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Genetic Engineering of a Zeaxanthin-rich Potato by Antisense Inactivation and Co-suppression of Carotenoid Epoxidation

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Zeaxanthin is an important dietary carotenoid but its abundance in our food is low. In order to provide a better supply of zeaxanthin in a staple crop, two different potato (*Solanum tuberosum* L.) varieties were genetically modified. By transformation with sense and antisense constructs encoding zeaxanthin epoxidase, zeaxanthin conversion to violaxanthin was inhibited. Both approaches (antisense and co-suppression) yielded potato tubers with higher levels of zeaxanthin. Depending on the transgenic lines and tuber development, zeaxanthin content was elevated 4 to 130-fold reaching values up to 40 $\mu g/g$ dry weight. As a consequence of the genetic manipulation, the amount of violaxanthin was diminished dramatically and in some cases the monoepoxy intermediate antheraxanthin accumulated. Between one and eight copies of the sense or antisense epoxidase gene fragments were integrated into the genome. In addition, most of the transformants with higher zeaxanthin levels showed also increased total carotenoid contents (up to 5.7-fold) and some of them exhibited reduced amounts of lutein. The increase in total carotenoids suggests that the genetic modification affects the regulation of the whole carotenoid biosynthetic pathway in potato tubers. Northern blot analysis demonstrated that upregulation of carotenogenesis in the transgenics is accompanied by substantial higher phytoene synthase transcript levels in 6-week-old tubers and a very slight increase of the β-carotene hydroxylase transcript. The amount of the deoxyxylulose 5-phosphate synthase mRNA was very similar in wild type and transformed tubers. Abscisic acid content of tubers remained unchanged whereas α-tocopherol was 2 to 3 fold elevated in the transformants.

Key Words: **carotenoids; potato tubers; transgenic plants; zeaxanthin accumulation.**

INTRODUCTION

Carotenoids are yellow to red pigments which are synthesized by microorganisms and plants. In the latter, they accumulate in the chloroplasts or in chromoplasts of flowers, fruit or other tissue. The typical cyclic plant carotenoids contain a conjugated double-bond system and carry ionone end groups which may be substituted by keto, hydroxy and epoxy groups at different positions (Sandmann, 2001a). Their prominent cellular function is as lipophilic antioxidants. They protect especially membranes and other lipophilic environments in the cell against oxidative damage by quenching of photosensitizers, interaction with singlet oxygen (Krinsky, 1994) and scavenging of peroxy radicals (Conn *et al.*, 1992). The important role of carotenoids in human health by preventing degenerative diseases may be due to these antioxidative properties. Another important feature of carotenoids with at least one unsubstituted β-ionone group is the provision of provitamin A.

Lutein and zeaxanthin are the major carotenoids of the yellow spot in the human retina (Landrum and Bone, 2001). There, these macular pigments prevent retinal damage by blue light and may also protect against singlet oxygen. Age-related macular degeneration is the main cause of blindness of elder people. The risk of age-related macular degeneration can be reduced by high dietary intake of zeaxanthin and lutein (Seddon *et al.*, 1994). While lutein is abundant especially in green vegetables, zeaxanthin levels in our diet are much lower (van den Berg *et al.*, 2000). A possible interconversion of lutein and zeaxanthin has been proposed (Khachik *et al.*, 1997), but is so far an unproven hypothesis (Landrum and Bone, 2001). The only significant natural sources of zeaxanthin in human diet are some maize cultivars (Quackenbush *et al.*, 1963) and special yellow/orange pepper varieties (Minguez-Mosquera and Hornero-Mendez, 1994). One way to overcome zeaxanthin deficiency in our diet could be by genetic

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manipulation of the carotenoid biosynthetic pathway of common food plants (Giuliana *et al*., 2000; Sandmann, 2001b). The enormous potential of metabolic engineering of plants for various products has just been reviewed by Hanson and Shanks (2002).

Transfer of carotenogenic genes was successfully used to elevate the content of another carotenoid, β-carotene with provitamin A activity, in crop plants. In tomato fruit, the carotenoid biosynthesis was shifted towards β-carotene (Römer *et al*., 2000; Rosati *et al*., 2000) and in canola seeds the β-carotene content could be 50-fold increased (Shewmaker *et al*., 1999). There is also an example of the establishment of β-carotene synthesis in non-carotenogenic tissue. By transfer of several genes encoding the initial steps of the carotenoid biosynthetic pathway to rice, β-carotene formation in the seeds was achieved (Ye *et al*., 2000). Very recently, tomato was successfully engineered for formation of xanthophylls including zeaxanthin (Dharmapuri *et al*., 2002).

