their structural features, carotenoids are divided into two major groups: carotenes and

xanthophylls. Carotenes are hydrocarbons that are either linear, like lycopene, or cyclized, like α-carotene and β-carotene. Xanthophylls are oxygenated carotenes, which can contain hydroxyl, epoxy or keto groups, and include lutein, zeaxanthin, violaxanthin and astaxanthin (Tian and DellaPenna 2001). Xanthophylls comprise most of the carotenoid pigment in the photosynthetic tissues of plants (Cunningham and Gantt 1998) and contribute to the assembly and stability of pigment-protein complexes, light-harvesting and photoprotection in chloroplasts (Hirschberg 2001; Rissler and Pogson 2001). Xanthophylls are also precursors for the biosynthesis of the phytohormone abscisic acid (ABA) (Giuliano et al. 2000).

Carotenoids are derived from the isoprenoid pathway in which the condensation of five-carbon isoprenoid units to form phytoene represents the first committed step in carotenoid biosynthesis (Taylor and Ramsay 2005). This is followed by the desaturation of phytoene to lycopene and two cyclization reactions converting lycopene to either α- or β-carotene (Cunningham and Gantt 1998). Hydroxylation of cyclic carotenes at the 3C, 3′C positions can be carried out by two types of hydroxylases: β- and ε-hydroxylase, which add a hydroxyl group to β- and ε-ring, respectively. Consequently, β-carotene is converted to zeaxanthin and α-carotene to lutein (Fig. 1; Giuliano et al. 2000; Hirschberg 2001; Tian and DellaPenna 2004). Lutein (3R, 3R′-β, ε-carotene-3, 3′-diol) is the most abundant carotenoid in all plant photosynthetic tissues and protects the photosynthetic system from damage by strong light. Zeaxanthin (3R, 3R′-β, β-carotene-3, 3′-diol) plays an important role in non-photochemical quenching, which helps to protect plants against damage from singlet oxygen and other reactive oxygen (Rissler and Pogson 2001). Zeaxanthin also acts as a lipid stabilizer and protects against lipid peroxidation (Rissler and Pogson 2001; Müller-Moulé et al. 2003). Transgenic *Arabidopsis thaliana* plants overexpressing β-carotene hydroxylase are more tolerant to conditions of high light and high temperature (Davison et al. 2002).

The genes encoding β-carotene hydroxylases have been cloned from bacteria, green algae and higher plants (Hundle et al. 1993; Sun et al. 1996; Bouvier et al. 1998; Linden 1999; Kim et al. 2001; Tian and DellaPenna 2001). Two β-carotene hydroxylases have been identified in *A. thaliana* (Sun et al. 1996; Tian and DellaPenna 2001) and both acted effectively on the β-ring but poorly on the ε-ring. Sun et al. (1996) suggested that β-carotene hydroxylase of *A. thaliana* normally associates with a second β-carotene hydroxylase, or with an ε-hydroxylase, to form a dimer, and a portion of the N-terminus mediates these interactions. Bouvier et al. (1998) isolated two β-carotene hydroxylases from pepper fruits. Both enzymes were shown to be ferredoxin dependent proteins, and the histidine motifs conserved among this group of enzymes were suggested to be the iron ligands essential for activity. In-vitro reconstitution experiments indicated that pepper β-carotene hydroxylase used iron-activated oxygen to break the C–H bond with the formation of a double bond or oxygen insertion (Bouvier et al. 1998).

Flowers of *Adonis aestivalis* are known to accumulate high levels of xanthophylls (Seybold and Goodwin 1959)

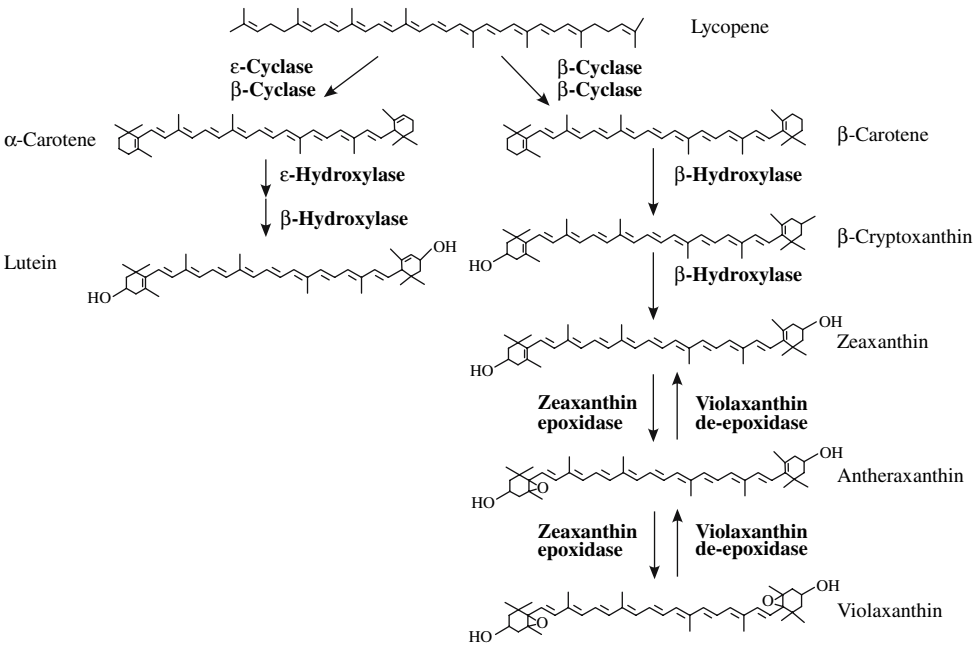

Fig. 1 Carotenoid biosynthesis pathway in plants

and are therefore an ideal source of genes involved in synthesizing and modifying these metabolites. However, no β-carotene hydroxylases have so far been cloned from this plant species. In an effort to develop tools and strategies for altering the carotenoid profiles of plants, we cloned a cDNA, *CrtH*1, from the flower petals of *A. aestivalis*. The *CrtH*1 cDNA encodes a predicted protein with homology to published β-carotene hydroxylases that catalyze the hydroxylation of the β-rings of β-carotene to give rise to zeaxanthin. The biochemical function of CrtH1 was determined by functional assays in a β-carotene-producing *E. coli* strain. The carotenoid profile of *A. thaliana* seeds was significantly altered upon the expression of *CrtH*1 in *A. thailana* wild type and a mutant deficient in both endogenous β-carotene hydroxylases (Tian et al. 2003). These results are discussed in relation to the possible use of *CrtH*1 to engineer carotenoid metabolism in plants.

Materials and methods

Plant materials

Seeds of *A. aestivalis* L. were purchased from Thompson & Morgan Ltd. (Ipswich, Suffolk, UK). *A. aestivalis* plants were grown in RediEarth (W.R. Grace & Co., Ajax, Canada) soil in a controlled greenhouse environment (16 h light/8 h dark, 20/17°C).

