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Synthesis of ketocarotenoids in the seed of *Arabidopsis thaliana*

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Summary

A cDNA coding for a gene necessary for synthesis of ketocarotenoids was cloned from the alga *Haematococcus pluvialis* and expressed in the seed of *Arabidopsis thaliana*. The expression of the algal *β-carotene-oxygenase* gene was directed to the seed by use of the 2S, seed storage protein promoter napA. Extracts from seeds of the transgenic plants were clearly red because of accumulation of ketocarotenoids, and free and esterified forms of ketocarotenoids were found in addition to the normal carotenoid composition in the seed. The major ketocarotenoids in the transgenic plants were: 4-keto-lutein (3,3′-dihydroxy-β-,ε-carotene-4-one), adonirubin (3-hydroxy-β-,β′-carotene-4,4′-dione) and canthaxanthin (β-,β′-carotene-4,4′-dione). 4-Keto-lutein differs from the more common adonixanthin only in the position of one double bond. To increase the substrate availability for the β-carotene-oxygenase, these transformants were crossed with transgenic plants overexpressing a construct of an endogenous *phytoene synthase* gene, also under the control of the napA promoter. The resulting crossings gave rise to seeds with a 4.6-fold relative increase of the total pigment, and the three major ketocarotenoids were increased 13-fold compared to seeds of transgenic plants carrying only the β-carotene-oxygenase construct.

Keywords: ketocarotenoids, canthaxanthin, phytoene, oxygenase, seed, *Arabidopsis*.

Introduction

All photosynthetic organisms produce carotenoids, and common carotenoids like β-carotene and lutein are structural and chemically active parts of the photosynthetic apparatus. The biosyntheses of oxygenated carotenoids have, in many cases, been characterised, and some xanthophylls and ketocarotenoids described in this study are depicted in Figure 1(a). In addition to the diverse colours of fruits and flowers, many fishes, crustaceans and birds are also pigmented by carotenoids. Carotenoids have antioxidative properties and have been shown to have positive effects in many aspects on human health as well as in prevention of oxidative damage of plant cells (Demmig-Adams and Adams, 2002). Specific qualities of certain carotenoids have also been reported. For example, gastric *Helicobacter pylori* infections can be inhibited by supplementation of the ketocarotenoid astaxanthin (3,3′-dihydroxy-β-,β′-carotene-4,4′-dione; Bennedsen *et al.*, 1999; Wang *et al.*, 2000), and in salmon, lack of ingestible astaxanthin seems to result in increased yolk sac mortality (M74 disease; Koski *et al.*, 1999; Pettersson and Lignell, 1999). Because of the potential of carotenoids to act as chemoactive agents and because of their use as pigments, industrial production of carotenoids is of great commercial interest. Synthetic as well as naturally produced carotenoids are readily used as neutraceuticals and supplements in food and fodder.

Introduction and *in vivo* synthesis of ketocarotenoids has successfully been achieved by genetic engineering in *Escherichia coli, Candida utilis* and *Nicotiana tabacum* through expression of either an algal or a bacterial *β-carotene-oxygenase* gene (Mann *et al.*, 2000; Misawa and Shimada, 1998; Miura *et al.*, 1998). These enzymes oxygenate the 4-position of the β-rings of carotenes (Figure 1a). The yield has so far been relatively low in these transgenic organisms, reaching levels of 0.4–0.5 mg g^{-1} DW, 0.4 mg g^{-1} DW and 0.084 mg g^{-1} FW ketocarotenoids, respectively. As a comparison, the levels of the ketocarotenoid astaxanthin, in the alga *Haematococcus pluvialis*,

are 100-fold higher (Boussiba *et al.*, 1999; Margalith, 1999). Nevertheless, better knowledge of the regulation of this rather complex pathway and manipulation of the early steps in the synthesis has resulted in higher levels of the carotenoid lycopene reaching 7.8 mg g^{-1} DW in *C. utilis* (Shimada *et al.*, 1998). By use of seed-specific promoters fused to the coding sequence of *phytoene synthase* genes, 43 and 50-fold increases of accumulated carotenoids have also been shown for seeds of *Brassica napus* and *Arabidopsis*, respectively (Lindgren *et al.*, 2003; Shewmaker *et al.*, 1999).

In the present study, we show that seed-specific expression of an algal *β-carotene-oxygenase* in *Arabidopsis* results in accumulation of free and esterified forms of ketocarotenoids, and that crossings of these plants to transgenic plants producing high levels of β-carotene greatly increased the levels of ketocarotenoids. The most abundant ketocarotenoids found in seeds of double transformants were 4-keto-lutein (3,3′-dihydroxy-β-,ε-carotene-4-one), canthaxanthin (β-,β′-carotene-4,4′-dione) and adonirubin (3-hydroxy-β-,β′-carotene-4,4′-dione).