In the present work, another transgenic strategy was followed to mediate the accumulation of zeaxanthin in the staple crop potato. The major carotenoids of potato tuber are lutein and violaxanthin (Iwanzik *et al*., 1983). The latter is formed from zeaxanthin by an epoxidase (Sandmann, 2001a). The synthesis of this enzyme was down-regulated specifically in potato tubers without interfering significantly with carotenoid biosynthesis in leaf chloroplasts by antisense technology and co-suppression approaches. This genetic manipulation of the carotenoid metabolism resulted in zeaxanthin-rich potato lines.

MATERIALS AND METHODS

Plant Material

Potato genotypes, *Solanum tuberosum* L. cv. Baltica and cv. Freya (SaKa-Ragis Pflanzenzucht GbR, Windeby, Germany), were used for all experiments. Plants were maintained in tissue culture with a 16 h light, 8 h dark regime on MS Medium (Murashige and Skoog, 1962) which contained 2% (w/v) sucrose. In the greenhouse, plants were grown either in 2 L pots under the same conditions with supplementary artificial light under a minimum of 250 μmol photons m^{-2} s^{-1} (mini-tuber production) or in 10 L pots under natural light conditions during the natural vegetation period. In the latter case, greenhouse temperature was controlled not to exceed 24°C during the light period and 15°C during the dark period.

Sense, Antisense Constructs and Preparation of Transgenic Potato Plants

Total RNA was extracted from leaves of sterily grown potato plantlets (*Solanum tuberosum* L., cv. Desiree) according to Kuntz *et al*. (1992). Two micrograms of RNA were reverse transcribed with M-MuLV reverse transcriptase (MBI Fermentas) in the presence of oligo dT primers. The resulting cDNA was purified (PCR purification kit from Roche, Mannheim, Germany) and used as template for PCR amplification of a cDNA fragment encoding zeaxanthin epoxidase. Amplification was carried out in a thermocycler (PCT-100, MJ Research Inc., Watertown, USA) with the degenerated primers zep1 TGG TAY TGY AAR TTY GAY ACI TTY AC and zep2 GCR TCR TCR TCY TCR AAC CAI based on zeaxanthin epoxidase cDNAs from the database which are all homologous to the first cloned cDNA from *Nicotiana plumbaginifolia* (Marin *et al*., 1996). PCR reaction was performed as outlined: 3 min denaturation followed by 30 cycles (1 min, 95°C, 1 min, 52°C, 1 min 72°C), final extension at 72°C for 5 min and a subsequent cooling step at 4°C. The PCR product (1000 bp) was directly cloned in the vector pCR2.1 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers protocol. The subcloned fragment was verified by sequencing. Two zeaxanthin epoxidase cDNA fragments in opposite orientation were isolated from the plasmid by restriction digest with the restriction enzymes *Bam*HI and *Xba*I and purified by agarose gel electrophoresis. The resulting inserts were subsequently cloned in the *Bam*HI/*Xba*I-digested binary vector pPGB121S which carries the *nptII* kanamycin resistance gene and the potato granule-bound starch synthase (GBSS) promoter (van der Steege *et al*., 1992). The final constructs pPGB-zep-sense and pPGB-zep-antisense were verified by restriction digest and sequencing. Then the plasmids were first introduced into *Agrobacterium tumefaciens* strain LBA 4404 according to Römer *et al*. (2000) which then was used for the transformation (Rocha-Sosa *et al*., 1989) of the potato cultivars Baltica and Freya, respectively. Transgenic plants were selected on kanamycin containing medium (Dietze *et al*., 1995). Stable transformation of independent regenerants was verified by PCR and after multiplication three plants per line were brought to the greenhouse for mini-tuber production.