Wild type *Arabidopsis thaliana* (ecotype Wassilewskija) and homozygous *A. thaliana* b1b2 mutant with T-DNA knockouts in two β-carotene hydroxylase genes (Tian et al. 2003) were grown in Co–Co soil-less mixture under controlled greenhouse environment (16 h light/8 h dark, 20°/17°C). Seeds of *A. thaliana* b1b2 mutant were kindly provided by Dr. Dean DellaPenna (Michigan State University, East Lansing, MI, USA). *A. thaliana* transformation was carried out according to the floral dip procedure described by Clough and Bent (1998). Transgenic plants were selected by spraying with the herbicide glufosinate at 150 g/l (Liberty), and the presence of the transgene was confirmed by PCR using forward primer, 5′-GCTTTC TTCATCGGTGATTGATTCC-3′ and reverse primer, 5′-GCGAGCTCTAAGGCATTCATACGCTTTAT TCTTC-3′.

Chemical standards

β-carotene and lutein were purchased from Sigma (Oakville, Canada). β-cryptoxanthin, zeaxanthin and violaxanthin were purchased from CaroteNature (Lupsingen, Switzerland).

Cloning of *CrtH*1 cDNA of *Adonis aestivalis*

Degenerate primers for amplifying *CrtH*1 were designed based on the conserved domains of β-carotene hydroxylases of various organisms: forward primer, 5′-GTTGG(C/T)GCTGC(C/T)GT(A/T)GG (A/T/G)ATGGA-3′; and reverse primer, 5′-(C/T)TT (A/G)TC(A/T)GTGTGGTG(A/T)AGCTGGTG(A/ G)G-3′. These primers were used to amplify a fragment of *CrtH*1 by PCR using as template cDNA synthesized from RNA isolated from the flower petals of *A. aestivalis* according to the manufacture's instruction for reverse transcriptase (Invitrogen, Burlington, Canada). A 363 bp PCR product was blunt-end cloned into the *Eco*RV site of pBluescipt KS (+) vector, and sequenced. The 5′- and 3′-ends of the *CrtH*1 cDNA were isolated by employing 5′- and 3′-rapid amplification of cDNA ends (RACE) techniques according to the manufacturers' instructions (5′-RACE system for rapid amplification of cDNA Ends, Version 2.0, Invitrogen; and 3′-RACE, First Choice RLM-RACE, Ambion, Austin, TX, USA).

Sequence analysis of *CrtH*1

Alignment of the predicted amino acid sequence encoded by the *CrtH*1 cDNA and its phylogenic relationship with β-carotene hydroxylases from various organisms was conducted using Vector NTI program (InforMax, Invitrogen). The chloroplast transit peptide was predicted using the online software Chloroplast v1.1 (http://www.cbs.dtu.dk/services/ChloroP/). Transmembrane helices were predicted using TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

DNA isolation and Southern-blot analysis

Total genomic DNA was isolated from leaves of *A. aestivalis* using DNeasy Plant Mini Kit (Qiagen, Mississauga, Canada). Approximately 10 μg of genomic DNA was digested with each of *Bam*HI, *Hind*III, *Kpn*I, *Spe*I and *Sst*I restriction endonucleases, separated on a 0.8% agarose gel and transferred to Hybond-XL membrane (Amersham Biosciences, Quebec, Canada). The blot was probed with a 201 bp ^{32}P-labeled *CrtH*1-specific fragment obtained by PCR using the forward primer 5′-AGAAGGAGATAG AGCGAAGAATAAAGCGT-3′, and the reverse primer 5′-ACATGTACACGTTGTGAGTACGAC TCTT-3′. Hybridization was performed with Church buffer (Church and Gilbert 1984) at 61°C for 22 h. The filter was washed twice in 2× SSC, 0.1% SDS for 10 min at 61°C and followed by washing twice in

0.2× SSC, 0.1% SDS for 10 min at 61°C. The filter was then exposed to an X-ray film with an intensifying screen at −70°C for 7 days.

RNA isolation and Northern-blot analysis

Total RNA was isolated from *A. aestivalis* leaves, flower petals, roots, stems and developing seeds as described by Carpenter and Simon (1998) with some modifications. After grinding in liquid nitrogen, about 100 mg of tissue was extracted with 600 ml of RNA extraction buffer (0.2 M Tris–HCl, pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS) and an equal volume of Tris–HCl buffered phenol (pH 7.9). Extraction was repeated twice with phenol and followed once with chloroform. Approximately 1/4 volume of 10 M LiCl was added to the aqueous layer, mixed well, stored at 4°C overnight and then centrifuged at 14,000*g* for 20 min. The pellet was resuspended in 0.3 ml of DEPC-treated dH_2O followed by 30 µl of 3 M sodium acetate (pH 5.3) and 0.7 ml of 95% ethanol. The mixture was chilled at −70°C for 10 min and then centrifuged at 14,000 *g* for 20 min. The pellet was washed with 75% ethanol and re-suspended in 20 µl of DEPC-treated dH_2O. A total of 15 µg of total RNA was electrophoresed on 1.2% agarose gel containing formaldehyde and transferred onto Hybond-XL membrane (Amersham Biosciences). The hybridization method and probe used were as described for Southern-blot analysis.

Vector construction for functional assays in *E. coli* and *A. thaliana*

Plasmid pACCAR16ΔcrtX containing *Erwinia uredovora* genes required to produce β-carotene was constructed by Misawa et al. (1995) and was kindly provided to us by Dr. Hartmut Linden (Universitaet Konstanz, Germany). This plasmid was introduced into *E. coli* JM101 strain by electroporation. The open reading frame (ORF) of *CrtH*1 was amplified by PCR using the forward primer, 5′-gc**ggatcc**aATGCTAGCT TCAATGGCAGCGGCA-3′, which incorporates a *Bam*HI site (in bold) and the reverse primer, 5′-gc**gagctc**CTATAAGGCATTCATACGCTTTATTCTTC-3′, which incorporates a *Sac*I site (in bold). The PCR product was directionally cloned between the *Bam*HI and *Sac*I sites of pBluescriptII KS (+) vector to produce plasmid pBS-CrtH1, and then introduced into the JM101 strain carrying plasmid pACCAR16ΔcrtX. The empty cloning vector pBluescriptII KS (+) was also introduced into the JM101 strain with plasmid pACCAR16ΔcrtX as a negative control.

For expression of *CrtH*1 in *A. thaliana* under the control of the seed-specific napin promoter from *Brassica napus* (Rask et al. 1998), the 930 bp ORF fragment of *CrtH*1 was amplified by PCR using the following forward and reverse primers having built-in *Bam*HI and *Sac*I sites, respectively: forward 5′-GCGGATCCAA TGCTAGCTTCAATGGCAGCGGCA-3′, and reverse 5′-GCGAGCTCCTATAAGGCATTCATACGCTT TATTCTTC-3′. The PCR product was digested with *Bam*HI and *Sac*I and ligated between the *Bam*HI and *Sac*I sites of pBluescriptII KS (+) vector, in which the napin promoter of *Brassica napus* was cloned between the *Hind*III and *Bam*HI sites. A ~2.1 kb fusion fragment of the napin promoter and the ORF of *CrtH*1 was then excised by digestion with *Hind*III and *Sac*I, and cloned between the *Hind*III and *Sac*I sites of an in house-built vector, p79–103, harbouring a *BAR* gene for glyphosinate selection in plants (Fig. 2).