Results

Generation of seeds that accumulate ketocarotenoids

To direct the production of ketocarotenoids to the seed, the cDNA of the algal *β-carotene-oxygenase* gene was cloned and fused to a seed-specific promoter napA (−1101) (Stalberg *et al.*, 1993). As the synthesis of carotenoids in flowering plants is localised to the chloroplast, the coding sequence of the *β-carotene-oxygenase* gene was truncated at nucleotide 264 and linked to the coding sequence of the *RBCS1a* genes transit peptide (Hand *et al.*, 1989; Holmstrom *et al.*, 1994; Figure 1b). By use of this construct,

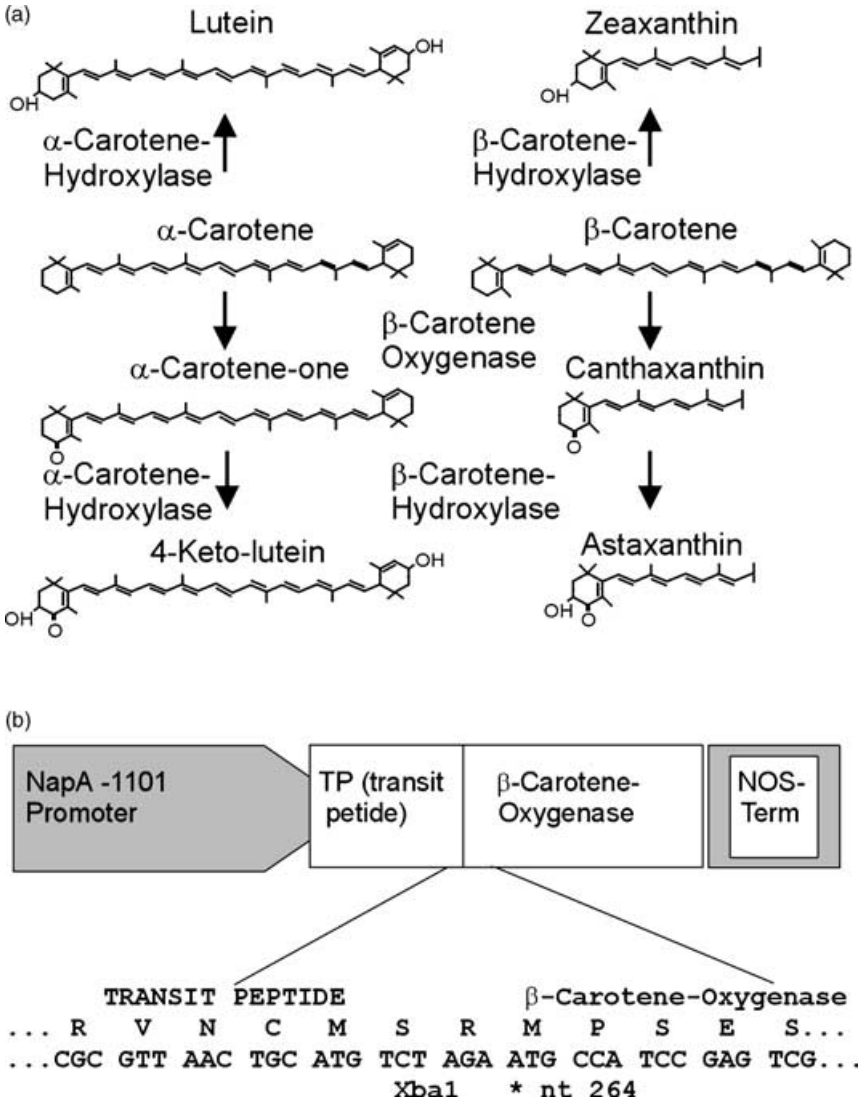

Figure 1. Schematic representation of part of the synthesis of ketocarotenoids and DNA construct used for transformation.
(a) Part of the biosynthetic pathway of ketocarotenoids and xanthophylls. Carotenoids having β-double bonds in both of its rings are represented as half molecules for simplification.
(b) A construct consisting of a promoter, a transit peptide sequence and an algal gene coding for a *β-carotene-oxygenase* from *H. pluvialis* used for transformation. The nucleotide sequence and amino acid sequence are given below. The introduced *Xba*I site and nucleotide 264 of the original algal sequence are indicated.