Isolation of Genomic DNA and Southern Analysis

Genomic DNA was isolated from potato leaves according to the nuclei lysis CTAB extraction method described by Sambrook *et al*. (1989). Twenty to 30 μg of genomic DNA were digested with the restriction enzyme *Eco*RV and the resulting fragments separated on a 0.8% (w/v)

Metabolic Engineering 4, 263–272 (2002)
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agarose gel in 1 × TAE buffer. The DNA fragments were then blotted onto a positively charged nylon membrane (Biodyne Plus, Pall, Dreieich, Germany) by capillary transfer. Hybridization was performed at 65°C overnight using a DIG-dUTP-labeled probe against the *nptII* gene encoding the kanamycin resistance gene. After hybridization, the probe was recovered and stored and the membrane subjected to post-hybridization washes (twice at room temperature with 2 × SSC, 0.1% (w/v) SDS for 10 min, then twice at 65°C with 0.1 × SSC, 0.1% (w/v) SDS for 15 min). The hybridization signal was obtained by chemoluminescence using CDP star (Roche, Mannheim) as outlined by the supplier after exposure to film (Hyperfilm ECL, Amersham Pharmacia Biotech).

Northern Blot Analysis

Expression studies were performed using total RNA of potato tubers from different developmental stages (6 or 8 weeks old). Ten or 15 μg of total RNA were loaded on a denaturing agarose/formaldehyde gel according to Kuntz *et al*. (1992). After capillary transfer of ribonucleic acids to a positively charged membrane (Roche, Mannheim, Germany) the filter was hybridized with digoxygenin-labeled probes at a temperature of 42°C overnight. Probes against phytoene synthase and β-carotene hydroxylase were generated by PCR following the instruction of the supplier of the DIG labeling reagent (Roche, Mannheim, Germany). Plasmid DNA of deoxyxylulose 5-phosphate synthase, phytoene synthase, β-carotene hydroxylase and 25 S rRNA served as template. After post-hybridization washes (twice with 2 × SSC, 0.1% (w/v) SDS at room temperature, twice with 0.5 × SSC, 0.1% (w/v) SDS at 62°C) transcript levels were detected by chemoluminescence using CDP star as outlined by the supplier (Roche, Mannheim, Germany). Bands were quantified using the Molecular Dynamics Imagequant program. All transcript values for carotenogenic genes were normalized with respect to the corresponding value of the 25 S rRNA. Expression values were given in relation to the expression level of the wild type at the 6 week of tuber development.

Carotenoid, Abscisic Acid and α-Tocopherol Analysis

For carotenoid analysis, two to four individual halves of mini tubers (1.5–4 cm in length) were freeze-dried. The whole material without the peel was rubbed through a sieve (425 μm pore size). To 150 mg of the fine powder 25 ml of acetone was added and heated at 50°C for 20 min. Extraction of potato leaves was with methanol containing 6% (w/v) KOH. The extracts were partitioned against 10% (v/v) diethyl ether in petrol and the total carotenoid content determined by measuring the absorbance of the upper phase at 450 nm. This pigment extract was evaporated in a stream of N_2, re-dissolved in acetone and the carotenoids separated by HPLC. HPLC was carried out on a 25 cm C_{18} Vydac 218TP54 column with methanol as mobile phase (Eppler *et al*., 1992) or on a 25 cm Spherisorb ODS-1 with an acetonitrile/methanol/hexane gradient as described by Gilmore and Yamamoto (1991). The distribution of the individual carotenoids was obtained by integration of the peak area and the total amounts used to calculate the contents of the different carotenoids. Reference carotenoids were isolated from cyanobacteria (Steiger *et al*., 1999) or pepper leaves (Simkin et al, 2000). Carotenoid data in Fig. 3 are given as means of 4–6 determinations of up to three different tubers together with the standard deviation.

For the determination of abscisic acid (ABA) 500 mg fresh tuber material was extracted first with 1 ml and then with 0.2 ml acetone. The combined extracts were evaporated and redissolved in 0.2 M Na-acetate buffer, pH 4, containing 15% (v/v) ethanol. The ABA content was determined by radioimmunoassay using antiserum against ABA with D,L-*cis*,*trans*-[G^3-H]ABA (Amersham TRK 644) as described by Weiler (1979).

α-Tocopherol was extracted from 400 mg or 1 g of fresh potato tubers ground in liquid nitrogen in four subsequent steps using each time 500 μl or 1 ml of acetone, respectively, and determined as described by Fraser *et al*. (2000) by HPLC on a C_{30}-column using a UV detector (Knauer, Germany) at 287 nm and a fluorescence detector (RF-530, Shimadzu) with an emission wavelength of 325 nm and an excitation wavelength of 295 nm.