Functional assay in *E. coli* and HPLC analysis

Assay of CrtH1 activity in *E. coli* and HPLC analysis were adapted from Misawa et al. (1995) and Shewmaker et al. (1999). Cultures of *E. coli* JM101 carrying different plasmids were grown in LB broth with antibiotics (34 µg/ml chloramphenicol and 150 µg/ml ampicillin) in the dark at 28°C overnight. For each *E. coli* strain, 2 ml of the overnight culture was used to inoculate 75 ml of LB broth media containing antibiotics. These cultures were grown in the dark at 28°C for 3 h, and then IPTG was added to a final concentration of 0.5 mM before further incubation for 24 h. Each culture was aliquoted into triplicates of 25 ml each, then

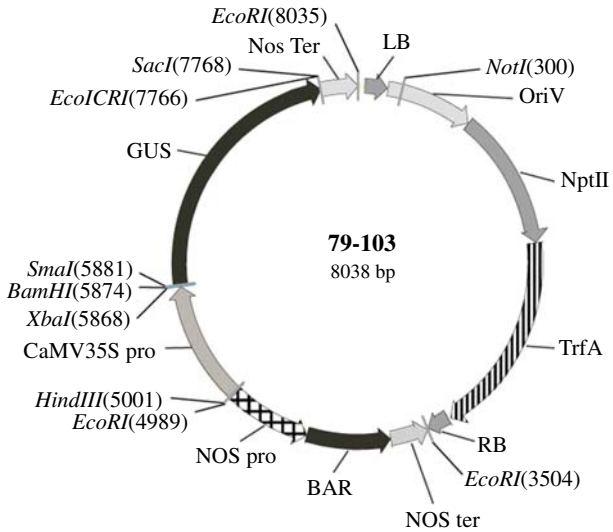

Fig. 2 Diagram of the p79–103 plant transformation vector. The BAR gene for glyphosinate selection in plants is driven by the NOS promoter

cells were harvested by centrifugation and the pellets were either immediately subjected to carotenoid extraction or were frozen at −80°C. Carotenoid extraction was carried out by adding 10 ml acetone to the pellet and mixing well by vortexing. The mixture was incubated at 55°C for 10 min and centrifuged at 11,000*g* for 10 min. The supernatant was transferred to a clean tube and dried down at room temperature under a stream of N_2 gas. The residue was re-suspended in 5 ml methanolic–KOH (10 g KOH per 100 ml in methanol/water, 80/20, v/v) and incubated at room temperature for 15 min to saponify the triacylglycerides (TAG) in the extract so they partition to the aqueous phase. A total of 2 ml H_2O and 3 ml petroleum ether were then added to extract carotenoids. Ether extraction was repeated once, and the extracts were pooled and dried down at room temperature under a N_2 gas stream. The residue was resuspended in 200 µl of acetonitrile/methylene chloride/methanol (50/40/10, by vol) with BHT (butylated hydroxytoluene, 0.5% (w/v) and filtered through a 0.2 µm pore size 4 mm nylon syringe filter into an HPLC sample vial. The extract was immediately subjected to analysis by HPLC-PDA. Aliquots of 20 µl were analyzed by HPLC employing a YMC "Carotenoid Column"—a reverse-phase C_{30}, 5 µm column (4.6 × 250 mm; Waters Ltd, Mississauga, Canada) with a column temperature of 35°C. Mobile phases consisted of methanol (A) and *tert*-methyl butyl ether (B). The gradient elution used with this column started at 95% A and 5% B, and then followed by a linear gradient to 35% A and 65% B in 25 min. A flow rate of 1.2 ml/min was used, and the eluate was monitored at 450 nm. Peaks were identified by their retention time and absorption spectra compared to those of known standards. Quantification of carotenoids was conducted using curves constructed from authentic standards.

Extraction of carotenoids from *A. thaliana* seeds and HPLC analysis

Approximately 200 mg of seed (weighed accurately to four decimal places) was combined with 3 ml of hexane/acetone/ethanol (50:25:25, by vol) extraction solvent in a scintillation vial (adapted from Shewmaker et al. 1999). A steel rod was put in the vial and the sample was pulverized for 30 min with rapid shaking. The sample was then centrifuged for 10 min at 1,800*g*, and the supernatant was transferred to a clean Kimax screw-cap culture tube. Another 3 ml of extraction solvent was added to the pellet and centrifugation was repeated. The extracts were pooled and dried down at room temperature under a stream of N_2 gas. The residue was re-suspended in 5 ml methanolic-KOH (10 g KOH per 100 ml in methanol/water, 80/20, v/v) and heated at 80°C for 1 h. Subsequent steps were the same as those described above for the extraction and analysis of carotenoids in the *E. coli* functional assay.

Results and discussion

*CrtH*1 encodes β-carotene hydroxylase in *Adonis aestivalis*

Alignment of the amino acid sequences of β-carotene hydroxylases of various organisms revealed two highly conserved amino acid motifs: VGAAVGME and AHQLHHTDK (Fig. 3). A pair of degenerate primers corresponding to the two conserved sequences was used to amplify a 363 bp fragment by RT-PCR using RNA extracted from the flower petals of *A. aestivalis*. The 5′- and 3′-ends of cDNA were then isolated using respective RACE techniques. The assembly of the three fragments produced a cDNA clone of 1,187 bp representing *CrtH*1. The cDNA encoded a predicted protein of 309 amino acids with a molecular weight of ~35 kDa and a pI of 9.15.

An alignment of the deduced protein sequence of CrtH1 with those of β-carotene hydroxylases from other plants showed that they shared high level of similarity, including ~70% amino acid identity, with most of the diverging sequence residing in the amino terminus (Fig. 4). However, CrtH1 showed much lower homology with β-carotene hydroxylases from bacteria (~33% identity; data not shown). Phylogenic analysis indicates that CrtH1 is most similar to the β-carotene hydroxylases from *Citrus unshiu*, and further highlights the divergence between the plant and prokaryotic enzymes (Fig. 5). The chloroplast transit peptide prediction software Chloroplast v1.1 (http://www.cbs.dtu.dk/services/ChloroP/) predicted a chloroplast transit peptide cleavage site at the amino terminus of CrtH1 between Val^{59} and Ala^{60}. This is consistent with the presumed plastid location of a carotenoid biosynthetic enzyme since carotenoid biosynthesis and modification in plants take place in plastids (Cunningham and Gantt 1998). Both β-carotene hydroxylase−1 and -2 of *A. thaliana* have chloroplast transit peptides in their N-terminus (Tian and DellaPenna 2001). The N-terminal region of about 100 amino acids in plant β-carotene hydroxylases is highly divergent, and is much longer than typical chloroplast transit peptide signals (Sun et al. 1996). It has been suggested that a portion of the amino terminus of the β-carotene hydroxylases of *A. thaliana* may be involved in mediating the formation