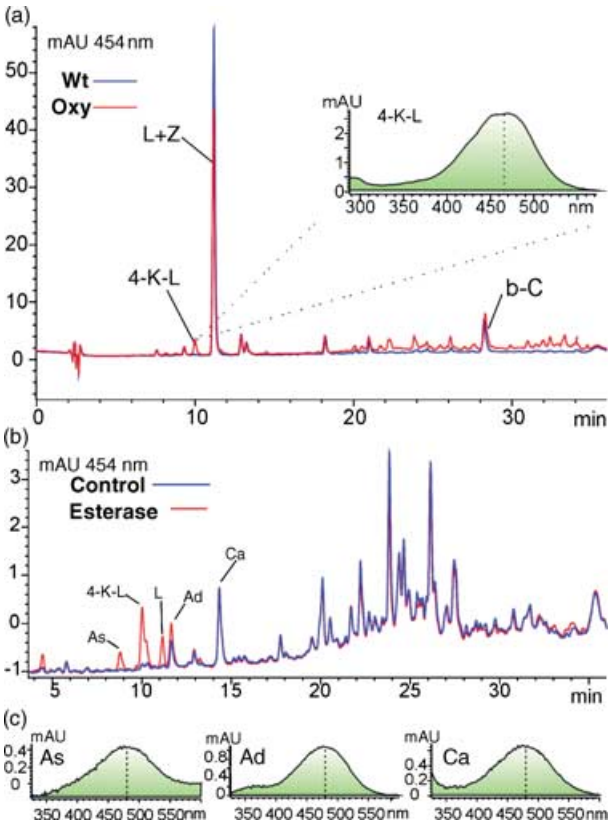

Figure 2. HPLC chromatograms and spectra obtained from extracts of seeds and esterase treatments.
(a) HPLC chromatogram of a control plant marked blue and transgenic plant Oxy marked red (a transgenic plant overexpressing *β-carotene-oxygenase*). Note the novel peak indicated as 4-keto-lutein in the transgenic plant and the inserted absorption spectrum from the peak of the putative 4-keto-lutein. The peak marked L + Z contains lutein and zeaxanthin because these carotenoids cannot be separated on this column; thus, a minor part of the content in this peak is zeaxanthin.
(b) Overlay of a fraction containing putative mono-esters of ketocarotenoids before (marked blue) and after treatment with cholesterol esterase (marked red). Inserted in the chromatogram are absorption spectra for different peaks of ketocarotenoids appearing after esterase treatment. Note that the canthaxanthin levels remained unaltered!
Abbreviations: 4-K-L, 4-keto-lutein; L + Z, lutein and zeaxanthin; b-C, β-carotene; As, astaxanthin; L, lutein; Ad, adonirubin; Ca, canthaxanthin.

135 kanamycin-resistant *Arabidopsis* plants were generated. HPLC analysis of extracts from seeds of individually selected kanamycin-resistant plants as well as seeds from batches of plants exhibited a unique peak that was not present in wild-type plants (Figure 2a). The novel peak displayed a symmetrical absorption spectrum, characteristic for ketocarotenoids (Figure 2a).

Esterified ketocarotenoids in the β-carotene-oxygenase transformants

Further HPLC analysis of the seed extracts revealed several small peaks with ketocarotenoid absorption spectra. These numerous small peaks eluted late when subjected to chromatography, indicating modifications rendering the compounds more hydrophobic in nature. To examine if these carotenoids were acylated by esterification, the seed extract was subjected to enzymatic cleavage by a *Pseudomonas* sp. cholesterol esterase. However, the digestion was poor on whole extracts, and no clear difference between a reaction with and a reaction without the esterase could be seen.

To overcome the inhibitory effects on the reaction, the total extract was further purified to yield oil and β-carotene in one fraction and the other two fractions with the putative fatty acid ester forms of carotenoids. By adding these fractions from the seed extracts to a mixture of algal extracts containing esterified astaxanthin and esterase, it could be verified that the seed oil and β-carotene containing fraction was highly inhibitory. The other fractions were subjected to enzymatic digestion by the cholesterol esterase, and a clear difference between non-esterase and esterase-treated samples could be shown for one of the fractions (Figure 2b). The peaks appearing in the esterase-treated sample were candidates to be free astaxanthin, lutein, adonirubin and 4-keto-lutein, respectively (Figure 2c).

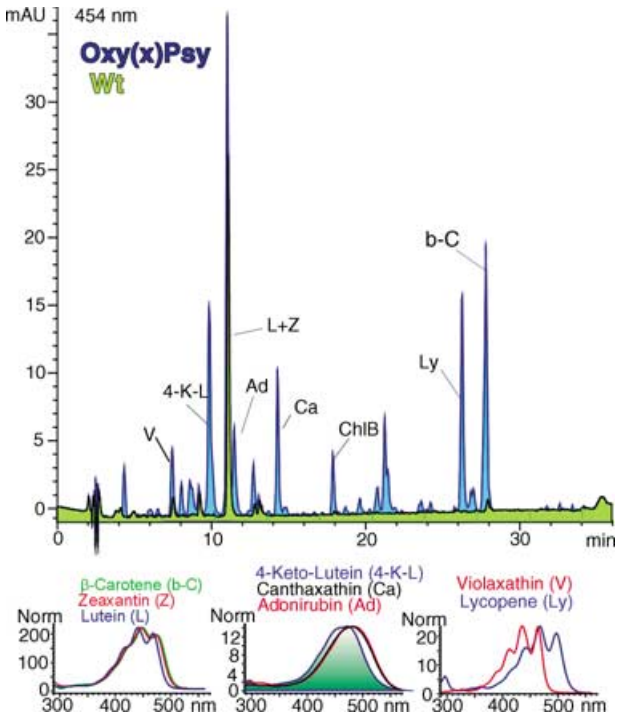

Figure 3. HPLC chromatograms and spectra from seed extracts.
The Oxy(x)Psy crossing was marked blue, and represented the extract from seeds of a transformant overexpressing β-carotene-oxygenase and phytoene synthase constructs. The wild type (Wt) is a control plant and was marked green. Overlay of spectra from different peaks containing carotenoids are indicated. The spectra of lutein and zeaxanthin are shown for comparison. Zeaxanthin spectrum was obtained from an zeaxanthin producing *E. coli* strain for comparison.
Abbreviations: V, violaxanthin; 4-K-L, 4-keto-lutein; L, lutein; Z, zeaxanthin; Ad, adonirubin; Ca, canthaxanthin; ChlB, chlorophyll B; Ly, lycopene; b-C, β-carotene.

