Research Article

# Metabolic engineering of ketocarotenoid biosynthesis in leaves and flowers of tobacco species

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Ketocarotenoids and especially astaxanthin are high-valued pigments used as feed additives. Conventionally, they are provided by chemical synthesis. Their biological production is a promising alternative. For the development of a plant production system, *Nicotiana glauca*, a species with carotenoid-containing yellow pigmented flower petals, was transformed with a cyanobacterial ketolase gene. The resulting plants accumulated 4-ketozeaxantin (adinoxanthin), which is the first ketocarotenoid synthesized in flower petals by genetic modification. Due to the very late flowering in this tobacco species, *N. tabacum* was used to optimize the yield and ketocarotenoid product pattern by metabolic engineering of the ketolation steps of carotenogenesis. The highly carotenogenic nectary tissue in the flowers represents a model of a flower chromoplast system. By expression of a ketolase gene, it was possible to engineer the biosynthetic pathway towards the formation of 3'-hydroxyechinenone, 3-hydroxyechinenone, 4-ketozeaxanthin, 4-ketozeaxanthin esters, 4-ketolutein and 4-ketolutein esters. Some of these ketocarotenoids were also formed in the leaves of the trangenic plants. In particular, by co-expression of the ketolase gene in combination with a hydroxylase gene under an ubiquitous promoter, the formation of total carotenoids in nectaries increased by more than 2.5-fold. In the nectaries of this type of transformants, more than 50% of the accumulating carotenoids were keto derivatives. In addition, the levels of ketocarotenoid esters were much lower and a higher percentage of the free ketocarotenoids accumulated. These results open new promising perspectives for a successful metabolic engineering of keto-hydroxy carotenoid production in carotenogenic flowers.

**Keywords:** Nectary carotenoids · *Nicotiana glauca* · *Nicotiana tabaccum* · 4-ketolutein/4-ketozeaxanthin/adinoxanthin · Ketolase genes *crtO* and *crtW*

## 1 Introduction

Carotenoids accumulate in plastids of leaves, flowers and fruit of plants. In photosynthetic tissue, they are involved in the light harvesting and in photoprotection mechanisms. In humans, different carotenoids exert health-promoting properties. β-Carotene and related derivatives are essential for the formation of vitamin A, and lutein together with zeaxanthin helps in protection from light-induced retinal damage and age-related macular degradation [1]. In addition, evidence is accumulating showing that diets rich in carotenoids prevent the onset of certain chronic disease and certain cancers [2, 3]. This may be related to the high anti-oxidative potential of carotenoids [4, 5]. Especially 4-ketocarotenoids are photo stable pigments with high anti-oxidative activities [6]. In this respect, ketocarotenoids are superior to non-ketolated analogues. However, with the exception of *Adonis* flowers [7], no higher plant source of ketocarotenoids exists. In contrast to various microorganisms, plants generally lack the biosynthetic potential for ketocarotenoid formation. Nevertheless, plants are attractive as ketocarotenoid production systems. The biosynthesis of novel compounds can

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**Abbreviation: dw**, dry weight

be manipulated by genetic modification of their metabolic capacity [8]. The carotenoid biosynthetic pathway can be extended to 4-ketocarotenoids by transformation with a ketolase gene. Orthologous ketolase genes of the *crtW/bkt* family from the alga *Haematococcus pluvialis* [9, 10] and the bacterium *Paracoccus* [11], the *crtO* gene from *Synechocystis* [12] or the P450 monooxygenase gene *asy* from *Xanthophyllomyces dendrorhous* [13] are available for genetic engineering of a ketocarotenoid pathway. All heterologous ketolase act on β-carotene yielding 4-keto derivatives followed by hydroxylation at position 3. This leads to a diverse array of potential products.

Several attempts have been made to genetically modify carotenogenesis towards the synthesis of keto derivatives in plants. For example, ketocarotenoid synthesis was achieved in tobacco nectary tissue and leaves [14, 15] and *Arabidopsis thaliana* seeds [16]. Alternatively, β-carotene-accumulating tomatoes were co-transformed simultaneously with a β-carotene ketolase and a hydroxylase gene [15], or high zeaxanthin-accumulating potato with the *crtO* ketolase gene [17].

In the present study, we report on successful attempts to generated ketocarotenoids in flower petals by genetic manipulation of *N. glaucum*. Due to faster growth and flower development, *N. tabacum* was then used as a model system for multiple engineering of carotenogenesis in order to apply these results to *N. glaucum*. Since the latter tobacco species offers the advantage of carotenoid biosynthesis in flower petals, it may be developed to an attractive ketocarotenoid plant source.