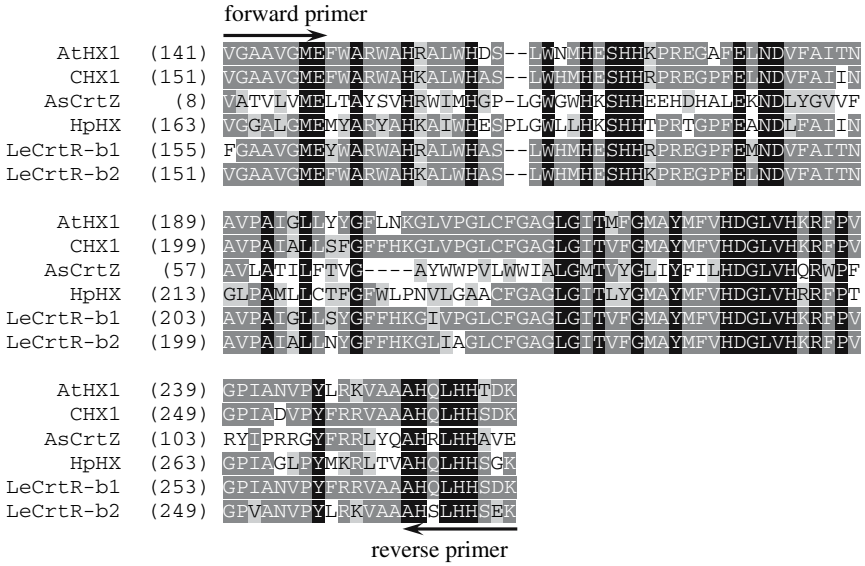

Fig. 3 Alignment of amino acid sequences of β-carotene hydroxylases of various organisms, and positions of the degenerate primers used to amplify the conserved 363 bp fragment. Identical and highly conserved amino acids in the six sequences are shown as *white letters* on *black* and *gray* backgrounds, and amino acids with similarity are indicated as *black letters* on a *gray* background. Amino acids with no similarity are shown as *black letters* on a *white background*. GenBank accession numbers of these sequences are as follows: *Lycopersicon esculentum LeCrtR*-b1 (Y14809) and *LeCrtR*-b2 (Y14810); *Alcaligenes sp. AsCrtZ* (D58422); *Arabidopsis thaliana AtHX*1 (AF370220); *Citrus unshiu CHX*1(AF296158); *Haematococcus pluvialis HpHX* (AF162276)

of dimers of β-carotene hydroxylase molecules (Sun et al. 1996), which is likely a common feature of plant hydroxylases.

Four transmembrane helical regions were predicted by TMHMM2.0 (http://www.cbs.dtu.dk/services/TMHMM/) in CrtH1 (Fig. 4). Four conservatively spaced histidine motifs were also present, HXXXXH and HXXH (Fig. 4). The histidine motifs are characteristic of membrane fatty acid desaturases and membrane hydrocarbon hydroxylases, where they are proposed to be involved in iron binding during hydroxylation reactions; mutations in any one of the ten histidine residues resulted in complete loss of the enzyme activity of β-carotene hydroxylase from pepper fruits (Bouvier et al. 1998). It is important to note that CrtH1 shares significant homology with the two β-carotene ketolases of *A. aestivalis*, AdKeto1 and AdKeto2, with ~70% amino acid identity (data not shown). However, neither AdKeto1 nor AdKeto2 showed either 4-ketolase or 3-hydroxylase activity when β-carotene was used as a substrate in *E. coli* functional assays. Both enzymes modified β-rings to form 4-keto-β-ring via an indirect route; keto-enol tautomerization (Cunningham and Gantt 2005).

CrtH1 is encoded by a multi-gene family in *Adonis aestivalis*

To estimate the *CrtH*1 gene copy number in the *A. aestivalis* genome, we carried out Southern-blot analysis on total genomic DNA digested with restriction endonucleases using a 201 bp *CrtH*1-specific fragment as a probe under high stringency conditions (Fig. 6). Digestion with *Bam*HI, *Hin*dIII, *Kpn*I, *Spe*I *and Sst*I, none of which cleaves within the ORF of *CrtH*1, resulted in the detection of at least three bands. The results suggest that *CrtH*1 belongs to a gene family of at least three members in the *A. aestivalis* genome (Fig. 6). This is similar to findings in other plants. In *A. thaliana*, β-carotene hydroxylase is encoded by two genes (Sun et al. 1996; Tian and DellaPenna 2001). The β-carotene hydroxylases, b1 and b2, of *A. thaliana* are functionally redundant, but with specialized functions (Tian et al. 2003). As well, two β-carotene hydroxylases, *Ca*1 and *Ca2*, have been isolated from pepper fruits (Bouvier et al. 1998), citrus (Kim et al. 2001) and tomato (Hirschberg 1998). This suggests that the existence of multi-copy gene families of β-carotene hydoxylases is likely of common occurrence in plants.

*CrtH*1 is differentially expressed in *A. aestivalis* organs

Northern-blot analysis was carried out to investigate the expression profile of the *CrtH*1 gene in different organs of *A. aestivalis*. The *CrtH*1 transcript (~1.35 kb) was highly abundant in flower petals, roots and stems with relatively much lower abundance in leaves and developing seeds (Fig. 7). This expression pattern is consistent with reports in the literature on