Astaxanthin and canthaxanthin had spectra and retention time similar to those of their authentic standards. Moreover, the peak containing canthaxanthin was unaltered between the treatments, as expected for a non-esterified ketocarotenoid (Figure 2b). The reason why the main part of the isolated carotenoids in this fraction remained unaltered after the cholesterol treatment was not further determined but might have been because of inhibition of the reaction.

Increased levels of carotenoids in crossings: identification of 4-keto-lutein, adonirubin and canthaxanthin

The most prominent of the novel peaks that appeared in the HPLC chromatogram from the single and double transformants was a good candidate for being a 4-keto-carotene (Figures 2a and 3). First, it eluted earlier than lutein, but later than astaxanthin, and second, it had a retention time and spectra similar to those of a ketocarotenoid found in an extract of *H. pluvialis*, but slightly shifted spectra compared to a peak occurring at a retention time similar to that obtained from an *E. coli* strain, containing the plasmids pACCAR25 and pHP51 (Kajiwara *et al.*, 1995), kindly provided by Dr Norihiko Misawa. The bacterially produced ketocarotenoid could not be 4-keto-lutein as this strain produced β-carotene only. The fractions containing the novel peaks from the seed extracts and the alga were sampled, and the mass was determined by MS analysis. The major peak was found at *m*/*z* 583.4 [M + H] in both samples and it implicated that these peaks consisted of either 4-keto-zeaxanthin or an unusual compound, 4-keto-lutein (3,3′-dihydroxy-β-,ε-carotene-4-one) also named α-doradexanthin. MS analyses of the peaks containing adonirubin and canthaxanthin supported the HPLC data, while MS analysis of the putative astaxanthin containing peak were inconclusive.

The carbonyl groups on ketocarotenoids like 4-keto-lutein, adonirubin and canthaxanthin can be reduced by sodium boro-hydride according to the scheme in Figure 4 and are used for chemico-physical identification. Purified fractions containing these carotenoids were isolated from the different transgenic plants and reduced by sodium boro-hydride to analyse the presence of putative keto groups according to Davies (1976) and Goodwin (1980). In general, conversion of 4-carbonyl moieties to 4-hydroxy groups drastically changes carotenoid spectra, as single-bond hydroxy groups in contrast to double-bond carbonyl groups have only a minor effect on the absorption spectrum of the molecule (Davies, 1976). Reduction of a fraction containing the putative 4-keto-lutein, according to the above-mentioned method, resulted in a spectral shift, and the absorption spectrum obtained exhibited a typical three-band spectrum. This coincided with the spectra of lutein/α-carotene and not the spectra of zeaxanthin/β-carotene (Figure 5a), thereby identifying the compound as 4-keto-lutein.

The putative adonirubin-containing peak eluted just after lutein in this system. When a fraction of this compound was reduced with sodium boro-hydride, the resulting carotenoid migrated slightly slower than reduced 4-keto-lutein (Figure 5a). The absorbance spectrum finally was changed from an astaxanthin-like spectrum to a spectrum coinciding with that of zeaxanthin/β-carotene, which strongly indicated that this ketocarotenoid was identical to adonirubin (Figure 5b). A similar shift in spectra and retention time was obtained for canthaxanthin, although the reduced canthaxanthin eluted close to lutein and zeaxanthin as expected for an di-hydroxy-β-carotene (Figure 5c).

Comparison of the relative carotenoid quantities in the different transformants

The vegetative part of the adult transgenic plant did not differ from the wild type in either morphology or pigmentation of the leaves. In contrast, seeds of plants transformed with the oxygenase construct, plants transformed with the phytoene synthase construct and crosses between these two transformants could be identified by the slight difference in their seed colour compared to the control plant (Figure 6). Moreover, when extracts were compared, all extracts had different colours, reflecting differences in

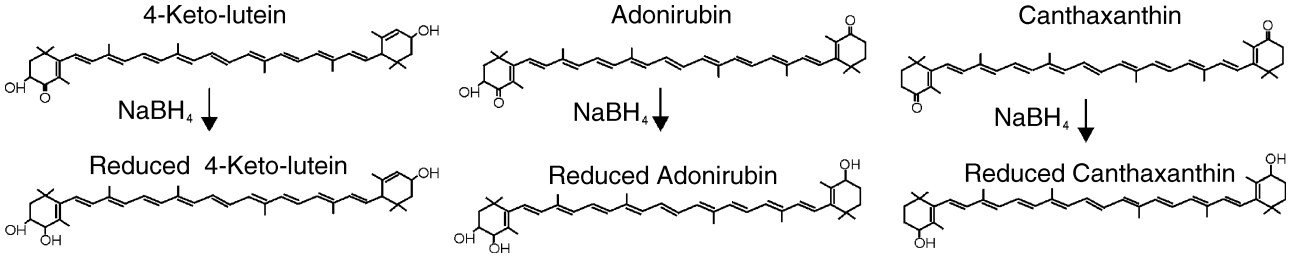

Figure 4. An outline of unreduced and reduced molecules of 4-keto-lutein, adonirubin and canthaxanthin.
Note that the number of carbonyl groups converted to hydroxy groups is different in 4-keto-lutein, adonirubin and canthaxanthin. In 4-keto-lutein, only one carbonyl group is converted to a hydroxy group that results in a smaller shift in absorption spectrum and shift in retention time than in adonirubin and canthaxanthin where two carobonyl groups are reduced to hydroxy groups.

