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β-Carotene accumulation induced by the cauliflower *Or* gene is not due to an increased capacity of biosynthesis

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Abstract

The cauliflower (*Brassica oleracea* L. var. *botrytis*) *Or* gene is a rare carotenoid gene mutation that confers a high level of β-carotene accumulation in various tissues of the plant, turning them orange. To investigate the biochemical basis of *Or*-induced carotenogenesis, we examined the carotenoid biosynthesis by evaluating phytoene accumulation in the presence of norflurazon, an effective inhibitor of phytoene desaturase. Calli were generated from young seedlings of wild type and *Or* mutant plants. While the calli derived from wild type seedlings showed a pale green color, the calli derived from *Or* seedlings exhibited intense orange color, showing the *Or* mutant phenotype. Concomitantly, the *Or* calli accumulated significantly more carotenoids than the wild type controls. Upon treatment with norflurazon, both the wild type and *Or* calli synthesized significant amounts of phytoene. The phytoene accumulated at comparable levels and no major differences in carotenogenic gene expression were observed between the wild type and *Or* calli. These results suggest that *Or*-induced β-carotene accumulation does not result from an increased capacity of carotenoid biosynthesis.
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Keywords: *Brassica oleracea* var. *botrytis*; Cauliflower; Brassicaceae; *Or* gene mutation; Carotenoids; Norflurazon

1. Introduction

Carotenoids are a diverse group of pigments widely distributed in nature. They fulfill many functions in plants such as in light harvesting and photoprotection (Demmig-Adams and Adams, 1996; Frank and Cogdell, 1996); in providing precursors for the biosynthesis of the plant hormone, abscisic acid (ABA) (Schwartz et al., 1997), and for production of the volatile fruit flavor/aroma (Simkin et al., 2004). In addition, carotenoids play an important role in human nutrition and health as the primary dietary source of pro-vitamin A (Combs, 1998) and in reducing the incidence of certain diseases (Giovannucci, 1999; Krinsky et al., 2003).

In plants, carotenoids are synthesized *de novo* within plastids (Fig. 1). The first committed step in carotenoid biosynthesis is condensation of two molecules of geranylgeranyl pyrophosphate (**1**) to produce phytoene (**2**). A series of desaturations and isomerizations converts phytoene (**2**) to lycopene (**4**). The latter is cyclized to yield α- and β-carotene (**5**) and (**6**). Subsequent oxygenation of these carotenes via addition of hydroxyl, epoxy or keto groups results in formation of xanthophylls, including lutein (**7**) from α-carotene (**5**), and zeaxanthin (**8**), violaxanthin and neoxanthin from β-carotene (**6**) (Cunningham and Gantt, 1998; Hirschberg, 2001; Fraser and Bramley, 2004). Lutein (**7**), β-carotene (**6**), violaxanthin and neoxanthin are the most abundant carotenoids in green tissue of many higher

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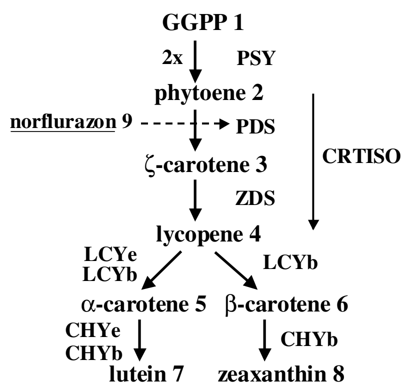

Fig. 1. An outline of the carotenoid biosynthetic pathway in plants. *Abbreviations*. GGPP, geranylgeranyl diphosphate; PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; LCYe, lycopene ε-cyclase; LYCb, lycopene β-cyclase; CHYe, ε-ring hydroxylase; CHYb, β-ring hydroxylase.

plants (Goodwin and Britton, 1988). The relative composition of these carotenoids is remarkably conserved and is required for optimal function of photosynthesis (Pogson et al., 1998).

Carotenoids are accumulated in large quantity in chromoplasts (Goodwin and Britton, 1988). They are responsible for the red, orange, and yellow colors found in many flowers, fruits, and roots. Chromoplasts are often derived from fully developed chloroplasts during fruit ripening. In many cases, they can also directly arise from proplastids in dividing tissues and from other non-photosynthetic plastids, such as leucoplasts and amyloplasts (Marano et al., 1993).

Carotenoid mutants provide excellent tools to study carotenoid metabolism (Tian et al., 2004; Isaacson et al., 2002; Park et al., 2002). The cauliflower (*Brassica oleracea* var. *botrytis*) *Orange* (*Or*) gene represents a rare carotenoid gene mutation (Crisp et al., 1975; Dickson et al., 1988). It confers a high level of β-carotene accumulation in many normally white tissues of the plant, turning them orange (Fig. 2a). Our previous studies showed that the orange color is due to a massive β-carotene (**6**) accumulation in one or two large chromoplasts per cell and the *Or* gene appears to represent a novel gene in regulating carotenoid accumulation (Li et al., 2001). We are working on functional analysis of the *Or* gene to gain insights into the regulatory control of carotenogenesis in the plant (Li et al., 2003).