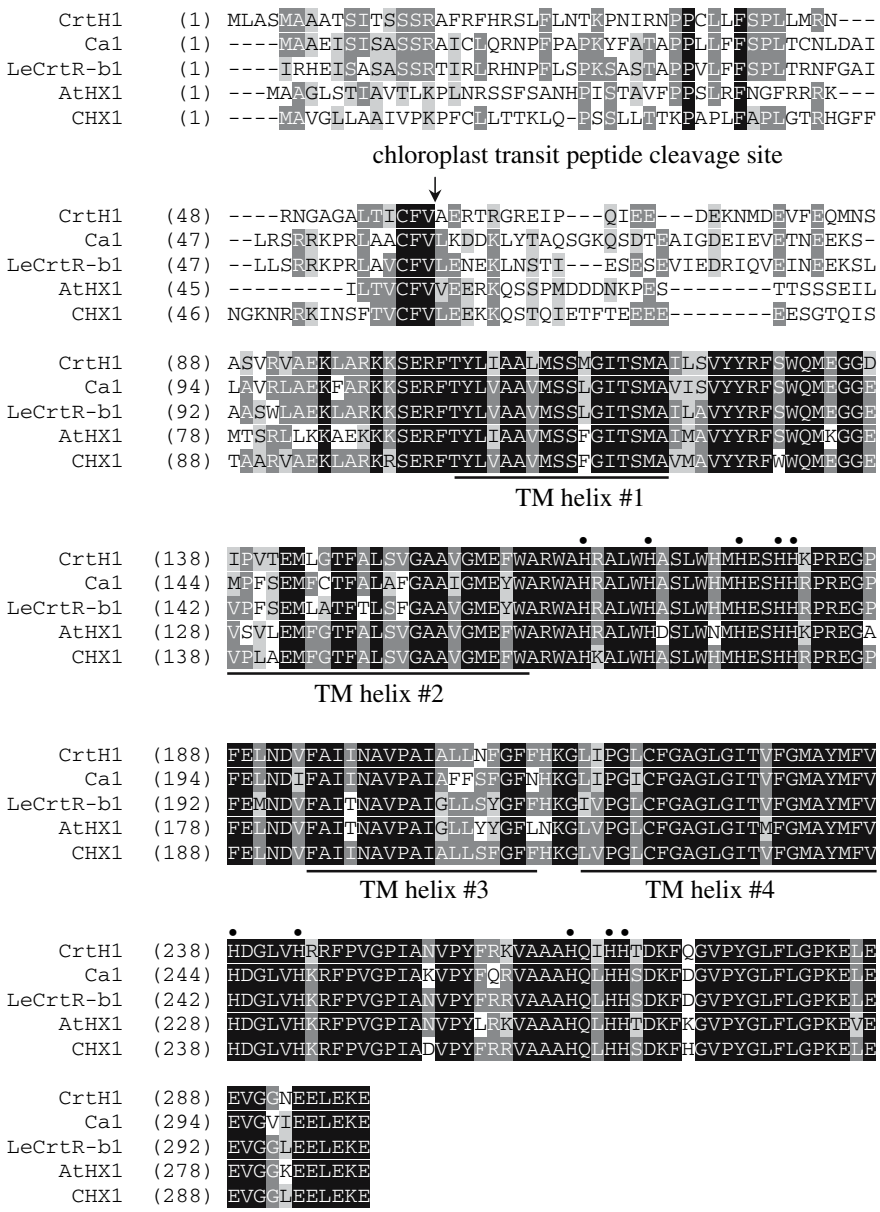

Fig. 4 Alignment of the deduced amino acid sequence of CrtH1 to those of β-carotene hydroxylases of various organisms. Shadings are as indicated in Fig. 3. GenBank accession numbers of these sequences are: *Arabidopsis thaliana AtHX*1(AF370220); *Capsicum annuum Ca*1 (Y09225); *Citrus unshiu CHX*1(AF296158); *Lycopersicon esculentum LeCrtR*-b1 (Y14809). *TM* transmembrane helices; *shaded circle* conserved β-carotene hydroxylase histidine residues

other β-carotene hydroxylases. For example, experiments in *A. thaliana* using antisense suppression of β-carotene hydroxylase indicated that only a few molecules of the total zeaxanthin in wild type plants were required for optimal chlorophyll fluorescence quenching in vivo (Rissler and Pogson 2001). This suggests that low level of β-carotene hydroxylase expression is likely sufficient in plant leaves. During the development of flowers, plastids differentiate into chromoplasts that accumulate high levels of carotenoids. In the flower petals of tomato plants, the process of plastid differentiation to chromoplasts resulted in more than eightfold increase in carotenoid content (Giuliano et al. 1993), and in pepper fruits chloroplast to chromoplast transition stage corresponded with strong induction of the expression of β-carotene hydroxylase (Bouvier et al. 1998). During marigold petal development, considerably higher level of β-carotene hydroxylase transcript accumulated in the petal than in the leaf (Moehs et al. 2001). Yellow carotenoids (xanthophylls) are responsible for the colors of petals and anthers (Giuliano et al. 1993). This suggests that more β-carotene hydroxylase is required during petal development, which is consistent with the relatively higher level of expression of *CrtH*1 in petals of *A. aestivalis*.

In addition to the *CrtH*1 transcript of ~1.35 kb, two other transcripts having sizes of ~2.37 and ~0.900 kb were also detected in petals, roots and stems, but not in leaves and seeds. The detection of three transcripts is in accordance with the Southern blotting data (Fig. 6), in which three *CrtH*1 homologues were predicted to be encoded by the *A. aestivalis* genome.

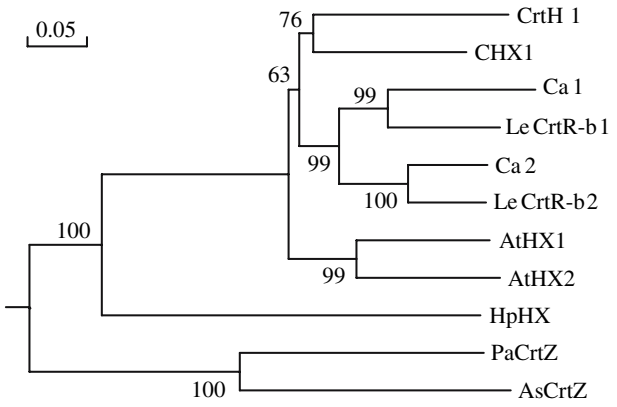

Fig. 5 Phylogenic relationship between the β-carotene hydroxylase of *Adonis aestivalis* (*CrtH*1) and those of other organisms. GenBank accession numbers of these sequences are the following: *Arabidopsis thaliana AtHX*1 (AF370220) and *AtHX*2 (NM_124636); *Citrus unshiu CHX*1 (AF296158); *Lycopersicon esculentum LeCrtR-b*1 (Y14809) and *LeCrtR-b*2 (Y14810); *Capsicum annuum Ca*1 (Y09225) and *Ca*2 (Y09722); *Haematococcus pluvialis HpHX* (AF162276); *Alcaligenes sp. AsCrtZ* (D58422); *Pantoea agglomerans PaCrtZ* (M87280). The number of bootstrap is 40,000 which was performed to test the reliability of the branches. The *bar* indicates the scale for branch length

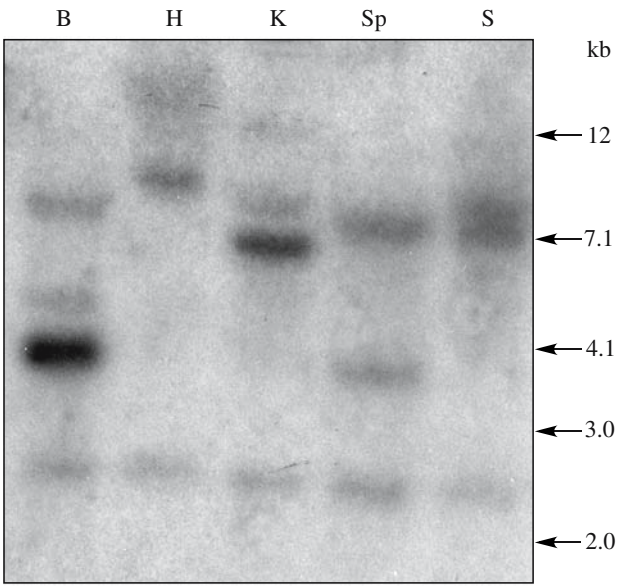

Fig. 6 Southern-blot analysis of the *CrtH*1 gene of *Adonis aestivalis*. Approximately 10 μg of genomic DNA was digested with *Bam*HI (*B*), *Hind*III (*H*), *Kpn*I (*K*), *Spe*I (*Sp*) and *Sst*I (*S*) restriction endonucleases, none of which cuts within *CrtH*1 cDNA sequence. Size markers (kb) are indicated on the right