## 2 Materials and methods

### 2.1 Biological material and growth conditions

The tobacco lines *N. tabacum* cv. SR1, *N. glauca* and *N. tabacum* cv. SR1 transformant crtZU3 [18] were grown at 25°C in a 16-h light and 8-h darkness cycle on MS20 media for transformation. Depending on the plasmid, transgenic plants were selected on kanamycin (100 mg/L) or hygromycin (10 mg/L) containing medium. After regeneration, transgenic *in vitro* plantlets were transferred and grown in a green house on soil at temperatures of about 20°–25°C during the day and 10°–15°C at night. Leaves and flowers of homozygous *N. tabacum* and *N. glauca* transformant lines were harvested after flower development.

*Agrobacterium tumefaciens* strain LBA 4404 was used for transformation. *Escherichia coli* DH5α was the host for subcloning and plasmid amplification. Both strains were grown on LB medium for 2 days at 28°C or overnight at 37°C, respectively. The following antibiotics were added: 50 mg/L kanamycin, 50 mg/L rifampicin, 50 mg/L streptomycin for *A. tumefaciens* and 100 μg/mL ampicillin for *E. coli*. [19].

### 2.2 Plasmids, construction and tobacco transformation

Plasmid pslr0088 [12] was the source of the *crtO* gene from *Synechocystis* (accession no. NP_442491). For construction of the plant transformation vector containing the ketolase gene *crtO* and a kanamycin resistance gene, *crtO* was amplified by PCR using primer *Sph*I-O 5'-TTGCCTCACCAC**CGATGT**TGTC-3' (generating a *Sph*I restriction site) and *Pst*I-O 5'-TTC**TGCAG**TTACCAAAAACGACGTTGTTG-3' (generating a *Pst*I restriction site). The fragment was subcloned into *Sph*I and *Pst*I-digested plasmid pTRA3XN [20], which harbors the transit peptide signal from the small subunit of ribulose bisphophate carboxylase from *Phaseolus vulgaris*. The transit-*crtO* fusion was cut out with *Xba*I and ligated into the *Xba*I-digested vector pGPTV-NPT-$P_{35S}$, a pBIN19 derivate [21], resulting in plasmid pGPTV-NPT-trcrtO with *crtO* expressed under the 35S cauliflower mosaic virus promoter and kanamycin as selection marker. The construction of pGPTV-HPT-trcrtO was carried out similarly to a recently described method [17]. The transit sequence was fused to the *crtW* gene (orf148) from *Nostoc* 73102 [22] by overlapping PCR in three PCR steps, with *crtW* and pTRA3XN [20] as template using two pairs of primers, F-TRA GGATCCATGGCTTCTATGATA and R148-TRA AACTGGATCATGCACTTTACTCTTCCAC, or TRA-148 GAGTAAAGTGCATGATCCAGTTAGAACAACCAC and E-148 GCTAAGGATCCTTCTATTTTGC. Both PCR products were fused and amplified using the first and last primer mentioned above. The reaction product with T-overhanging ends was ligated into the *Xcm*I-digested vector pMON3820 [23], cut out with *Bam*HI and ligated into the *Bam*HI-digested vector pBI121GS [24]. Plasmid pCrtZ-kan [18] and pGPTV-NPT-trcrtO [17] originate from previous publications. The structure of the three ketolase plasmids and the hydroxylase plasmid are shown in Fig. 1. *Agrobacterium*-mediated transformation of both tobacco species was performed according to the leaf disc method of Horsch *et al*. [25]. The *crtZ/crtO* double transformant was obtained using the transgenic tobacco [18] carrying the integrated plasmid pCrtZ-kan (Fig. 1), which was over-transformed with plasmid pGPTV-HPT-tcrtO (Fig. 1) carrying hygromycin resistance. Plants regenerated either on kanamycin or hygromycin were immediately analyzed by PCR with primers specific for *crtO* or *crtW* to demonstrated the integration of the transgene. This was later confirmed by Southern analysis with DIG-labeled *crtO* and *crtW* probes (data not shown). For the blots, genomic DNA was isolated from leaf tissue using the GenElute Plant Genomic DNA Miniprep Kit from Sigma (Straubenhard, Germany).

### 2.3 Pigment extraction and analysis

Chlorophyll was extracted from freeze-dried leaves (2 mg) with methanol at 60°C for 15 min. The content was quan-