To investigate whether the *Or* gene caused an increased carotenoid accumulation by altering the capacity of biosynthesis, we examined the carotenoid biosynthesis in wild type (WT) and *Or* callus cultures. Our initial tissue culture experiments revealed that calli derived from the *Or* mutant plant unexpectedly exhibited this orange phenotype like the *Or* mutant plants. Therefore, we took the advantage of this simplified system and investigated the

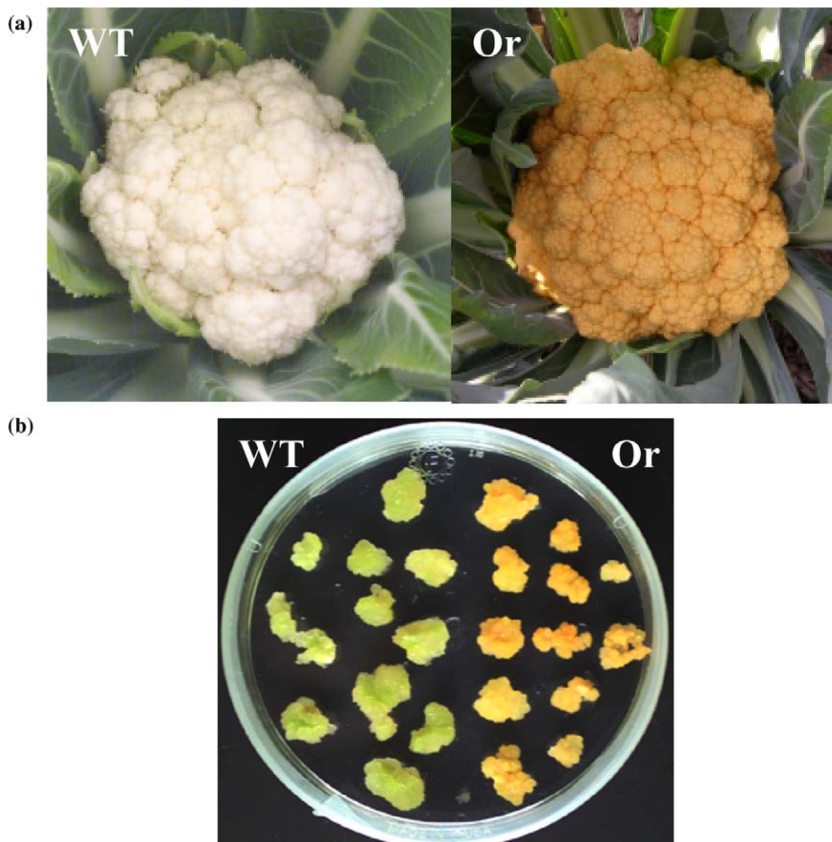

Fig. 2. Effect of the *Or* gene on the curds and calli of cauliflower. (a) Curds of the WT and *Or* mutant cauliflower. *Or*, commercial hybird cauliflower (b) Callus cultures derived from WT and *Or* mutant seedlings. The calli were grown under a 16 h light/8 h dark cycle.

effect of a phytoene desaturase inhibitor, norflurazon (**9**) in blocking phytoene (**2**) desaturation in the carotenoid biosynthetic pathway (Fig. 1) (Boeger and Sandmann, 1998). Evaluating the accumulation of phytoene (**2**) in the presence of norflurazon (**9**) provides a convenient way to monitor carotenoid biosynthesis from overall carotenoid metabolism (Simkin et al., 2003). Thus, we compared phytoene (**2**) accumulation between WT and *Or* calli in the presence of norflurazon (**9**). Our results showed that the *Or*-induced β-carotene (**6**) accumulation in the callus cultures is not associated with an increased capacity of carotenoid biosynthesis.

2. Results and discussion

2.1. The Or gene confers distinctive orange coloration in the callus cultures

To obtain uniform starting materials for the biochemical study of the effects of the cauliflower (*Brassica oleracea* var. *botrytis*) *Or* gene on carotenoid metabolism, as well as in providing a simpler system than whole plants for future cellular and molecular studies, we generated callus cultures from both young seedlings of an *Or* homozygous line (1227) and a wild type control line "Stovepipe". In this regard, calli formed quickly from the explants on the callus-generating BCS medium, as a uniform healthy-looking calli were usually obtained after three to four subcultures.