RESULTS

A cDNA fragment encoding a zeaxanthin epoxidase from potato was cloned into the pBin19 related (Bevan, 1984) transformation vector pPGB121S in sense and antisense orientation, respectively. The fragment was inserted between the granule-bound starch synthase promoter (van der Steege *et al*., 1992) and the nopaline synthase terminator sequence in order to drive tuber-specific expression. The resulting constructs pPGB-zep-sense and pPGB-zep-anti were used to transform potato genotypes Baltica and Freya via *Agrobacterium* mediation (Rocha-Sosa *et al*., 1989). In total, 127 individual transgenic lines resulting from six transformation series were transferred to the greenhouse and grown in 2 liter pots.

Mature mini tubers were harvested, initially screened for flesh color and subsequently analyzed for the carotenoid composition of the tuber. Since the desired transformants

were those with elevated zeaxanthin content, the carotenoid content only of positive lines are listed in Table 1. In comparison to the wild type, the increase of the zeaxanthin level (including minor portions of zeaxanthin fatty acid esters) of series 47 (Baltica, sense construct) varied from 4- to 134-fold. Also in series 48 (Baltica, antisense construct),

TABLE 1

Carotenoid Content of Mini Tubers from Transgenic Potato Lines (μg/g dw)

Line[a]	Total carotenoids	Neox	Viol	Anth	Lut	Zeax	βCry	βCar
Baltica co-suppression								
47-WT	10.6	0.6	3.5	0.3	2.7	0.3	0.6	0.7
47-2	21.4	0.3	1.9	6.2	1.0	10.9	0.4	0.6
47-17	35.0	0	0.1	1.5	1.4	29.5	1.2	1.3
47-18	60.8	0.7	1.7	14.9	5.2	40.1	2.3	2.4
47-36	35.5	1.7	16.2	4.5	7.3	1.4	1.4	2.6
47-51	31.8	0.7	5.1	8.6	3.2	11.3	0.7	1.9
47-52	33.9	1.8	15.4	3.0	7.0	1.4	1.3	1.2
47-53	44.3	0.7	4.0	12.8	2.0	22.5	1.0	1.3
47-73	50.7	1.7	7.6	16.2	5.0	18.7	0.5	1.0
Baltica, antisense								
48-WT	15.6	1.5	9.3	0.4	4.1	0.2	0.5	0.3
48-5	27.4	1.0	13.2	1.1	7.3	1.5	1.7	1.6
48-17	44.2	1.3	10.7	12.1	2.6	16.5	0.5	0.4
48-18	26.6	1.2	14.1	1.9	5.8	2.5	0.6	0.6
48-19	25.1	0.8	11.5	1.9	6.6	2.7	0.8	0.9
48-20	42.2	0.5	4.4	13.2	3.4	17.2	1.6	1.8
48-27	19.0	0.5	5.3	0.9	6.0	0.7	2.6	3.0
48-36	43.0	1.4	22.4	2.0	11.6	2.4	1.5	1.7
48-39	38.3	1.2	18.1	2.4	10.2	4.3	0.5	1.5
52-WT	22.3	0.7	11.1	0.7	6.9	0.8	1.2	0.9
52-7	33.8	0.8	14.1	1.8	8.5	3.0	2.7	2.9
52-13	42.2	1.3	19.4	7.5	7.2	3.9	0.7	2.1
52-25	53.2	2.0	27.2	5.8	10.0	2.9	1.4	3.4
52-26	41.1	0.0	13.5	12.9	5.1	7.0	0.3	1.4
Freya, co-suppression								
49-WT	20.0	1.0	7.4	0.7	5.3	0.2	1.6	1.6
49-2	24.9	0.8	9.7	2.2	5.5	0.8	1.5	1.9
49-4	35.4	1.9	16.6	4.5	5.8	1.5	1.1	2.2
60-15	24.0	0.4	1.7	4.2	4.1	11.2	0.6	1.9
60-47	38.0	0.8	2.8	7.6	5.6	17.4	1.0	2.8
60-85	34.2	0.3	2.2	7.8	4.3	17.8	0.4	1.5
60-119	43.9	0.8	6.9	9.8	6.3	16.4	1.8	1.9
60-154	43.7	0.6	3.1	9.7	5.2	22.8	1.1	1.4
Freya, antisense								
50-WT	23.0	1.2	9.4	1.1	5.5	1.3	1.6	2.9
50-12	41.7	1.1	11.9	4.4	13.4	7.0	3.1	0.8
50-24	32.3	1.5	13.6	1.0	6.8	6.6	1.4	1.4
WT	24.9	1.3	8.3	4.4	6.3	1.4	1.2	2.0
59-37	28.0	0.8	8.7	5.8	6.2	4.3	0.8	1.4
59-48	21.3	0.6	6.0	4.0	5.5	3.6	0.5	1.2
59-49	43.1	0.7	10.4	6.2	13.0	10.0	1.4	1.4