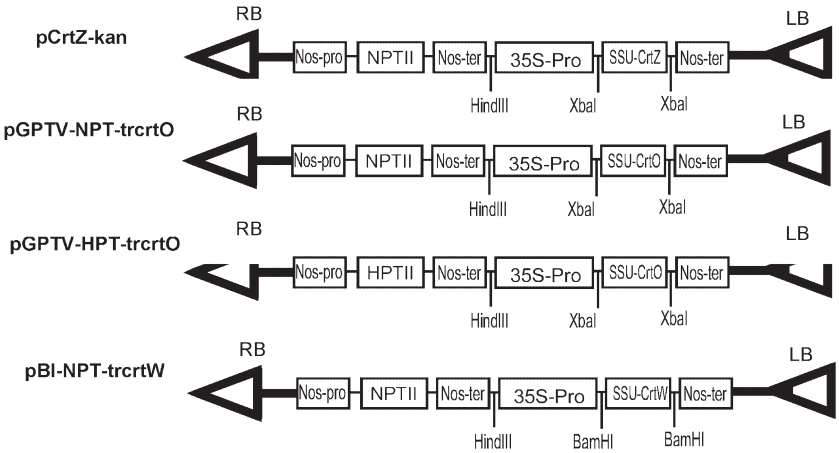


**Figure 1.** Binary vector constructs for transformation of tobacco species.

titated from the absorbance at 650 and 665 nm according to Mackinney [26]. The carotenoids from freeze-dried leaves, petals or nectary tissue (10 mg) were extracted in the same way. Alternatively, methanol containing 6% KOH was used for leaf carotenoid extraction. The extracts were partitioned against 10% diethyl ether in petrol. The carotenoids were determined by HPLC analysis. The system used was a Hypersil HyPurity Elite C18 column with 5-µm particle size run with acetonitrile/2-propanol/methanol/water 83:10:5:2 at 32°C. Standards were isolated from *X. dendrorhous* [27] and *E. coli* transformed with appropriate carotenogenic genes for keto carotenoid production and their molecular weight confirmed by mass spectroscopy [28]. All carotenoids mentioned were identified by co-chromatography with these reference compounds and comparison of their spectra. For quantitation, the extinction coefficients were taken from Davies [29]. In case of 4-keto-lutein and ketocarotenoids with absorbance spectra similar to echineneone, the extinction coefficient of the latter carotenoid was used. 4-Ketolutein and 4-ketozeaxanthin (adinoxanthin) were reduced with $NaBH_4$ to the corresponding alcohols according to Eugster [30].

### 2.4 Determination of photosynthetic efficiency

Chlorophyll fluorescence by photosystem (PS) II was measured with a PAM 101 fluorometer (Walz, Effeltrich, Germany). Leaves were measured without detachment from the plants. The initial fluorescence yield (*F*0*)* in weak modulated light was recorded followed by the maximum fluorescence yield (*F*m*)* after a saturating light pulse (4000 µmol/$m^2$ s). The photosynthetic efficiency *F*v/*F*m ratio of dark-adapted leaves was calculated automatically.

## 3 Results and discussion

The majority of plants are devoid of ketocarotenoids. Nevertheless, they are very useful for heterologous ketocarotenoid production in their different carotenogenic tissues. So far, ketocarotenoid biosynthesis has been successfully engineered into fruit, seeds and tubers (see Sandmann *et al*. [31] for a recent review). Although many flowers are pigmented by carotenoids, no reported attempt has been made yet to express a ketolase gene to modify carotenoid biosynthesis in carotenogenic flower petals. Most of these plants are very difficult to transform. Therefore, we used a tobacco species *N. glauca*, which unlike the well-know *N. tabaccum* develops carotenoid-pigmented yellow flowers [32], and applied to this species the standard transformation protocol for *N. tabaccum*. Our goal was to genetically engineer a novel ketocarotenoid pathway into flower petals [33]. After transformation of *N. glauca* with the *crtW* gene using the binary plasmid pBI-NPT-trcrtW (Fig. 1), 130 PCR-positive transformants were raised. Among them, 116 plants started flowering within 1 year. According to their ketocarotenoid formation in flowers, we could group them into those with trace amounts (37% of transgenic flowering plants), low ketocarotenoid formation of 5–30 µg/g dry weight (dw) (21%) and higher ketocarotenoid formation of up to 60 µg/g dw (22%). In the flowers of the remaining plants, ketocarotenoids were below detection. Figure 2 shows a HPLC separation of the carotenoids in petals of transformant NgCrtW#111. It belongs to the higher ketocarotenoid-forming transformants with a 4-ketozeaxanthin content in flower petals of 56 µg/g dw. In the non-transformed wild type, lutein (peak 2) together with a lutein *cis* isomer (peak 2') is the dominant carotenoid (trace 2A). Furthermore, substantial amounts of β-carotene (peak 3 and 3') and violaxanthin (peak 1) in addition to an array of different violaxanthin fatty acid esters indicated by an asterisk are present. In the *crtW* transfor-

 

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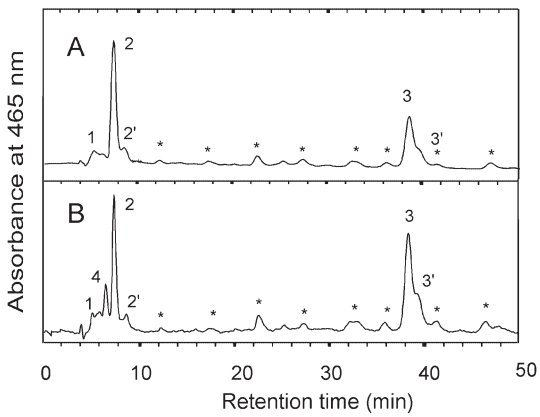


**Figure 2.** HPLC separation of carotenoids from leaves of *N. glauca* wild type (A) and transformant NgCrtW#111 (B). Peaks were identified as: 1, violaxanthin; 2, lutein; 2', lutein cis isomer; 3, β-carotene; 3', β-carotene cis isomer; 4, 4-ketozeaxanthin. Asterisks indicate different violaxanthin fatty acid esters.