The most noticeable effect of the *Or* gene mutation on cauliflower plants is the alteration of the color of curds, especially the very outer surface of the rapidly dividing cells of the curd tissue (Fig. 2a) (Crisp et al., 1975; Dickson et al., 1988). The *Or* gene mutation also exerts strong effect on other tissues, such as on the vegetative shoot apical meristems and leaf base interiors (Li et al., 2001). Interestingly, while calli derived from roots, hypocotyls, and leaves of the white cauliflower young seedlings showed green color under a 16 h light/8 h dark cycle, the calli derived from various tissues of young *Or* seedlings all exhibited an intense orange coloration (Fig. 2b). When they were grown in the dark over 2–3 weeks, the wild type calli turned pale white and the *Or* calli became light orange. However, the overall growth of the calli was not as healthy or vigorous as those grown under light. In a preliminary study, we also isolated protoplasts from mature leaves of the *Or* mutant plants. Calli derived from protoplasts also showed an intense orange coloration (L. Li, D. Garvin, E. Earle, unpublished results). Thus, the *Or* gene exerts its effect on callus cultures derived from both photosynthetic and non-photosynthetic tissues.

Since callus cultures consist of undifferentiated cells, and the cauliflower curds of the very outer surface and the apical shoots represent inflorescence meristems and apical meristems, respectively, it suggests that the *Or* gene has a strong effect on tissues containing large numbers of dividing cells. Normally, the shoot meristematic cells contain many proplastids (Pyke, 1999) and the cells of the outer surface of cauliflower curds are enriched with leucoplasts (Paolillo et al., 2004). The *Or* mutation changes the plastid type in these cells and induces carotenoid accumulation in one or two large chromoplasts per cell in the affected tissues of the plant (Li et al., 2001). As suggested previously (Paolillo et al., 2004), it appears that in the *Or* mutant plant, the *Or* gene is capable to initiate differentiation of chromoplasts at an early stage in the apical meristematic cells, converting non-colored plastids into chromoplasts. The *Or* gene may extend its effect on the differentiation of chromoplasts in the outer surface of the curds as the curd tissue could be regarded as a proliferation of apical meristems (Sadik, 1962). Evidently, in green tissues, the developmental program overcomes the effect imposed by *Or* and redifferentiates chromoplasts into the chloroplasts, as shown in other studies (Hormaetxe et al., 2004). In the callus cultures, because the development of plastids is relatively less synchronized in comparison to shoot meristems (Kumar and Neumann, 1999), the apparent effect of the *Or* gene on plastid differentiation in the tissue is maintained, leaving the *Or* calli orange. In plants, chromoplasts have been shown to be able to derive from proplastids and other non-photosynthetic plastids (Marano et al., 1993).

2.2. Calli derived from the Or mutant synthesized predominantly β-carotene

The *Or* gene mutation causes carotenoid accumulation in the low-pigmented tissues of cauliflower plants with β-carotene (**6**) as the major accumulated carotenoid (Li et al., 2001). To examine the pigments synthesized in the callus tissues, carotenoids from both WT and *Or* calli were extracted and analyzed by reversed-phase HPLC. Like the carotenoid composition in green tissues of almost all higher plants (Goodwin and Britton, 1988), the green calli derived from wild type cauliflower young seedlings contained lutein (**7**) (40%), β-carotene (**6**) (30%), violaxanthin (25%), and neoxanthin (5%). Chlorophylls were also present in the WT calli (Fig. 3a). In contrast, the orange calli derived from *Or* mutant plants accumulated predominantly β-carotene (**6**) with small amount of lutein (**7**) (Fig. 3b). No chlorophylls were detected. The total carotenoids accumulated in the *Or* calli were calculated to be over 2.5-fold higher than those in the WT calli.

Since the pigment composition of the WT callus cultures was similar to a typical carotenoid composition of green leaf tissues (Goodwin and Britton, 1988), the green color in the WT calli implies that chloroplast differentiation occurred during callus cultures. Such a differentiation has also been observed in other callus cultures (Abe et al., 2002). When the *Or* gene was present in the callus tissue, no chlorophyll pigments were detected. The result suggests that the possible effect of the *Or* gene on chromoplast differentiation is incapable to be overcome by the

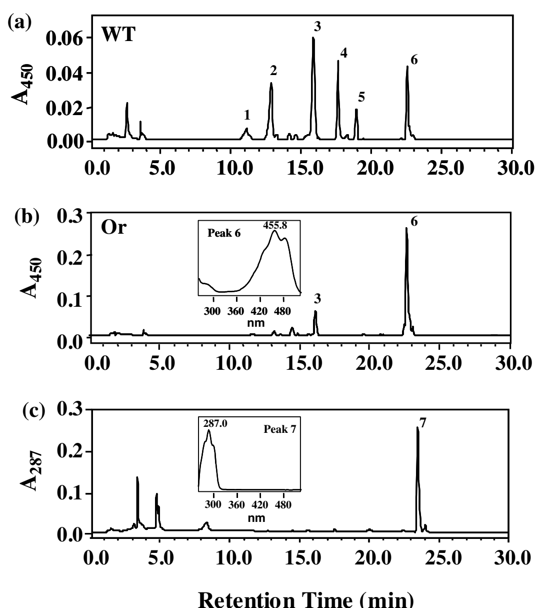

Fig. 3. Typical HPLC elution profiles of pigments extracted from cauliflower callus cultures. (a) Pigments from WT calli. (b) Carotenoids from the *Or* calli. (c) Phytoene from the WT or *Or* calli after exposure to norflurazon for 2 weeks. Peak 1, neoxanthin; peak 2, violaxanthin; peak 3, lutein; peak 4, chlorophyll b; peak 5, chlorophyll a; peak 6, β-carotene; peak 7, phytoene. Inserts represent the absorption spectra of peaks.

developmental program of chloroplast differentiation in the *Or* callus cultures. Despite the significant amount of total carotenoids deposited in the WT calli due to chloroplast differentiation, the *Or* calli accumulated significantly more carotenoids. Thus, the *Or* calli with distinctive orange phenotype provide us with a much simplified, but fully functional model system to study carotenoid metabolism.