[a] Series 47 and 53 (Baltica, co-suppression) with 26 and 26 tested transgenic lines, series 48 and 52 (Baltica, antisense) with 37 and 22 tested transgenic lines series 49 and 60 (Freya, co-suppression) with 10 and 67 tested transgenic lines, series 50 and 59 (Freya, antisense) with six and 47 tested transgenic lines. WT indicates a non-transgenic control. Tuber carotenoid content from 10 individual Baltica transformants with the vector containing the GBSS promoter but without the zeaxanthin epoxidase sense or antisense sequence was 12.2 ± 2.4 μg/g dw. The difference between total carotenoid content and sum of individual carotenoids is due to variable amounts of carotenoid degradation products.

Metabolic Engineering **4,** 263–272 (2002)
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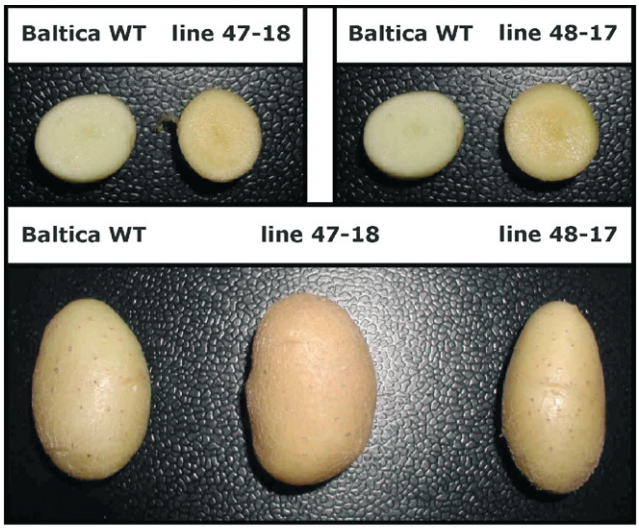

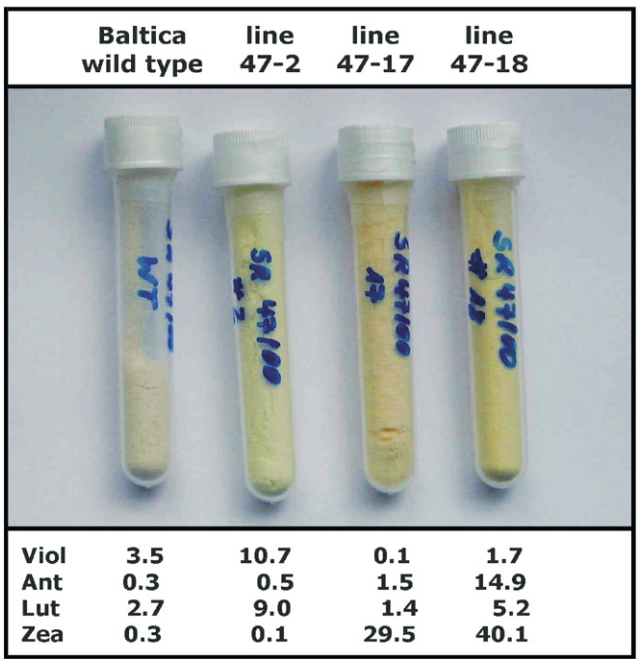

FIG. 1. Color of mini tubers derived from either Baltica wild-type plants or transgenic lines 48-17 and 47-18 as well as coloration of freeze-dried potato powder from wild type and transgenic lines 47-2, 47-17 and 47-18. The content of the major carotenoids is given below.