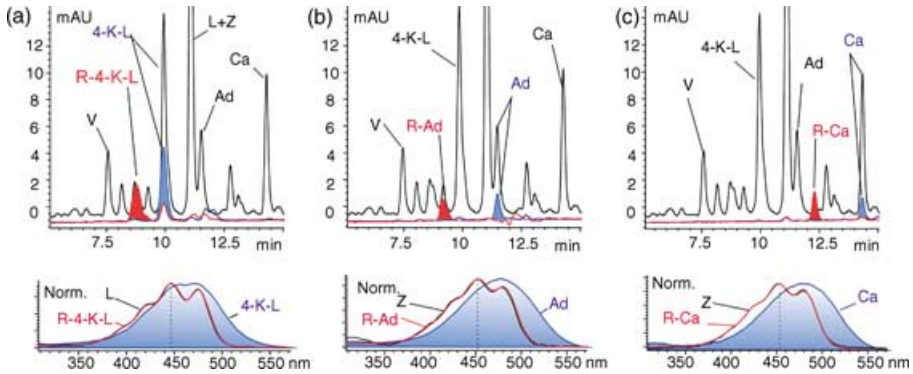

Figure 5. HPLC diagrams of sodium borohydride reduction of 4-keto-lutein, adonirubin and canthaxanthin are shown.
(a) 4-Keto-lutein.
(b) Adonirubin.
(c) Canthaxanthin.
Reduction converts keto groups to hydroxy groups that result in a shift in absorption spectra from the rounded keto-type spectrum to an α- or β-carotene type of spectrum. Reduction also results in a shorter retention time of the molecules because of an increase in hydrophilicity. The reduced carotene is marked red and unreduced marked blue, and spectrum after respective reduction of carotenoids are shown below for comparison.
Abbreviations: 4-K-L, 4-keto-lutein; R-4-K-L, reduced 4-keto-lutein; Ad, adonirubin; R-Ad, reduced adonirubin; Ca, canthaxanthin; R-Ca, reduced canthaxanthin; V, violaxanthin; L, lutein; Z, zeaxanthin.

concentration and composition of carotenoids, and in particular the extract from seeds of double transformants was clearly reddish (Figure 6). The expression pattern determined by immunostaining of sections of immature seeds was characteristic for the expression from the napin promoter (data not shown).

The relative amount of ketocarotenoids formed in β-carotene oxygenase/phytoene synthase double transformants was estimated to be 38% of the total pigment content. This figure was obtained by subtracting all mAU areas of HPLC chromatogram peaks of the phytoene synthase plants from all corresponding new peaks having ketocarotenoid absorption spectra, formed in seeds of the double transformants. A similar number was obtained by subtracting the amount of unidentified carotenoids of the phytoene synthase-overexpressing plants from the unidentified carotenoids and adding this difference to the percentage of identified ketocarotenoids in the double transformant. Among the carotenoids unidentified in wild-type plant, HPLC spectral data suggested that antheraxanthin and two peaks having lutein-like spectra contributed the most to this fraction (approximately 11% of the total mAU areas). Comparing extracts from seeds of transgenic plants and control plants revealed a change in the distribution of the carotenoids. The major carotenoids were changed from 84 to 63% in the primary transgenic plant overexpressing the algal gene, whereas the total carotenoid content was not