2.3. The Or-induced β-carotene accumulation in the callus cultures is not associated with an increased capacity of biosynthesis

Carotenoid accumulation is a net result of biosynthesis, degradation, and sequestration of the end products. Mechanisms such as modifications of substrate abundance, changes in carotenogenic activity, reduction of catabolism, or facilitation of a metabolic sink formation could all account for a high level of carotenoid accumulation in the *Or* mutant. For example, although our previous studies indicated that *Or* does not encode a carotenogenic enzyme and the *Or* mutation did not alter the gene expression of β-carotene hydroxylase (Li et al., 2001), the immediate downstream gene of β-carotene (**6**) accumulation, there is the possibility that *Or* is responsible for modification or impairing of the β-carotene hydroxylase activity, which results in blocking downstream metabolism and leading to the accumulation of β-carotene (**6**). In addition, the post-transcriptional modification of the other carotenoid enzymes could lead to an increased catalytic activity of this pathway, resulting in enhanced carotenoid biosynthesis. There is evidence that post-transcriptional regulation of carotenogenic enzymes is involved in enzyme activation in plants. In daffodil flowers, the soluble forms of phytoene synthase and phytoene desaturase are enzymatically inactive and only become functional when they are associated with chromoplast membrane (Al Babili et al., 1996; Schledz et al., 1996). Post-transcriptional modification of enzymes has been demonstrated to be a general and important control mechanism in many other cases in plants (Chae et al., 2003; Devarenne et al., 2002; Tiessen et al., 2002; Smith et al., 2004).

Radiolabeled precursors are very useful for determining the rate of biosynthesis and turnover of metabolites. Since cauliflower WT curds do not accumulate carotenoids and there is limited information available for the colorless apocarotenoid production, except ABA, it is difficult to monitor the rate of biosynthesis or degradation of carotenoids in WT curds following radioactive labeling in order to compare with that in the *Or* mutant. Further, ABA, the catalytic product, may not be a good indicator of catabolism of carotenoids as completely blocking of carotenoid pathway by lycopene cyclase inhibitor CPTA still resulted in increased concentration of ABA (Al Babili et al., 1999). To investigate whether the *Or*-induced carotenoid accumulation resulted from an increased capacity of carotenogenesis, we sought an alternative approach by evaluating the accumulation of phytoene (**2**) in the callus cultures in the presence of phytoene desaturase inhibitor, norflurazon (**9**). This herbicide inhibits phytoene desaturase, leading to the accumulation of the colorless carotenoid metabolic intermediate phytoene (**2**). The site and mechanism of norflurazon (**9**)-mediated inhibition have recently been elucidated to be due to competition of the cofactors of phytoene desaturase (Breitenbach et al., 2001). To ensure maximum inhibition of phytoene desaturase activity, a high concentration of norflurazon (**9**) (0.1 mM) was employed in this study (Simkin et al., 2003).

When the calli from WT and *Or* mutant seedlings were exposed to norflurazon (**9**) in the BCS medium, the growing calli became white under a 16 h light/8 h dark condition due to the blocking of the biosynthesis of colored carotenoids. Nevertheless, the calli appeared to grow as vigorously as those grown on norflurazon-free medium during the norflurazon (**9**) treatment period. HPLC analysis of the carotenoids showed that both WT and *Or* calli accumulated phytoene (**2**) in the presence of norflurazon (**9**) (Fig. 3c). The relative concentration of phytoene (**2**) increased with extended period of exposure to norflurazon (**9**) (Fig. 4). Although the *Or* calli accumulated significantly higher levels of total carotenoids than the WT controls, comparable amounts of phytoene (**2**) accumulation were observed between the WT and *Or* calli. A similar result was also obtained when the calli of WT and *Or* were grown and treated in dark despite that the WT calli synthesized only trace amounts of carotenoids in dark (data not shown). In contrast, when white, a conventional