the highest zeaxanthin value was 55-fold higher than in the corresponding wild type. In total, series 47 resulted in 26 zeaxanthin transformants; series 48 yielded 37 zeaxanthin transformants and series 49 (Freya, sense construct) gave rise to 10 zeaxanthin overaccumulators. In series 50 (Freya, antisense construct) and 52 (Baltica, antisense construct) only a few positive lines were found which exhibited just a moderate 4- to 7.5-fold increase of zeaxanthin. For series 53 (Baltica, sense construct), no line with higher zeaxanthin levels was obtained. However, from the latter series only a relatively small number of total transformants with integrated gene constructs could be regenerated and analyzed. In the wild type control tuber, violaxanthin was the major carotenoid and also high levels of lutein were found. In addition, neoxanthin, antheraxanthin, zeaxanthin, β-cryptoxanthin and β-carotene could be detected in trace amounts. In contrast, zeaxanthin was the dominating carotenoid present in positive transformants such as for example line 47-17. Whereas the lutein content was similar to the control, the amount of violaxanthin was decreased and that of antheraxanthin increased. We also analyzed the carotenoid content of the leaves of lines 47-17, 47-18, 48-17, 48-20 and 50-12. In no case an elevation in the amount of zeaxanthin or a significant change of the leaf carotenoid composition could be detected (data not shown).

Determination of the total carotenoid content in mini tubers revealed that the amount of carotenoids in several transformants is up to 5.7-fold higher than in the wild type or in control transformants containing just the GBSS promoter binary construct without any zeaxanthin epoxidase sense or antisense sequence. This effect was already clearly visible in the potato tuber itself by its dark-yellow to orange color or in the powdered material (Fig. 1). An increase of color intensity was observed in lines with higher zeaxanthin concentrations (47-17) and in those without enhanced zeaxanthin formation (47-36) but elevated total carotenoid content. In the latter, violaxanthin and lutein were the enriched carotenoids. The highest zeaxanthin level was found in mini tubers of transformant 47-18 with a value of 40.1 μg/g dry weight (Table 1).

Growth, development and tuber yield of the transgenic plants were not different compared to the wild type. No phenotypic divergence was evident. Since the phytohormon ABA originates from neoxanthin and violaxanthin (Seo and Koshiba, 2002), the ABA content in 6- and 8-week-old tubers was analyzed. The concentrations were around 223 pmol/g fresh weight with a standard deviation of less than 5% of this value. No significant differences were found between transformants and wild type. In 13-week-old potato tubers the α-tocopherol concentrations were 0.42 μg/g fresh weight for the wild type and increased to 1.29 μg/g in transformant 47-18 and 1.08 μg/g in line 48-20. Standard deviations were less than 5% of these values.

Accumulation of total carotenoid and zeaxanthin of selected lines was determined during tuber development under optimum greenhouse conditions in the natural vegetation period which comes as close as possible to field conditions (Fig. 2). In the wild type, a maximum of total carotenoid content is reached after 10 weeks. When the

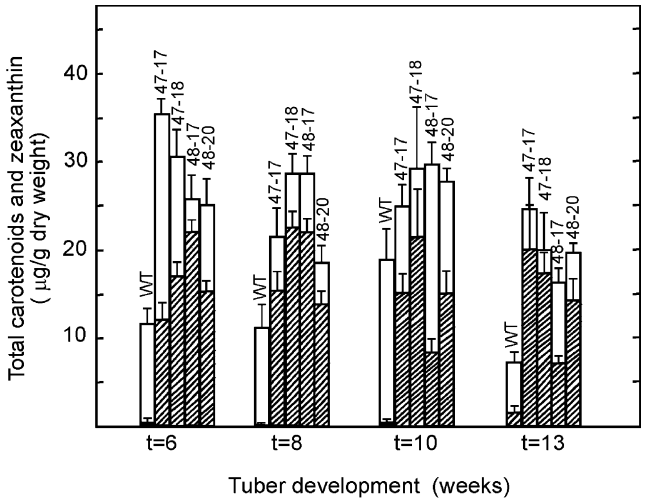

FIG. 2. Accumulation of total carotenoids and zeaxanthin (hatched boxes) in developing tuber. Bars represent standard deviations.