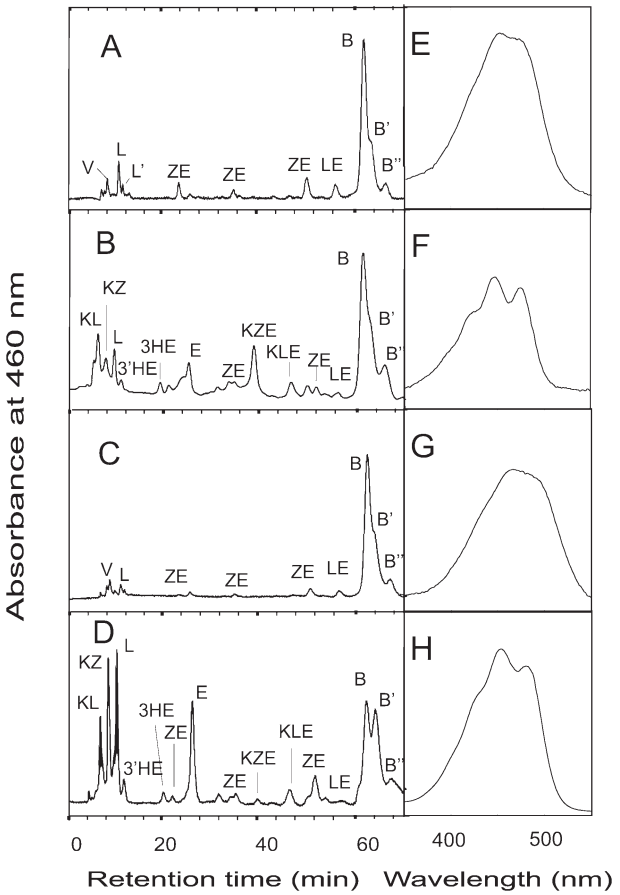


**Figure 3.** HPLC separation of carotenoids from nectary tissue of *N. tabacum* and carotenoids spectra. (A) wild type, (B) NtCrtO1/2, (C) NtCrtZ#U3, (D) NtCrtZO#4. Spectra of ketolutein (E), ketolutein reduced to HO-ketolutein (F), 4-ketozeaxanthin (G) and 4-ketozeaxanthin reduced to 4-HO-zeaxanthin (H). Peaks are labeled as B, β-carotene with cis isomers B' and B''; E, echinenone; 3HE, 3-HO-echinenone; 3'HE, 3'-HO-echinenone; KL, 4-ketolutein; KLE, 4-ketolutein fatty acid ester; KZ, 4-ketozeaxanthin (adonixanthin); KZE, 4-ketozeaxanthin fatty acid ester; L, lutein with L' cis isomer; LE, lutein fatty acid esters; V, violaxanthin and ZE, zeaxanthin fatty acid esters.

mant, an additional peak at 6.8 min with a typical ketocarotenoid spectrum and a maximum at 465 nm (see Fig. 3G) was found. The spectral properties and co-chromatography with an authentic standard identified peak 4 as 4-ketozeaxanthin. This is the first demonstration of engineered ketocarotenoid biosynthesis in flower petals. However, the yield of 7–8% in the best transformants is still low but comparable to the ketocarotenoid content of *N. tabacum* leaves and nectaries transformed with a similar bacterial ketolase gene [15] or in potato tubers [17]. Therefore, further attempts should be made to overcome the low ketocarotenoid production rates and to reach complete ketolation of both β-ionone end groups of β-carotene with astaxanthin as the final product. A promising strategy is to improve the activities of β-carotene 3-hydroxylation and 4-ketolation [15].

Flower formation of *N. glauca* depends very much on the season even in a green-house. The plant prefers long day conditions and high intensity sun light. Thus, it may take up to 1 year after seedling transfer to soil before the first flowers can be analyzed. Therefore, we have chosen the faster growing and flowering *N. tabacum* as a model to improve ketocarotenoid biosynthesis in leaves and flowers by transformation with a ketolase and hydroxylase genes. The nectary in the flowers of *N. tabacum* is the tissue of choice to study manipulation of carotenogenesis in flowers [14]. In addition to a hydroxylase gene from *Erwinia uredovora*, which was highly functional in tobacco [18], we have used the cyanobacterial ketolase gene *crtO*. This gene was successfully used to engineer the biosynthesis of the di-keto derivative astaxanthin in zeaxanthin-accumulating potato tubers [17]. Therefore, it may offer a better chance than crtW for the synthesis of astaxanthin, especially in tobacco crtZU3 with an engineered zeaxanthin background [18].