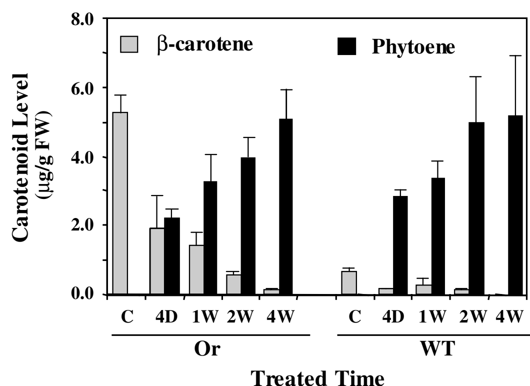

Fig. 4. β-carotene (**6**) and phytoene (**2**) concentration in the *Or* and WT calli treated with or without norflurazon. Phytoene (**2**) levels were expressed as β-carotene (**6**) equivalents of μg/g FW (fresh weight). C, non-treated control; 4D, treated for 4 days; 1W, 2W, and 4W, treated for 1, 2, and 4 weeks. The values are averages of two repeated experiments with at least three replications each experiment. Error bars indicate standard deviation.

yellow, and a deep yellow potato tubers which exhibit different levels of carotenoid accumulation (kindly provided by Dr. W. De Jong, Cornell University) were treated with norflurazon (**9**), the accumulation of phytoene (**2**) was positively correlated with the total carotenoids synthesized in the non-treated tubers. The deep yellow potato tubers accumulated significantly more phytoene (**2**) than the white ones (data not shown), suggesting a different capacity of carotenoid biosynthesis in these cultivars. Thus, the fact that a comparable phytoene (**2**) accumulation occurred in the WT and *Or* calli offers clear evidence that the *Or*-induced carotenoid accumulation is not due to an increased capacity of carotenoid biosynthesis.

Taken together, these results suggest that carotenoid accumulation is not dependent solely upon the capacity of biosynthesis. Control of other metabolic processes may play an important role in regulating carotenoid accumulation. Indeed, previous studies have shown that sequestration of carotenoid end products play a significant role for carotenoid accumulation (Vishnevetsky et al., 1999). In some cases, it has been established that carotenoid accumulation is more directly associated with the formation of carotenoid sequestering structures than with the changes of carotenoid biosynthetic gene expression or enzyme abundances (Al Babili et al., 1999; Rabbani et al., 1998). Experiments are under way to decipher the control mechanism by which *Or* induces carotenogenesis.

2.4. Expression of carotenoid biosynthetic genes in response to norflurazon (9) treatment

To examine the regulation of carotenoid biosynthesis in the callus cultures, the expression of carotenogenic genes in the norflurazon-treated calli was analyzed by RT-PCR. A very similar pattern of expression was obtained between WT and *Or* calli. Examination of the

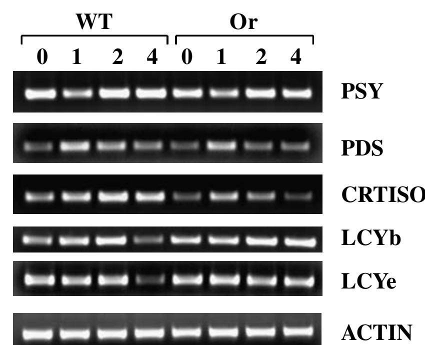

Fig. 5. Expression of carotenoid biosynthetic genes in the *Or* and WT calli treated with or without norflurazon. The transcript levels of the carotenoid genes were examined by RT-PCR from total RNA isolated from 0, 1, 2, 4 weeks post norflurazon (**9**) treatment. The amplification products were separated on 1.5% agarose gels and stained with ethidium bromide. *Actin* was used as an internal control.

effect of norflurazon (**9**) on carotenogenic gene expression showed that while the transcript levels of phytoene desaturase and carotenoid isomerase appear to be slightly stimulated during initial norflurazon treatment, the mRNA levels of other carotenogenic genes seem to remain consistent. In tomato, a significant upregulation of phytoene desaturase was observed in norflurazon-treated plants, and it was suggested that the gene expression is regulated by the end-product of carotenoid biosynthesis (Corona et al., 1996; Giuliano et al., 1993). However, in *Arabidopsis* and pepper, the expressions of carotenogenic genes are not affected by norflurazon (**9**) treatment (Wetzel and Rodermel, 1998; Simkin et al., 2000). It appears that in the cauliflower callus cultures, the effect of norflurazon (**9**) treatment on the expression of carotenogenic genes was minimal (see Fig. 5).

3. Conclusions

The *Or* gene mutation exerts its strong effect on tissues containing large number of dividing cells and results in tissue-specific carotenoid accumulation mainly in the curds and apical shoots of the cauliflower plant. As the cells of the *Or* young seedlings become undifferentiated during callus cultures, the *Or* gene induces high levels of β-carotene (**6**) accumulation, turning the calli orange. The discovery of the *Or* calli showing the orange phenotype of the *Or* mutant facilitated investigation of the biochemical basis of *Or* in controlling carotenoid accumulation. Our results obtained here clearly demonstrate that the *Or* gene does not directly affect on the capacity of carotenoid biosynthesis. The ultimately unveiling of the biochemical and molecular basis of *Or*-induced carotenogenesis should provide important information for a better understanding of the regulatory mechanisms underlying carotenoid accumulation in plants. In addition, the information should aid the identification of potential control points to enrich carotenoid contents in important crops.