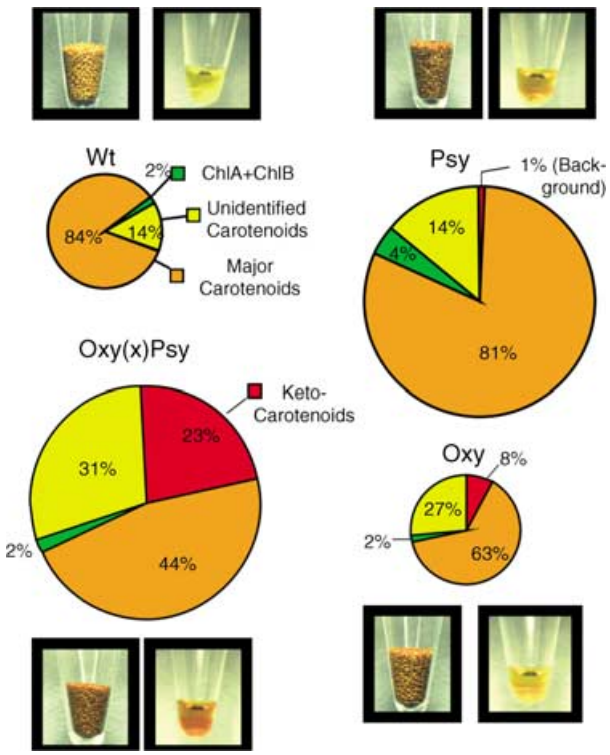

Figure 6. The relative carotenoid content, colour of seeds and extracts of different transformants.
The tubes containing hexane-extracted oil and seeds from a wild-type (Wt) plant, and the different transgenic plants are inserted to show the differences in the colour of the oil. The distribution and percentage peak areas from HPLC chromatograms of the pigments indicated are drawn as pie diagrams for each plant, and the area of the pie represent the relative total pigment content. In *Psy*, small peaks with similar retention time as the three major ketocarotenoids not containing ketocarotenes were present, and this background were 1% of the total pigments. Indicated percentages were the average of four individual transgenic plants except the single *Oxy* transformants that were the average from three individual transgenic plants. Abbreviations: Wt, wild type; Oxy, plant transformed with a *β-carotene-oxygenase* construct; Oxy(x)Psy, a crossing between transgenic plant overexpressing β-carotene-oxygenase and phytoene synthase constructs. *Psy* is a transgenic plant overexpressing a phytoene synthase construct. Pigments indicated: major carotenoids are: violaxanthin, lutein, zeaxanthin, lycopene and β-carotene; ChlA + ChlB, chlorophyll *A* and *B*; ketocarotenoids include 4-keto-lutein, adonirubin, canthaxanthin.

significantly changed (Figure 6). The difference of the sum of the major carotenoids violaxanthin, lutein, zeaxanthin and β-carotene was compensated in seeds of transgenic plants by increased areas of unidentified carotenoids and ketocarotenoids (Figure 6). A considerable part of the ketocarotenoids found eluted late, and this part was also shown to contain esterified forms of ketocarotenoids (Figure 2a).

Crossings between plants transformed with the β-carotene-oxygenase construct and plants transformed with an endogenous phytoene synthase construct were made to find out whether it was possible to increase the yield of ketocarotenoids by providing the β-carotene-oxygenase with more of suitable substrates during seed development. The first generation of these crossings were selfed, and the average from four individual plants of the next generation from these crossings produced 4-keto-lutein (11%), adonirubin (5.3%) and canthaxanthin (6.4%), respectively, calculated as the percentage of its total mAU areas. The identified ketocarotenoids in the double transformants increased 13-fold and the total carotenoid content 4.6-fold, compared to those in plants transformed with the β-carotene-oxygenase construct only (Figure 6). The chosen plants that overexpressed the endogenous *phytoene synthase* used for these crossings had 0.088 mg g^{-1} FW β-carotene. This was approximately 33% of the relative β-carotene content of the highest levels previously reported by Lindgren *et al.* (2003), indicating that there might be a potential to further increase the levels of ketocarotenoids.

Discussion

Arabidopsis thaliana is a model plant belonging to the large family of *Brassicaceae*, a family that includes many important oilseed crops. The relationship between *Arabidopsis* and other oleiferous plants in this family makes *Arabidopsis* useful as a test system to obtain preliminary results that later can be used to produce a crop with specific qualities. By overexpression of an algal construct in combination with overexpression of a phytoene synthase construct, the carotenoid content was altered and increased in *Arabidopsis*. Seeds from the transgenic plant only overexpressing the algal gene contained several ketocarotenoids such as 4-keto-lutein, canthaxanthin, adonirubin and astaxanthin, but just as in wild-type extracts; the major carotenoid found in seeds was lutein. The low levels of β-carotene and lack of a detectable level of α-carotene in the wild type and the *β-carotene-oxygenase* transformants indicated that both α- and β-rings were efficiently hydroxylated. The fact that hydroxylated carotenes have been shown to be less efficiently carbonylated *in vitro* (Fraser *et al.*, 1998) makes it reasonable to speculate that competition between hydroxylation and carbonylation of α- and β-carotene determines the type of carotenoids that accumulate. Other explanations for the incomplete formation of ketocarotenoids could be differences in temporal expression of the introduced genes in relation to endogenous biosynthesis of carotenoids or that suitable substrates were not available for the β-carotene-oxygenase because of differences in compartmentalization. Low levels of co-factors such as oxygen and Fe^{2+} required for the ketocarotenoid-forming activity could also explain the result. Nevertheless, the formation of free and esterified forms of ketocarotenoids in the primary β-carotene-oxygenase-overexpressing plants resulted in substantial reduction of the levels of the major carotenoids, while the total peak areas of the pigments were not significantly changed.

The relative amount of ketocarotenoids produced in seeds of *Arabidopsis* greatly increased by simultaneous overexpression of a cDNA construct of an endogenous *phytoene synthase* gene. The proportion of free ketocarotenoids increased while ketocarotenoids eluted after β-carotene remained more constant. It is interesting that there is an enzyme capable of esterifying ketocarotenoids in seeds of *Arabidopsis* that are normally devoid of these modified carotenoids, and it seems reasonable to assume that the ketocarotenoids are more easily modified than the normal carotenoids in seeds. Whether this is because of differences in location of the ketocarotenoids or differences in their structure is currently not known. The presence of naturally occurring, plastidic enzymes with esterifying properties is common and likely to be important for sequestration of carotenoids in fruits and flowers. In another study, a homologue to the algal *β-carotene-oxygenase* gene used in this study was fused to the phytoene desaturase promoter and its corresponding transit peptide sequence. This resulted in accumulation of mainly esterified forms of astaxanthin, adonirubin and 4-keto-zeaxanthin in the nectary tissue of tobacco. In species like *Adonis astaevalis* and *Tagetes erecta*, respectively, esterified astaxanthin and lutein accumulate in the chromoplasts in petals (Kamata and Simpson, 1987; Moehs *et al.*, 2001). Thus, such enzymes involved in the esterification/binding of fatty acids to carotenoids have, to our knowledge, not been defined, although esterified carotenoids are abundant in tissues/cells with high levels of carotenoids. The accumulation and deposition of high levels of astaxanthin in *H. pluvialis* might depend on the activity of a putative acyltransferase, as it has been shown that the accumulation of ketocarotenoids depends on an active synthesis of fatty acids in the alga (Schoefs *et al.*, 2001).