CrtH1 converts β-carotene to β-cryptoxanthin and zeaxanthin in *E. coli*

Amino acid alignment showed that CrtH1 shared sequence similarity with β-carotene hydroxylases of other plants (Fig. 4). Therefore, we undertook to determine whether CrtH1 exhibited β-carotene hydroxylase activity. A functional assay was conducted in an *E. coli* strain possessing plasmid pBS-CrtH1 for the expression of the CrtH1 ORF and plasmid pACCAR16ΔcrtX, which expresses the genes required for the biosynthesis of β-carotene (Misawa et al. 1995). HPLC analysis to determine the carotenoid profiles of the *E. coli* strains showed that cells harboring plasmids pBS-CrtH1 and pACCAR16ΔcrtX produced β-carotene, β-cryptoxanthin and zeaxanthin (Fig. 8). Of total detectable carotenoids in triplicate samples, β-cryptoxanthin and zeaxanthin accounted for 24.5% (±0.4%) and 38.9% (±0.7%), respectively. As expected, the negative control *E. coli* strain harboring plasmids pBluescriptII KS (+) and pACCAR16ΔcrtX accumulated only β-carotene (Fig. 8). Compounds eluting at different peaks were identified by their retention time and absorption spectra compared to the corresponding authentic standards as well as by co-chromatography with authentic standards. This analysis confirms that *CrtH*1 encodes an enzyme with β-carotene hydroxylase activity that converts β-carotene to zeaxanthin via β-cryptoxanthin (mono-hydroxylated β-carotene). The accumulation of a high level of β-cryptoxanthin in the *E. coli* expression system suggested asymmetric introduction of hydroxyl groups to the β-rings of β-carotene by the CrtH1 enzyme. This could be due to the possibility that CrtH1 expressed in *E. coli* may have a slightly altered configuration that confers a different mechanism for substrate recognition that allows for asymmetric hydroxylation of the β-rings of β-carotene. It is also possible that, in *E. coli*, CrtH1 could not form a stable dimer that is deemed necessary for the full function of β-carotene hydroxylases (Sun et al. 1996). For two-step carotenogenic enzymes, dimerization is considered essential for catalysis of the second reaction (Römer et al. 2002). CrtH1 protein may also be unstable in *E. coli*, and only a small number of dimers are formed at low cellular concentrations of CrtH1. This could explain the accumulation of high levels of monohydroxyl β-cryptoxanthin in the *E. coli* assay. The

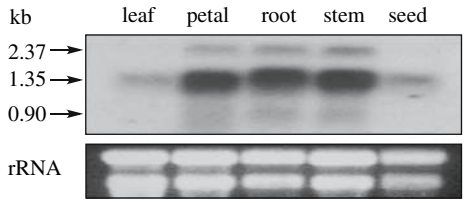

Fig. 7 Expression profile of *CrtH*1 in different organs of *Adonis aestivalis*. Approximately 15 μg of total RNA was electrophoresed on 1.2% agarose/formaldehyde gel and blotted onto Hybond-XL membrane. Probe was the same as that used for Southern analysis in Fig. 6

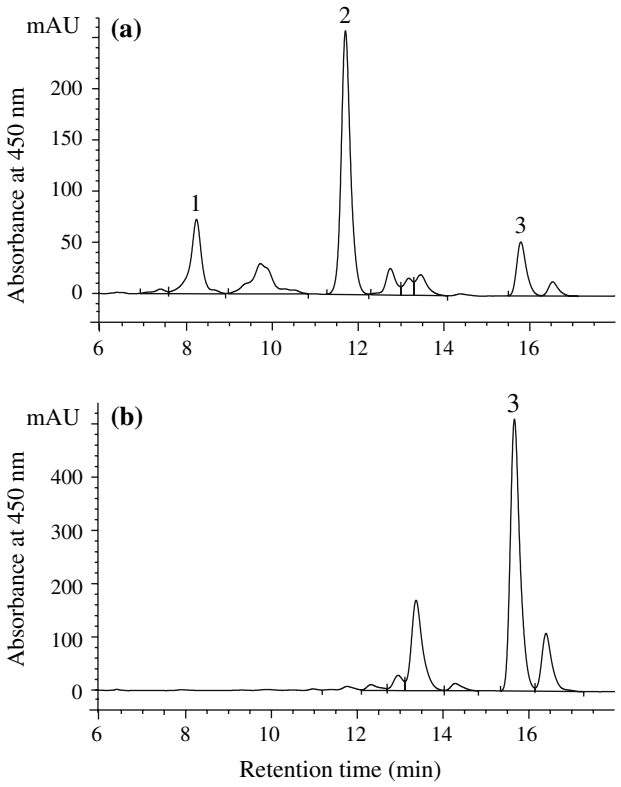

Fig. 8 HPLC profiles of carotenoids extracted from *E. coli* cells carrying plasmids. **a** pBS-CrtH1 and pACCAR16ΔcrtX, **b** empty cloning vector pBluescriptII KS (+) and pACCAR16ΔcrtX. Peaks numbered *1*, *2* and *3* correspond to zeaxanthin, β-cryptoxanthin and β-carotene, respectively

catalytic properties of CrtH1 are different from those of *A. thaliana* β-carotene hydroxylases which could convert β-carotene to ~90% zeaxanthin and ~6% β-cryptoxanthin (Sun et al. 1996; Tian and DellaPenna 2001) using similar functional assays in *E. coli*. The β-carotene hydroxylase CrtR from the cyanobacterium *Synechocystis* sp. PCC6803 also showed symmetric hydroxylation reaction towards the two β-rings of β-carotene in *E. coli* (Masamoto et al. 1998).