4. Experimental

4.1. General experimental procedures

The solvents used for carotenoid analysis were HPLC grade from Fisher Scientific Inc. (Pittsburgh, PA). Norflurazon (**9**) was purchased from Crescent Chemical Co. Inc. (Islandia, NY).

4.2. Establishment of callus cultures

Seeds of a cauliflower (*Brassica oleracea* L. var. *botrytis*) white variety "Stovepipe" and an *Or* homozygous mutant line (1227) were surface-sterilized with 70% ethanol for 1 min, 30% Clorox for 20 min, and then rinsed five times with sterile H_2O. The sterilized seeds were placed in Magenta boxes containing 0.5 × MS salts (Murashige and Skoog, 1962) with 3% sucrose, and germinated in an incubator at 24 °C with a 16 h light/8 h dark photoperiod for 7–10 days. Callus cultures were initiated from germinating young seedlings by cutting the roots, hypocotyls, and leaves into small pieces and placing them directly onto plates of BCS medium containing MS salts supplemented with 3% sucrose, 1 mg/l NAA, 5 mg/l BAP, 0.2 mg/l 2,4-D, 200 mg/l casein hydrolysate, and 2.2 g/l Gelrite (Brants and Earle, 2001). Callus tissues were subcultured onto fresh BCS plates every two weeks.

4.3. Norflurazon (9) treatment

To treat the callus cultures with the phytoene desaturase inhibitor, norflurazon (**9**), two-month-old calli from both WT and the *Or* mutant cauliflowers were cut into small pieces at approximately the same size and placed onto BCS plates with or without 0.1 mM norflurazon (**9**). The calli were allowed to grow for various times in the incubator, harvested, and stored at −80 °C until use. The calli were transferred onto fresh plates after two weeks of treatment.

4.4. Carotenoid extraction and analysis

The procedure for extraction and analysis of carotenoids was performed essentially following the method of Norris et al. (1995). Callus samples (approximately 0.3 g) of at least three replicates were ground in acetone–H_2O (4:1, 0.6 ml), extracted with ethyl acetate (0.4 ml), and partitioned with the addition of H_2O (0.4 ml). The mixture was centrifuged at 12,000*g* for 5 min. The carotenoid-containing upper phase was transferred to new tubes, dried under a stream of nitrogen or with a Speed-Vac (Labconco, Kansas City, MO), and redissolved in ethyl acetate (200 μl). Carotenoids (40 μl) were analyzed on a Spherisorb ODS2 (5 μm particle size) reversed-phase C18 column using a Waters HPLC system (Waters, Milford, MA) equipped with a photodiode array detector. The pigments were separated by a linear gradient between solvent A (acetonitrile:H_2O:triethylamine at 9:1:0.01) and solvent B (100% ethyl acetate) over 30 min at a flow rate of 1.0 ml/min. Carotenoids were identified by their characteristic absorption spectra and their retention times in comparison with standards and published spectra. Quantification of individual carotenoids was carried out based on peak areas and calibration curves generated with the commercial β-carotene (**6**) and lutein (**7**) standards (Sigma, St. Louis, MO). Phytoene (**2**) levels were calculated as relative concentration to β-carotene (**6**) due to lack of pure phytoene standard.

4.5. Carotenoid gene expression by RT-PCR

Total RNA from the callus tissues was extracted using Trizol reagent according to the manufacturer's instruction (Invitrogen, Carlsbad, CA). Reverse transcription of total RNA (1 μg) was carried out using oligo-dT (Promega, Madison, WI) as a primer and SuperScript™ III reverse transcriptase (Invitrogen, Carlsbad, CA) to generate the first strand cDNA templates. The synthesized cDNAs were normalized using primers for *Actin*. Amplification of samples with cauliflower carotenogenic gene specific primers (Table 1) included 2 min denaturation at 94 °C, followed by 25 or 30 cycles of 30 s at 94 °C, 30 s at various temperatures depending on gene-specific primers, and 1 min at 72 °C. PCR products were analyzed by agarose gel.

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Table 1
Primers used for amplifying fragments of carotenoid biosynthetic genes from calli of cauliflower

Gene	Primers	
	Forward (5′ → 3′)	Reverse (5′ → 3′)
Actin	ccgagagaggttacatgttcaccac	gctgtgatctctttgctcatacggtc
PSY	gaggaagagtttatctgccccaagatgag	ctgatttagcataagccaatggcagagctg
PDS	ggagaacttgggatcaatgatcggttgc	ctcgcctgctttcgcatccattgttc
CRTISO	cctccaggagctctcccagattca	ggcacttttcctgtcaagaccttccc
LCYb	ggagagataagcatcttgacgcgtaccc	cgtttgcaactatcggagcagctgc
LCYe	ggctattcagttgtgagatctttgtctgaagc	gatgttaatgttgatcctagaaaccctctccac

References

Abe, T., Ii, N., Togashi, A., Sasahara, T., 2002. Large deletions in chloroplast DNA of rice calli after long-term culture. J. Plant Physiol. 159, 917–923.