Normally, the carotenoid content in carotenoid-accumulating tissues in higher plants is in the range of hundreds of micrograms per gram FW, but in the alga *H. pluvialis* as much as 43 mg g^{-1} FW astaxanthin accumulates in lipid bodies outside the chloroplast (Boussiba *et al.*, 1999; Grunewald *et al.*, 2001; Margalith, 1999). The mechanisms that allow the alga to accumulate these high levels of esterified astaxanthin are still not clear. Although a suggestion

regarding this mechanism has recently been presented by Grunewald *et al.* (2001). They found that the main activity of the β-carotene-oxygenase was in the cytosolic lipid vesicles. It is believed that the early steps in the synthetic pathway are localised to the thylakoids of the chloroplast, because of which one of the early enzymes, the phytoene desaturase (Grunewald *et al.*, 2000), has been detected by immunolocalisation to this compartment. Thus, the authors proposed that transport of early substrate intermediates to the site of accumulation in cytoplasmic vesicles must occur. Recently, it was also discovered that in the liverwort *Marchantia polymorpha*, the complete synthesis of carotenoids occurs outside the plastid in oil bodies in the cytoplasm (Suire *et al.*, 2000). This clearly shows that carotenoid synthesis is not limited to the chloroplast in photosynthesising organisms. Further studies on the carotenoid biosynthesis pathway and the mechanisms for deposition of the products are crucial to establish plants that can accumulate high levels of these lipophilic molecules.

Experimental procedures

DNA constructs and transformation

The transit peptide RBCS1a sequence was obtained by PCR amplification using the 5′ primer AGACGGATCCTCAGTCACACAAAGAGTA homologous to −28 of the leader sequence and the 3′ primer GTTCGAGCTCTCTAGACATGCAGTTAACGC homologous to the opposite strand of the transit peptide ending at the cleavage site. Both primers were synthesised with an additional four nucleotides complementary to its template to maintain a functional cleavage site. This generated, after cleavage, a 5′ *Bam*HI site and 3′ *Xba*I/*Sac*I site to the ends of the Rubisco transit peptide sequence. The fragment was inserted into a −1101 napA promoter construct, thus replacing the *Bam*HI/*Sac*I GUS fragment (Stalberg *et al.*, 1993). A truncated cDNA from a *β-carotene-oxygenase*, homologous to the sequence isolated by Kajiwara *et al.* (1995), Accession no. D45881, was cloned by RT-PCR from mRNA preparations of *H. pluvialis*, using the 5′ and 3′ primer-pair ACAGTCTAGAATGCCATCCGAGTCGTCA and CACCGAGCTCCATGACACTCTTGTGCAGA, respectively. The cDNA was fused to the transit peptide by insertion into the *Xba*I/*Sac*I sites. The fusion generated the construct described in Figure 1. The *Hin*dIII/*Eco*R1 construct consisting of the seed-specific napin promoter, transit peptide, β-carotene-oxygenase and a termination sequence (napA-TP-β-carotene-oxygenase-Nos termination) was inserted into the *Hin*dIII/*Eco*R1 sites of the pGA581 binary vector from An (1987). The phytoene synthase construct and plants used in this study were described by Lindgren *et al.* (2003). *A. thaliana* (Wassilewskija) plants were transformed by vacuum infiltration according to the Green lab protocol (Bariola *et al.*, 1999). Kanamycin-resistant *Arabidopsis* plants transformed with β-carotene-oxygenase construct were selected and put on soil for growth at 16-h light, 22°C and 8-h dark, 20°C. Twenty of these plants were grown individually, and seeds were collected at maturity and stored at −20°C. The remaining 115 kanamycin-resistant plants were divided in four batches, and seeds were collected and marked as batch 1–4. Second generations of crossings between plants overexpressing the cDNA from the algal gene and the endogenous phytoene synthase construct were selected on kanamycin and spectinomycin and by differences in their seed colour. Seeds from 77 individual plants were generated, and four of these having the highest ketocarotenoid content were analysed in detail.

Extraction and purification of carotenoids

Screening for ketocarotenoid accumulation in 19 individual primary transformants expressing the algal *β-carotene-oxygenase* was performed by grinding 5 mg of seeds using a nylon pestle in 150 μl of buffer A (82% methanol, 8% water and 10% ethyl acetate), avoiding exposure to light. The homogenate was centrifuged for 3 min, and the pellet was re-extracted by adding 150 μl of buffer B (20% methanol, 1% water and 79% ethyl acetate). All other extractions were performed according to Fraser *et al.* (2000). For esterase treatments, 50 mg of seeds were homogenized in 300 μl of pure hexane with a nylon pestle in a microfuge tube. The tube was centrifuged at 13 000 ***g*** for 1 min, and the supernatant was taken off and loaded on top of 0.5 g of silica 60, mesh 230–400, in a Pasteur pipette. The column was pre-wet with 1 ml of hexane before loading. The extract was eluted by stepwise addition of increasing concentrations of diethylether in hexane: 3 × 1 ml of 2%; 3 × 0.5 ml of 15%; and 4 × 0.5 ml of 20%. Adding 100% diethylether eluted the remainder when appropriate. 0.5–1-ml fractions were taken and evaporated under nitrogen. β-Carotene and oil eluted in tube 5–6 and the esterified carotenoids in the following tubes.