Due to the ubiquitous CaMV 35S promoter, expression of *crtO* in wild-type tobacco resulted in the formation of ketocarotenoids in leaves and in the flower nectaries (Table 1). The transformants showed lower leaf carotenoid content and concurrently lower chlorophyll concentrations. However, photosynthetic efficiency was not impaired. The newly formed ketocarotenoids in the leaves were echinenone derived from β-carotene as precursor and 4-ketolutein. Obviously, ketocarotenoids are formed in leaves by mono ketolation from the carotenoids with the highest pools. The total share of these ketocarotenoids was 4.9%. As in tomato [15] and potato [17], echinenone was one of the major ketocarotenoids in tobacco leaves. However, 4-ketolutein has never been detected in leaves before.

**Table 1.** Chlorophyll and carotenoid content in leaves and nectaries of wild-type and transgenic *N. tabacum* transformed with pGPTV-NPT-trcrtO and photosynthetic efficiency[a)]

| | Leaves | | Nectary tissue | |
|---|---|---|---|---|
| | **Wild type** | **NtCrtO1/2** | **Wild type** | **NtCrtO1/2** |
| Chlorophyll (mg/g dw) | 20.3 ± 1.7 | 15.0 ± 1.9 | 0 | 0 |
| Carotenoids (μg/g dw) | 1634.1 ± 185.6 | 1118.4 ± 118.2 | 315.8 ± 89.4 | 596.5 ± 170.4 |
| Distribution (%) | | | | |
| Violaxanthin | 7.9 | 9.1 | 2.6 | 1.7 |
| Antheraxanthin | 1.0 | 1.6 | 0 | 0 |
| Lutein | 34.3 | 37.9 | 3.8 | 9.1 |
| *cis*-Lutein | 3.8 | 5.1 | 0 | 0 |
| β-Carotene | 49.9 | 39.2 | 74.5 | 33.2 |
| *cis*-β-Carotene | 3.2 | 2.1 | 6.5 | 8.1 |
| Zeaxanthin esters | 0 | 0 | 7.7 | 0 |
| Luteinesters | 0 | 0 | 5.1 | 2.2 |
| Echinenone | 0 | 2.3 | 0 | 8.3 |
| 3'-Hydroxyechinenone | 0 | 0 | 0 | 1.3 |
| 3-Hydroxyechinenone | 0 | 0 | 0 | 1.4 |
| 4-Ketozeaxanthin | 0 | 0 | 0 | 6.7 |
| 4-Ketolutein | 0 | 2.6 | 0 | 8.3 |
| 4-Ketozeaxanthin esters | 0 | 0 | 0 | 12.5 |
| 4-Ketolutein esters | 0 | 0 | 0 | 5.0 |
| Photosynthetic efficiency | 0.81± 0.02 | 0.81 ± 0.02 | – | – |

a) All values are means ± standard deviation from three different tissues.

Nectary tissue contains chromoplasts instead of chlorophyll-containing chloroplasts [34]. Their carotenoid composition is different to the composition found in leaves. β-Carotene is the dominating pigment together with lutein and zeaxanthin fatty acid esters (Table 1). The esters were identified by their spectra and conversion to the free carotenoids upon saponification with KOH. Expression of *crtO* led to an increase of total carotenoid synthesis almost by a factor of two. In addition, more than half of all carotenoids were ketolated. Thus, the concentration of all non-ketolated carotenoids was close to the absolute values in the wild type. A much broader spectrum of keto derivatives was detectable in nectaries than in leaves. Their separation is shown in Fig. 3B in comparison with the wild type. In addition to the β-carotene-derived 4-ketozeaxanthin and its esters, echinenone and 3-hydroxy as well as 3'-hydroxyechinenone, α-carotene-derived 4-ketolutein and its ester were found (see Fig. 4 for their structures). All spectra of these mono keto-β-carotenes resemble that of Fig. 3G. The spectrum of free or esterified 4-ketolutein is shown in Fig. 3E. The ketocarotenoids were identified by standard carotenoids and the change in their spectra upon reduction to the 4-hydroxy derivatives (Figs. 3F and H). This is the first time that 4-ketolutein could be identified in leaves and nectaries of tobacco transformed with a ketolase gene, including its esterified form in the latter. Previously, its synthesis could be demonstrated in *A. thaliana* seeds [16] and in potato tuber [17] both engineered for formation of ketocarotenoids.