Al Babili, S., Hartung, W., Kleinig, H., Beyer, P., 1999. CPTA modulates levels of carotenogenic proteins and their mRNAs and affects carotenoid and ABA content as well as chromoplast structure in *Narcissus pseudonarcissus* flowers. Plant Biol. 1, 607–612.

Al Babili, S., von Lintig, J., Haubruck, H., Beyer, P., 1996. A novel, soluble, form of phytoene desaturase from *Narcissus pseudonarcissus* chromoplasts is Hsp70-complexed and competent for flavinylation, membrane association and enzymatic activation. Plant J. 9, 601–612.

Boeger, P., Sandmann, G., 1998. Carotenoid biosynthesis inhibitor herbicides – mode of action and resistance mechanisms. Pestic. Outlook 9, 29–35.

Brants, A., Earle, E.D., 2001. Transgenic tobacco cell cultures expressing a *Trichoderma harzianum* endochitinase gene release the enzyme into the medium. Plant Cell Rep. 20, 73–78.

Breitenbach, J., Zhu, C., Sandmann, G., 2001. Bleaching herbicide norflurazon inhibits phytoene desaturase by competition with the cofactors. J. Agric. Food Chem. 49, 5270–5272.

Chae, H.S., Faure, F., Kieber, J.J., 2003. The *eto*1, *eto*2, and *eto*3 mutations and cytokinin treatment increase ethylene biosynthesis in Arabidopsis by increasing the stability of ACS protein. Plant Cell 15, 545–559.

Combs Jr., G.F., 1998. The Vitamins: Fundamental Aspects in Nutrition and Health. Academic Press, San Diego.

Corona, V., Aracri, B., Kosturkova, G., Bartley, G.E., Pitto, L., Giorgetti, L., Scolnik, P.A., Giuliano, G., 1996. Regulation of a carotenoid biosynthesis gene promoter during plant development. Plant J. 9, 505–512.

Crisp, P., Walkey, D.-G.A., Bellman, E., Roberts, E., 1975. A mutation affecting curd colour in cauliflower (*Brassica oleracea* L. var. *botrytis* DC). Euphytica 24, 173–176.

Cunningham, F.X., Gantt, E., 1998. Genes and enzymes of carotenoid biosynthesis in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. 49, 557–583.

Demmig-Adams, B., Adams, W.W., 1996. The role of xanthophyll cycle carotenoids in the protection of photosynthesis. Trends Plant Sci. 1, 21–26.

Devarenne, T.P., Ghosh, A., Chappell, J., 2002. Regulation of squalene synthase, a key enzyme of sterol biosynthesis, in tobacco. Plant Physiol. 129, 1095–1106.

Dickson, M.H., Lee, C.Y., Blamble, A.E., 1988. Orange-curd high carotene cauliflower inbreds, NY 156, NY 163, and NY 165. Hortscience 23, 778–779.

Frank, H.A., Cogdell, R.J., 1996. Carotenoids in photosynthesis. Photochem. Photobiol. 63, 257–264.

Fraser, P.D., Bramley, P.M., 2004. The biosynthesis and nutritional uses of carotenoids. Prog. Lipid Res. 43, 228–265.

Giovannucci, E., 1999. Tomatoes, tomato-based products, lycopene, and cancer: review of the epidemiologic literature. J. Natl. Cancer Inst. 91, 317–331.

Giuliano, G., Bartley, G.E., Scolnik, P.A., 1993. Regulation of carotenoid biosynthesis during tomato development. Plant Cell 5, 379–387.

Goodwin, T.W., Britton, G., 1988. Distribution and analysis of carotenoids. In: Goodwin, T.W. (Ed.), Plant Pigments. Academic Press, London, pp. 61–132.

Hirschberg, J., 2001. Carotenoid biosynthesis in flowering plants. Curr. Opin. Plant Biol. 4, 210–218.

Hormaetxe, K., Hernandez, A., Becerril, J.M., Garcia-Plazaola, J.I., 2004. Role of red carotenoids in photoprotection during winter acclimation in *Buxus sempervirens* leaves. Plant Biol. 6, 325–332.

Isaacson, T., Ronen, G., Zamir, D., Hirschberg, J., 2002. Cloning of *tangerine* from tomato reveals a carotenoid isomerase essential for the production of β-carotene and xanthophylls in plants. Plant Cell 14, 333–342.

Krinsky, N.I., Landrum, J.T., Bone, R.A., 2003. Biologic mechanisms of the protective role of lutein and zeaxanthin in the eye. Annu. Rev. Nutr. 23, 171–201.

Kumar, A., Neumann, K.H., 1999. Comparative investigations on plastid development of meristematic regions of seedlings and tissue cultures of *Daucus carota* L. J. Appl. Bot. 73, 206–210.

Li, L., Lu, S., O'Halloran, D.M., Garvin, D.F., Vrebalov, J., 2003. High-resolution genetic and physical mapping of the cauliflower high-beta-carotene gene *Or* (Orange). Mol. Gen. Genomics 270, 132–138.