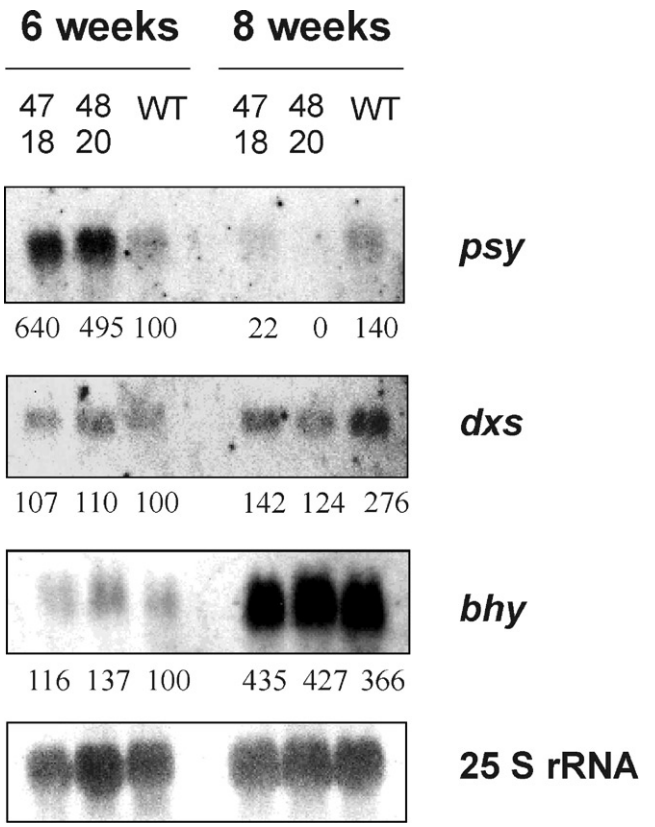

FIG. 3. Northern analysis of the transcripts of *dxs*, *psy* and *bhy* in 6- and 8-week-old potato tubers from wild type and transformants 47-18 (co-suppression), 48-20 (antisense) and Baltica control of different developmental stages. The bottom gel shows the hybridized 25 S rRNA as loading reference. The values which quantitate the transcripts relative to the wild type were set to 100% for wild type at 6 weeks after normalization with respect to the corresponding rRNA.

tubers grow older, the concentration of total carotenoids decreases. This is also the case for most transformants. The fact that starch synthesis still occurs during later tuber development leading to an increase in tuber weight (Khurana and McLaren, 1982), may explain why the carotenoid values based upon dry weight are decreasing as tuber development proceeds (even when the carotenoid amounts stay more or less constant). In general, the maximum zeaxanthin content of the transgenic tubers is reached after approximately 8 weeks. However, the zeaxanthin accumulation pattern varies in the individual transformants. It is either diminished in later stages of tuber development (48-17) or remains almost constant over the whole period (48-20). The maximum amounts in these normal size tuber were around 20 μg/g dry weight for all transformants.

To investigate the impact of the genetic manipulation on endogenous gene expression, expression studies were performed for three selected genes of isoprenoid biosynthesis using tuber material of two transformants (one sense, one antisense) and wild type Baltica (Fig. 3) at different developmental stages. The genes under examination were (1) *dxs* encoding the initial enzyme of plastidic terpenoid biosynthesis deoxyxylulose 5-phosphate synthase, (2) *psy* encoding the initial enzyme of the specific carotenogenic pathway, phytoene synthase, and (3) *bhy* encoding the enzyme β-carotene hydroxylase which catalyzes the formation of zeaxanthin. The most pronounced difference was observed for *psy* transcript levels which were about 5- or 6-fold higher in 6-week-old tubers of transformants 47-18 and 48-20, respectively. After 8 weeks of tuber development, the *psy* transcript was down-regulated in the transformants to very low levels. For the *dxs* transcript level little variation was observed between transformants and wild type in 6-week-old tubers. The amount of *dxs* mRNA increased in all cases after 8 weeks, however, to a lesser extent in the transformants. The β-carotene hydroxylase gene *bhy* showed a slightly elevated expression level after 6 weeks in both transformants compared to the wild type, but was strongly induced after 8 weeks in transgenics as well as wild-type controls (Fig. 3).