Leaves of the *crtZ/crtO* double transformants possess lower β-carotene and lutein pools than the *crtO* single transformant (Table 2). The same was observed for β-carotene in the nectaries. Simultaneously, both tissues

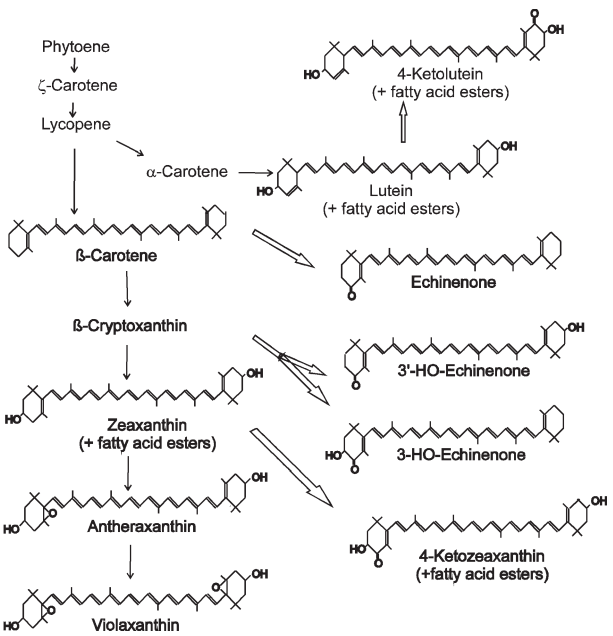


**Figure 4.** Carotenoid biosynthesis in leaf and nectary tissue of tobacco species. Open arrows indicate the reactions mediated by a ketolase gene in the transgenic plants.

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**Table 2.** Chlorophyll and carotenoid content in leaves and nectaries of transgenic *N. tabacum* transformed with pCrtZ-kan or pCrtZ-kan together with pGPTV-HPT-trcrtO, and photosynthetic efficiency[a)]

| | Leaves | | Nectary tissue | |
|---|---|---|---|---|
| | NtcrtZ | NtCrtZO#4 | NtcrtZ | NtcrtZO#4 |
| Chlorophyll (mg/g dw) | 17.6 ± 1.7 | 15.5 ± 1.6 | 0 | 0 |
| Carotenoids (μg/g dw) | 1163 ± 186 | 998 ± 144 | 325.9 ± 356 | 839.2 ± 83 |
| Distribution (%) | | | | |
| Violaxanthin | 9.0 | 7.9 | 2.9 | 0 |
| Antheraxanthin | 0.9 | 0 | 0 | 0 |
| Lutein | 37.8 | 26.2 | 2.2 | 16.2 |
| *cis*-Lutein | 4.1 | 3.8 | 1.1 | 0 |
| β-Carotene | 44.9 | 26.7 | 86.5 | 20.5 |
| *cis*-β-Carotene | 3.2 | 13.4 | 5.9 | 16.2 |
| Zeaxanthin esters | 0 | 0 | 0.7 | 6.1 |
| Lutein esters | 0 | 0 | 0.8 | 0.7 |
| Echinenone | 0 | 8.0 | 0 | 11.6 |
| 3'-Hydroxyechinenone | 0 | 5.2 | 0 | 1.8 |
| 3-Hydroxyechinenone | 0 | 0 | 0 | 2.1 |
| 4-Ketozeaxanthin | 0 | 0 | 0 | 12.9 |
| 4-Ketolutein | 0 | 8.8 | 0 | 8.5 |
| Ketozeaxanthin esters | 0 | 0 | 0 | 1.0 |
| Ketolutein esters | 0 | 0 | 0 | 2.9 |
| Photosynthetic efficiency | 0.80 ± 0.01 | 0.76 ± 0.3 | – | – |

a) All values are means ± standard deviation from three different tissues.

produce a higher amount of ketocarotenoids. In nectaries, less ketocarotenoids are esterified and an increase of the hydroxy-keto derivatives 3'-hydroxyechinenone and 4-ketozeaxanthin were observed yielding 136 and 108 μg/g dw, respectively (Fig. 3D). The only comparable data from nectary tissue on a dw basis are those from Ralley *et al.* [15], who co-expressed bacterial *crtW* and *crtZ* genes and predominantly obtained esterified ketocarotenoids. The concentrations were similar to our values in Table 2 for ketocarotenoid esters but our non-esterified ketocarotenoids exceed their total ketocarotenoid amounts by a factor of 4.

In leaves and nectaries, ketocarotenoids other than ketolutein are synthesized at the expense of the β-carotene pool (Fig. 4). However, we can see that in the nectaries of either the *crtO* or the *crtZ/crtO* transformants the absolute β-carotene concentrations stayed constant even though additional ketocarotenoids are formed (Tables 1 and 2). Their levels are maintained by an up-regulation of the entire carotenogenic pathway, which has been observed before [14, 15]. This makes similar chromoplast systems very useful for ketocarotenoid production with little limitation due to exhaustion of precursors. Formation of 4-ketozeaxanthin negatively affects the lutein pool in leaves. When the concentration drops below a certain threshold, it may impair photosynthetic productivity. Since photosynthetic efficiency was unaffected in leaves of the *crtO* and the *crtZ* transformants although chlorophyll concentrations were lower (Tables 1 and 2), the decrease in photosynthetic efficiency in the *crtZ/crtO* transformant may be attributed to lower lutein levels rather than to the alteration of the chlorophyll content. Even after its decrease, the β-carotene concentration of the *crtZ/crtO* transformant is substantial enough not to account for a limitation for the assembly of functional PSII reaction centers.