Li, L., Paolillo, D.J., Parthasarathy, M.V., DiMuzio, E.M., Garvin, D.F., 2001. A novel gene mutation that confers abnormal patterns of β-carotene accumulation in cauliflower (*Brassica oleracea* var *botrytis*). Plant J. 26, 59–67.

Marano, M.R., Serra, E.C., Orellano, E.G., Carrillo, N., 1993. The path of chromoplast development in fruits and flowers. Plant Sci. 94, 1–17.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiol. Plant 15, 473–497.

Norris, S.R., Barrette, T.R., Dellapenna, D., 1995. Genetic dissection of carotenoid synthesis in Arabidopsis defines plastoquinone as an essential component of phytoene desaturation. Plant Cell 7, 2139–2148.

Paolillo, D.-J.J., Garvin, D.F., Parthasarathy, M.V., 2004. The chromoplasts of *Or* mutants of cauliflower (*Brassica oleracea* L. var. *botrytis*). Protoplasma 224, 245–253.

Park, H., Kreunen, S.S., Cuttriss, A.J., DellaPenna, D., Pogson, B.J., 2002. Identification of the carotenoid isomerase provides insight into carotenoid biosynthesis, prolamellar body formation, and photomorphogenesis. Plant Cell 14, 321–332.

Pogson, B.J., Niyogi, K.K., Bjorkman, O., Dellapenna, D., 1998. Altered xanthophyll compositions adversely affect chlorophyll accumulation and nonphotochemical quenching in *Arabidopsis* mutants. Proc. Natl. Acad. Sci. USA 95, 13324–13329.

Pyke, K.A., 1999. Plastid division and development. Plant Cell 11, 549–556.

Rabbani, S., Beyer, P., von Lintig, J., Hugueney, P., Kleinig, H., 1998. Induced beta-carotene synthesis driven by triacylglycerol deposition in the unicellular alga *Dunaliella bardawil*. Plant Physiol. 116, 1239–1248.

Sadik, S., 1962. Morphology of the curd of cauliflower. Am. J. Bot. 49, 290–297.

Schledz, M., Al Babili, S., Lintig, J., Haubruck, H., Rabbani, S., Kleinig, H., Beyer, P., 1996. Phytoene synthase from *Narcissus pseudonarcissus*: functional expression, galactolipid requirement, topological distribution in chromoplasts and induction during flowering. Plant J. 10, 781–792.

Schwartz, S.H., Tan, B.C., Gage, D.A., Zeevaart-Jan, A.D., McCarty, D.R., 1997. Specific oxidative cleavage of carotenoids by VP14 of maize. Science 276, 1872–1874.

Simkin, A.J., Breitenbach, J., Kuntz, M., Sandmann, G., 2000. In vitro and in situ inhibition of carotenoid biosynthesis in *Capsicum annuum* by bleaching herbicides. J. Agric. Food Chem. 48, 4676–4680.

Simkin, A.J., Schwartz, S.H., Auldridge, M., Taylor, M.G., Klee, H.J., 2004. The tomato carotenoid cleavage dioxygenase 1 genes contribute to the formation of the flavor volatiles beta-ionone, pseudoionone, and geranylacetone. Plant J. 40, 882–892.

Simkin, A.J., Zhu, C., Kuntz, M., Sandmann, G., 2003. Light–dark regulation of carotenoid biosynthesis in pepper (*Capsicum annuum*) leaves. J. Plant Physiol. 160, 439–443.

Smith, S.M., Fulton, D.C., Chia, T., Thorneycroft, D., Chapple, A., Dunstan, H., Hylton, C., Zeeman, S.C., Smith, A.M., 2004. Diurnal changes in the transcriptome encoding enzymes of starch metabolism provide evidence for both transcriptional and posttranscriptional regulation of starch metabolism in Arabidopsis leaves. Plant Physiol. 136, 2687–2699.

Tian, L., Musetti, V., Kim, J., Magallanes-Lundback, M., Dellapenna, D., 2004. The Arabidopsis LUT1 locus encodes a member of the cytochrome P450 family that is required for carotenoid epsilon-ring hydroxylation activity. Proc. Natl. Acad. Sci. USA 101, 402–407.

Tiessen, A., Hendriks, J.H.M., Stitt, M., Branscheid, A., Gibon, Y., Farre, E.M., Geigenberger, P., 2002. Starch synthesis in potato tubers is regulated by post-translational redox modification of ADP-glucose pyrophosphorylase: a novel regulatory mechanism linking starch synthesis to the sucrose supply. Plant Cell 14, 2191–2213.

Vishnevetsky, M., Ovadis, M., Vainstein, A., 1999. Carotenoid sequestration in plants: the role of carotenoid-associated proteins. Trends Plant Sci. 4, 232–235.

Wetzel, C.M., Rodermel, S.R., 1998. Regulation of phytoene desaturase expression is independent of leaf pigment content in *Arabidopsis thaliana*. Plant Mol. Biol. 37, 1045–1053.