Expression of *CrtH*1 in *A. thaliana* leads to the accumulation of violaxanthin

In plants, a key enzymatic step in the biosynthesis of lutein and β-carotene-derived xanthophylls is the hydroxylation reaction carried out by β-carotene hydroxylase. The aforementioned functional assay in *E. coli* demonstrated that CrtH1 exhibited β-carotene hydroxylase activity. To investigate the effectiveness of using CrtH1 to alter carotenoid biosynthesis in plants, a transformation construct for overexpressing this gene under the control of the seed-specific napin promoter was made, and used to transform wild type *A. thaliana* (wt) and the b1b2 mutant deficient for endogenous β-carotene hydroxylase activities (Tian et al. 2003). To assess the level of *CrtH*1 expression in transgenic *A. thaliana*, Northern-blot analysis was conducted on total RNA isolated from immature siliques of select transgenic lines. The transgenic lines had significant levels of *CrtH*1 expression, whereas no expression could be detected in untransformed control plants (Fig. 9). No obvious visible phenotypic differences were observed among *A. thaliana* wt, b1b2 mutant and transgenic lines expressing *CrtH*1 (data not shown). Mature seeds were assessed for the presence and levels of carotenoids. HPLC traces of representative wt and b1b2 mutant lines expressing *CrtH*1, as well as those of wild type and b1b2 mutant are illustrated in Fig. 10, which shows the heterologous expression of *CrtH*1 in *A. thaliana* caused an increase in the level of violaxanthin (peak 1). Table 1 shows HPLC quantification of carotenoids in seeds from representative *A. thaliana* lines. Decreases in the range of 15–78% in the amount of β-carotene were observed in most of the transgenic lines compared to untransformed plants; this is expected as a result of enhanced hydroxylation and conversion of β-carotene to xanthophylls. Expression of *CrtH*1 in the wild type line resulted in 44–64% increase in lutein content, except for the BY275 and BY284 lines, which did not show any significant difference in lutein level. With the exception of the BY330 line, all b1b2 mutant lines expressing *CrtH*1 showed increases in lutein levels ranging from14 to 72% as compared to b1b2 mutant. The hydroxylation of the β-ring of α-carotene is required for its conversion to lutein. Therefore, enhanced biosynthesis of lutein in lines expressing *CrtH*1 would be expected if this gene encodes a β-carotene hydroxylase. It is noteworthy that β-carotene-derived xanthophylls continued to be detected in the b1b2 mutant, possibly as a result of functional overlap between β- and ε-hydroxylases (Tian et al. 2003). β-cryptoxanthin (mono-hydroxylated β-carotene), an intermediate in the conversion of β-carotene to zeaxanthin, was undetectable in wild type, but was detected in the b1b2 mutant as a result of incomplete hydroxylation of the β-rings of β-carotene. β-cryptoxanthin was also undetectable in wild type lines expressing *CrtH*1 except in the BY293 line where trace amounts were detected. Levels of β-cryptoxanthin in b1b2 mutant lines expressing *CrtH*1 were slightly reduced or undetectable as a result of its conversion to zeaxanthin and then violaxanthin. The only exception was the BY341 line, which showed approximately 41% increase in β-cryptoxanthin, presumably due to its inefficient hydroxylation to zeaxanthin. This result is in contrast to that obtained in the functional assay of CrtH1 in *E. coli* where β-cryptoxanthin accumulated at

Table 1 Concentrations of carotenoids in *Arabidopsis thailana* seeds from select lines transformed with CrtH1 gene

Plant line	Violaxanthin μg/g dw	Lutein μg/g dw	Zeaxanthin μg/g dw	β-Cryptoxanthin μg/g dw	β-Carotene μg/g dw
wt	0.694 ± 0.056	13.380 ± 0.729	0.955 ± 0.051	UD	1.890 ± 0.504
wt+CrtH1					
BY275	3.186 ± 0.544	13.413 ± 3.354	0.776 ± 0.193	UD	1.009 ± 0.188
BY280	5.47 ± 0.342	21.631 ± 0.941	1.162 ± 0.036	UD	0.422 ± 0.033
BY281	6.162 ± 0.416	20.684 ± 0.623	1.067 ± 0.033	UD	0.4 ± 0.007
BY284	2.939 ± 0.199	12.678 ± 1.367	0.642 ± 0.100	UD	0.718 ± 0.081
BY287	7.572 ± 0.284	22.016 ± 0.483	1.077 ± 0.011	UD	0.591 ± 0.068
BY293	5.611 ± 0.216	21.49 ± 0.597	0.895 ± 0.034	0.098 ± 0.002	1.491 ± 0.142
BY294	3.975 ± 0.12	19.306 ± 0.95	0.883 ± 0.016	UD	1.015 ± 0.074
b1b2 mutant	0.881 ± 0.036	26.214 ± 2.690	0.711 ± 0.082	0.153 ± 0.014	8.238 ± 1.019
b1b2 mutant +CrtH1					
BY317	24.741 ± 2.37	42.523 ± 2.275	0.727 ± 0.023	UD	2.473 ± 0.439
BY330	5.522 ± 1.295	25.912 ± 4.857	0.544 ± 0.048	0.139 ± 0.043	6.41 ± 0.081
BY334	8.713 ± 1.092	34.809 ± 2.493	0.611 ± 0.039	0.156 ± 0.006	8.942 ± 0.88
BY341	11.005 ± 0.548	33.705 ± 2.331	0.526 ± 0.061	0.216 ± 0.075	5.78 ± 0.435
BY342	20.62 ± 0.688	45.199 ± 0.709	0.731 ± 0.025	UD	3.099 ± 0.228
BY346	15.111 ± 1.103	36.464 ± 1.591	0.584 ± 0.037	0.164 ± 0.013	3.262 ± 0.11
BY347	3.871 ± 0.163	30.08 ± 1.882	0.616 ± 0.014	0.148 ± 0.015	6.985 ± 0.515

UD undetectable, *dw* dry weight, Each value is the mean result from triplicate ±SD

a higher level. This indicates that CrtH1 may be more efficient at fully hydroxylating β-carotene rings in plants than in *E. coli*. Most of the wild type and b1b2 mutant lines expressing *CrtH*1 showed either a slight decrease or no statistically significant change in levels of zeaxanthin when compared to untransformed wild type and b1b2 mutant. However, elevated levels of violaxanthin (at least a threefold increase) were observed in all *CrtH*1 expressing lines when compared to their untransformed counterparts. In wild type lines expressing *CrtH*1, violaxanthin level increased by up to tenfold. Of all the b1b2 mutant plants expressing *CrtH*1, the BY317 line had the highest level of violaxanthin with a 27-fold increase compared to the b1b2 mutant. The lack of zeaxanthin accumulation combined with enhanced violaxanthin levels in transgenic lines is an indication that zeaxanthin epoxidase is active enough to convert excess zeaxanthin to violaxanthin in seeds of *A. thaliana*. In plants, there exists a mechanism involving a set of reactions in the xanthophyll cycle; the reversible interconversion of zeaxanthin and violaxanthin through the intermediate product antheraxanthin (Fig. 1; Davison et al. 2002), which rapidly optimizes the concentration of zeaxanthin (Demmig-Adams and Adams 2002). Therefore, more violaxanthin would be expected if the zeaxanthin biosynthesis was enhanced by β-carotene hydroxylase. The xanthophyll cycle helps to protect against light-induced damage by converting violaxanthin into zeaxanthin which participates in thermal dissipation of excessively absorbed light energy (Malkin and Niyogi 2002). It is noteworthy that seeds from b1b2 mutant expressing *CrtH*1 had on average higher level of violaxanthin than those from wild