Cholesterol esterase reaction

Acetone (0.6 ml) was added to each tube containing dried purified extracts from 25 mg of seeds, and the carotenoids were dissolved. A pre-made reaction mix of 0.22 ml containing 20 U ml^{-1} cholesterol esterase from *Pseudomonas* sp. (sterol-ester acylhydrolase, Enzyme Commission Number: 3.1.1.13, Molecular Design Limitid (MDL) number: MFCD00071052, 9026-00-0), 0.05 M Tris–HCl (pH 7), kept on ice, was added to the mixture and incubated at 37°C for 1 h at slow shaking. After incubation, 0.4 ml diethylether was added and the tubes were vortexed for 20 sec. Following centrifugation, the upper phases were removed to new tubes. This was repeated once. The upper phases were pooled in tubes containing 0.2 g Na_2SO_4 and centrifuged. The Na_2SO_4 pellet was re-extracted once. The upper phases were pooled and transferred to a new tube for evaporation under nitrogen.

HPLC analysis

The screen of the carotenoid content of individual transformants was according to a procedure described by Pettersson and Lignell (1999). The method was performed as follows: an end-capped ReproSil 120 C18-Aq, particle size 5 μm, protected with an A 103X Stainless steel Frit (Upchurch Scientific Inc., Knivsta, Sweden), was used for reversed phase chromatography. The HPLC system was an HP Agilent 1100 series, and signal detection was set to 280 and 454 nm bandwidth (bw) 10 nm. Data were collected from 260 to 700 nm for UV/Vis identification. Forty-five to ninety microlitres of the extracts was injected and subjected to a linear gradient starting with 100% buffer A to 100% buffer B at a flow rate of 1.2 ml min^{-1}. After 20 min, buffer B was maintained for 2 min, and then a linear gradient for 2 min to 100% buffer A was adopted. The column was equilibrated with 100% of buffer A for 6 min before next run.

Further analysis of extracts was performed by the HPLC analysis as mentioned above, but modified as follows: a 25-min linear gradient from 80% methanol, 10% water and 10% ethyl acetate

to 30% methanol, 5% water and 65% ethyl acetate was executed. Then, a 7-min linear gradient to 29% methanol, 1% water and 70% ethyl acetate and a 3-min linear gradient back to starting conditions were accomplished. All peak spectra were stored ranging from 260 to 700 nm at steps of 2 nm and a threshold of 0.1 mAU. Signal detection was set to 280 and 454 nm bw 10 nm, and the temperature was 20°C. For injection, 25–30 μl of the extracts was used.

Mass analysis

Mass spectrometric nanomole analysis was performed by use of a Micromass Q-TOF electrospray instrument (Micromass, Manchester, UK) or a MALDI-TOF. A total of 0.6 ml from HPLC fractions of the peak containing 4-keto-lutein from transgenic plants was evaporated under nitrogen to reduce the volume to approximately 20 μl or complete dryness. The amount was estimated by comparison of absorption spectra to the absorption spectra of a known amount of canthaxanthin or β-carotene, and purity of the fraction was analysed by HPLC. These fractions were diluted by adding an equal volume of buffer A or acetone; 2 μl of this sample contained approximately 5 pmol of the unknown fraction and was then infused for MS analysis on the Micromass Q-TOF. MS spectra were collected, and the obtained 583.4 and 565.4 peaks were fragmented in order to investigate the relatedness of the peaks. Samples were also analysed on a Bruker Reflex IV MALDI-TOF instrument with 2,5-dihydroxy-benzoic acid dissolved acetone as matrix. Matrix solution (0.5 μl) was applied on Ankor Targets 800 μm (Bruker Daltonics, Germany) followed by 0.5 μl (approximately 35 pmol) of each sample. Mass spectrometer was operated in reflector mode and calibrated with peptide standard mixture from Bruker Daltonics.

Chemical analysis

HPLC fractions containing approximately 50 pmol of the peaks containing the putative 4-keto-lutein, adonirubin and canthaxanthin were collected and evaporated under nitrogen for chemical analysis. The fractions were dissolved in 25 μl of methanol and chemically modified by adding a pinch (<1 mg), with the point of a scalpel, of sodium borohydride (Lancaster Synthesis, Morecambe, UK). After the reaction had reached completion, 25 μl of chloroform was added and 30 μl of the sample was used for injection in the HPLC. An equal amount of untreated sample was used as a control.

The canthaxanthin standard was obtained as a gift from Hoffmann-La Roche (lot no. 0303342), and the astaxanthin standard was bought from Sigma–Aldrich (AA9335). Zeaxanthin was obtained from an *E. coli* strain, containing the plasmids pACCAR25 and pHP51 (Kajiwara *et al.*, 1995), kindly provided by Dr Norihiko Misawa.

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