## 4 Concluding remarks

We have engineered, for the first time, ketocarotenoid biosynthesis into flower petals. A ketolase gene was successfully expressed in *N. glauca*. With *N. tabacum* as a model, we could demonstrate that engineering a combination of carotenoid ketolase and hydroxylase genes is a promising strategy to increase total ketocarotenoid production in a flower chromoplast system. It should also be applicable to the chromoplasts of *N. glauca* petals. The last ketolation converting 4-ketozeaxanthin to astaxanthin is still crucial. This is the limiting step in all plant systems. The reason may be the competition between foreign and endogenous ketolases or other foreign hydroxylases rather than a necessary cooperation of these enzymes. A strategy that may help to overcome this problem is the use of a new type of hydroxylase/ketolase gene from *X. dendrorhous* [13]. The product of this astaxanthin synthase gene has the advantage of using β-carotene as the direct substrate catalyzing sequentially both the 4-ketola-

 

tion and the 3-hydroxlation reactions. This gene should be combined with a strong chromoplast-specific promoter, which was already successfully introduced into tobacco [14]. On the long term, engineering of the carotenogenic pathway with suitable ketolase and hydroxylase genes will increase the chance to use cultured plants like *N. glauca* as a cost-efficient source of the high-value product astaxanthin [35].

## 5 References


[1] Landrum, J. T., Bone, A. R., Lutein, zeaxanthin and the macular pigment. *Arch. Biochem. Biophys.* 2001, *385*, 28–40.

[2] Giovannucci, E., Lycopene and prostate cancer risk. Methodological considerations in the epidemiologic literature. *Pure Appl. Chem.* 2002, *74,* 1427–1434.

[3] Sharoni, Y., Agbaria, R., Amir, H., Ben-Dor, A. *et al.,* Modulation of transcriptional activity by antioxidant carotenoids. *Mol. Aspects Med.* 2003, *24,* 371–384.

[4] Rice-Evans C. A., Sampson J., Bramley, P. M., Holloway, D. E., Why do we expect carotenoids to be antioxidants *in vivo*? *Free Radical Res.* 1997, *26*, 381–398.

[5] Stahl, W., Sies, H., Antiocidative activity of carotenoids. *Mol. Aspects Med.* 2003, *24,* 345–351.

[6] Woodall, A. A., Britton, G., Jackson, M. J., Carotenoids and protection of phospholipids in solution or in liposomes against oxidation by peroxyl radicals: relationship between carotenoid structure and protective ability. *Biochim. Biophys. Acta* 1997, *1336,* 575–586.

[7] Seybold, A., Goodwin, T. W., Occurence of astaxanthin in the flower petals of *Adonis annua* L. *Nature* 1959, *184,* 1714–1715.

[8] Lessard, P. A., Kulaveerasingam, H., York, G. M., Strong, A. *et al.,* Manipulation gene expression for the metabolic engineering of plants. *Metab. Eng.* 2002, *4*, 67–79.

[9] Kajiwara, S., Kakizono, T., Saito, T., Kondo, K. *et al.,* Isolation and functional identification of a novel cDNA for astaxanthin biosynthesis from *Haematococcus pluvialis*, and astaxanthin synthesis in *Escherichia coli*. *Plant Mol. Biol.* 1995, *29,* 343–352.

[10] Misawa, N., Satomi, Y., Kondo, K., Yokoyama, A. *et al.,* Structure and functional analysis of a marine bacterial carotenoid biosynthesis gene cluster and astaxanthin biosynthetic pathway proposed at the gene level. *J. Bacteriol.* 1995, *177*, 6575–6584.

[11] Lotan, T., Hirschberg, J., Cloning and expression in *Escherichia coli* of the gene encoding beta-C-4-oxygenase, that converts beta-carotene to the ketocarotenoid canthaxanthin in *Haematococcus pluvialis*. *FEBS Lett.* 1995, *364,* 125–128.

[12] Fernandez-Gonzalez, B., Sandmann, G., Vioque, A., A new type of asymmetrically acting beta-carotene ketolase is required for the synthesis of echinenone in the cyanobacterium *Synechocystis* sp. PCC 6803. *J. Biol. Chem.* 1997, *272,* 9728–9733.