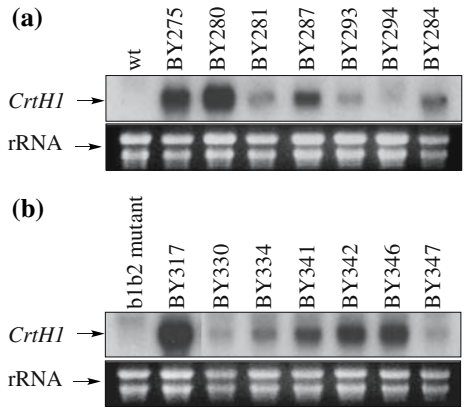

Fig. 9 **a, b** Northern analysis of *CrtH*1 in immature siliques of transgenic *Arabidopsis thaliana*. **a** Wild type (wt) and wild type expressing *CrtH*1 (BY275 to BY284) , **b** b1b2 mutant, and b1b2 mutant expressing *CrtH*1 (BY317 to BY347). Approximately 10 μg of total RNA was electrophoresed on 1.2% agarose/formaldehyde gel and blotted onto Hybond-XL membrane. The blot was hybridized with a 350 bp fragment from the 5′-end of the *CrtH*1 ORF

type plants expressing *CrtH*1. This might be due to the co-supression of the endogenous β-carotene hydroxylase in wild type lines expressing *CrtH*1, because of the high nucleotide homology between *CrtH*1 and the ORFs of the two β-carotene hydroxylases of *A. thaliana* (AF370220 and NM_124636). Alignment of the three ORFs revealed an overall homology level of 72%, but a region of about 600 bp showed a homology of 89% (data not shown); a level of homology sufficient to result in co-suppression of the endogenous genes.

No correlation could be drawn between levels of accumulation of different species of carotenoids and

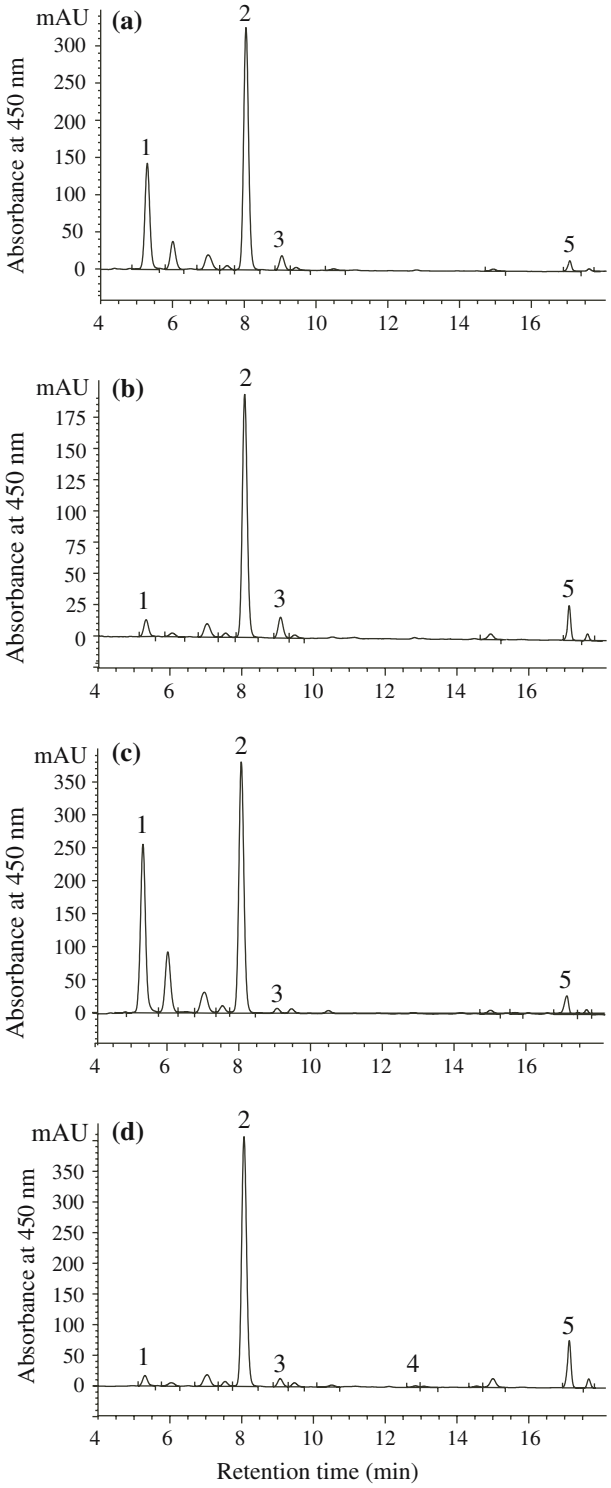

Fig. 10 HPLC profiles of carotenoids extracted from seeds of *Arabidopsis thaliana*. **a** Wild type expressing *CrtH*1 (BY287line), **b** wild type, **c** b1b2 mutant expressing *CrtH*1 (BY317line) and **d** b1b2 mutant. Peaks numbered *1*, *2*, *3*, *4* and *5* correspond to violaxanthin, lutein, zeaxanthin, β-cryptoxanthin and β-carotene, respectively

levels of *CrtH*1 expression in different transgenic *A. thaliana* lines. This suggests a differential role for *CrtH*1 expression in the regulation of carotenoid biosynthesis in individual transformants. This also indicates that the β-carotene hydroxylase activity of CrtH1 may be constrained by factors such as substrate availability or feedback inhibition.

When a β-carotene hydroxylase gene was constitutively overexpressed in *A. thaliana*, the transformants were more tolerant to conditions of high light and high temperature (Davison et al. 2002). Also, transgenic tobacco overexpressing a bacterial β-carotene hydroxylase showed an enhanced UV photoprotection (Götz et al. 2002). Whether *A. thaliana* plants expressing *CrtH*1 are more tolerant to stress conditions requires further investigation.

Conclusions

We present data supporting that *CrtH*1 encodes a β-carotene hydroxylase in *Adonis aestivalis*. The predicted amino acid sequence of CrtH1 showed strong homology to β-carotene hydroxylases of other organisms. Features of the translated protein are consistent with a plastid-localized β-carotene hydroxylase. These features include a plastid transit peptide, transmembrane helices and conserved histidine motifs. Functional assays in *E. coli* showed that CrtH1 could convert β-carotene to its hydroxylated derivatives, β-cryptoxanthin and zeaxanthin. Expression of *CrtH*1 gene in *A. thaliana* wild type and the b1b2 mutant, which has T-DNA knockouts in the two β-carotene hydroxylase genes, caused a marked increase in the level of violaxanthin. The *CrtH*1 gene is a tool that may be useful in modifying the biosynthetic pathway of carotenoids in higher plants, especially cruciferous species, such as *A. thaliana* and Brassica oilseeds. As these species are evolutionarily distant from *A. aestivalis*, *CrtH*1 would be expected to cause less co-suppression than its homologues from crucifers. Because of the economic importance of carotenoids, metabolic engineering of their biosynthesis has been explored in several plant species (Shewmaker et al. 1999; Ye et al. 2000; Römer et al. 2002; Dharmapuri et al. 2002; Ravanello et al. 2003; Ralley et al. 2004). Changes in carotenoid profiles in *A. thaliana* seeds caused by the expression of *CrtH*1 provide information that may be useful in engineering carotenoid biosynthesis in related plant species.

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