[13] Ojima, K., Breitenbach, J., Visser, H., Setoguchi, Y. *et al.*, Cloning of the astaxanthin synthase gene from *Xanthophyllomyces dendrorhous (Phaffia rhodozyma)* and its assignment as a beta-carotene 3-hydroxylase/4-ketolase. *Mol. Gen. Genet.* 2006, *275*, 148–158.

[14] Mann, V., Harker M., Pecker, I., Hirschberg, J., Metabolic engineering of astaxanthin production in tobacco flowers. *Nat. Biotechnol.* 2000, *18*, 888–892.

[15] Ralley, L., Enfissi, E. M., Misawa, N., Schuch, W. *et al.,* Metabolic engineering of ketocarotenoid formation in higher plants. *Plant J.* 2004, *39*, 477–486.

[16] Stalberg, K., Lindgren, O., Ek, B., Hoglund, A., Synthesis of ketocarotenoids in the seed of *Arabidopsis thaliana*. *Plant J.* 2003, *36,* 771–779.

[17] Gerjets, T., Sandmann, G., Ketocarotenoid formation in transgenic potato. *J. Exp. Bot.* 2006, *57,* 3639–3645.

[18] Götz, T., Sandmann, G., Römer, S., Expression of a bacterial carotene hydroxylase gene (*crtZ*) enhances UV tolerance in tobacco. *Plant Mol. Biol.* 2002, *50,* 127–140.

[19] Sambrook, J., Fritsch, E., Maniatis, F. (Eds.), *Molecular cloning: a laboratory manual,* 2nd edn, Cold Spring Harbour Laboratory Press, New York 1989.

[20] Wagner, T., Windhövel, U., Römer, S., Transformation of tobacco with a mutated cyanobacterial phytoene desaturase gene confers resistance to bleaching herbicides. *Z. Naturforsch.* 2002, *57c,* 671–679.

[21] Bevan, M., Binary *Agrobacterium* vectors for plant transformation. *Nucleic Acids Res.* 1984, *12,* 8711–8721.

[22] Steiger, S., Sandmann, G., Cloning of two carotenoid ketolase genes from *Nostoc punctiforme* for the heterologous production of canthaxanthin and astaxanthin. *Biotechnol. Lett.* 2004, *26,* 813–817.

[23] Borovkov, A. Y., Rivkin, M. I., XcmI-containing vector for direct cloning of PCR products. *BioTechniques* 1997, *22,* 812–814.

[24] Misawa, N., Yamano, S., Linden, H., de Felipe, M. R. *et al.,* Functional expression of the *Erwinia uredovora* carotenoid biosynthesis gene *crtI* in transgenic plants showing an increase of beta-carotene biosynthesis activity and resistance to the bleaching herbicide norflurazon. *Plant J.* 1993, *4*, 833–840.

[25] Horsch, R. B., Fry, J. E., Hoffmann, N. L., Eichholtz, D. *et al.*, A simple and general method for transferring genes to plants. *Science* 1985, *227,* 1229–1231.

[26] Mackinney, G., Absorption of light by chlorophyll solutions, *J. Biol. Chem.* 1941, *140*, 315–322.

[27] Andrewes, A. G., Phaff, H., Starr, M. P., Carotenoids of *Phaffia rhodozyma*, a red-pigmented fermenting yeast. *Phytochemistry* 1976, *15,* 1003–1007.

[28] Sandmann, G., Combinatorial biosynthesis of carotenoids in a heterologous host: a powerful approach for the biosynthesis of novel structures. *Chem. Biochem.* 2002, *3,* 629–635.

[29] Davies, B. H., Carotenoids, in: Goodwin, T. W. (Ed.). *Chemistry of Plant Pigments,* Academic Press, London 1976.

[30] Eugster, C. H., Chemical derivatization: microscale tests for the presence of common functional groups in carotenoids, in: Britton, G., S. Liaaen-Jensen, S., Pfander H. (Eds.). *Carotenoids,* Vol. 1 A: *Isolation and Analysis*, Birkhäuser, Basel 1995.

[31] Sandmann, G., Römer, S., Fraser, P. D., Understanding carotenoid metabolism as a necessity for genetic engineering of crop plants. *Metab. Eng.* 2006, *8,* 291–302.

[32] Marcotrigiano, M., Experimentally synthesized plant chimeras. 3. Qualitative and quantitative characteristics of the flowers of interspecific nicotians chimeras. *Ann. Bot.* 1986, *57*, 435–442.

[33] Daniell, H., Streatfield, S. J., Wycoff, K., Medical molecular farming: production of antibodies, biopharmaceuticals and edible vaccines in plants. *Trends Plant Sci.* 2001, *6,* 219–226.

[34] Fahn, A., Secretory tissues in vascular plants. *New Phytologist* 1988, *108,* 229–257.

[35] Giddings, G., Allison, G., Brooks, D., Carter, A., Transgenic plants as factories for biopharmaceuticals. *Nat. Biotechnol.* 2000, *18,* 1151–1155.


 