Southern hybridization experiments were carried out to demonstrate the integration of the sense and antisense zeaxanthin epoxidase gene fragments into the potato genome and to determine the number of insertions in selected transgenic lines (Fig. 4). The hybridization probe was directed against the kanamycin resistance gene of pPGB 121S. Since the restriction enzymes *Hin*dIII and *Eco*RV do not cut in the kanamycin resistance gene (*npt II*) used as selection marker the number of bands obtained by

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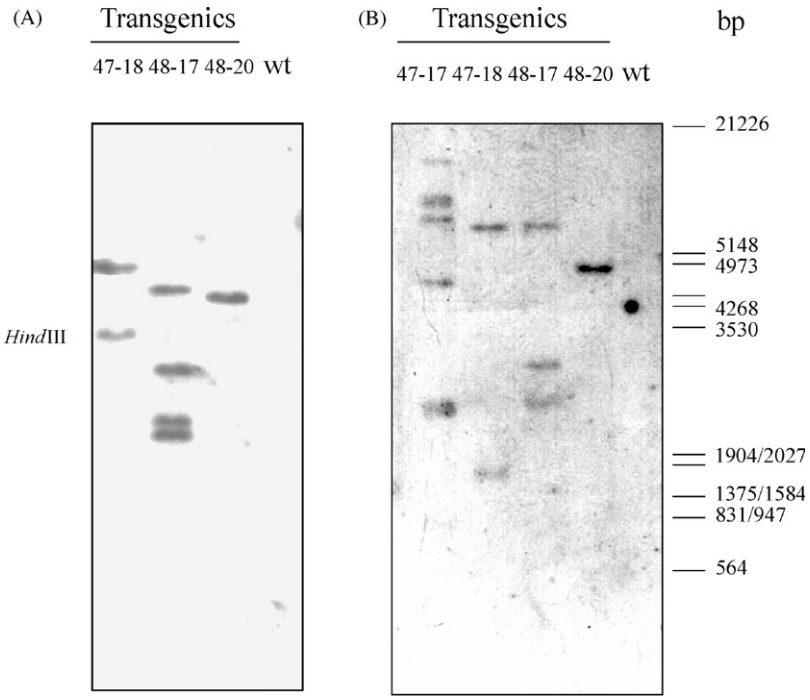

FIG. 4. Southern analysis of genomic DNA of selected transformants restricted with *Hin*dIII (A) or *Eco*RV (B) and probed against the *nptII* gene.

Southern hybridization of *Hin*dIII- and *Eco*RV-digested genomic DNA with a *npt II* probe represent the number of transgene insertions. Eight copies were determined in line 47-17, two in 47-18, and least one in 48-20 (Fig. 4). Southern blot analysis of line 48-17 showed four bands when genomic DNA was digested with *Hin*d III. Three clearly visible bands were found after digestion with *Eco*RV in addition to a faint one of approximately 17 kb which could hardly be detected in the current reproduction of the Southern blot.

DISCUSSION

Dietary carotenoids including zeaxanthin have become increasingly important due to their benefits in the prevention of degenerative diseases (van den Berg *et al.*, 2000). Only a few vegetables are known with a high zeaxanthin content. To overcome the shortage of zeaxanthin supply in our diet, we have transformed potato, an important staple crop which is accessible to genetic manipulations (Davies, 1996), with the endogenous gene coding for zeaxanthin epoxidase under the control of a tuber-specific promoter. In contrast to previous experiments using constitutive expression of carotenoid genes in tomato (Fray *et al.*, 1995) no negative effects on plant growth and vigor could be detected in our transgenics and the concentration of the phytohormone abscisic acid was also not affected in tubers. Although moderate suppression of the zeaxanthin epoxidase mRNA was also detected in leaves of the sense and antisense transformants 47-18 and 48-20 by Northern blot analysis, the leaf carotenoid content of all transformants analyzed was very similar to the control plants (data not shown). This relative specificity of the GBSS promoter for tuber as compared to leaf (van der Steege *et al.*, 1992) obviously avoided changes of the carotenoid composition of the photosynthetic apparatus where violaxanthin is important as a light-harvesting pigment especially under low light conditions (Ruban *et al.*, 1996). From this point of view, photosynthesis should not be negatively affected and the risk of impaired crop yield minimized.

The carotenoid biosynthetic pathway in potato tuber is shown in Fig. 5. The carotenoids in the dotted box are the dominating carotenoids in the wild type. Due to their carotenoid patterns, various types of transformants can be found. This result suggests a differential interference with the regulation of carotenoid biosynthesis in the transformants which we can assign to three groups with respect to their quantitative carotenoid composition. Some lines exhibit an enhanced formation of all carotenoids, not only of zeaxanthin but also of violaxanthin and lutein. As indicated by a zeaxanthin to violaxanthin ratio <0.1, the epoxidase reaction is only slightly inactivated and the major effect is a flow of precursors into cyclic carotenoids

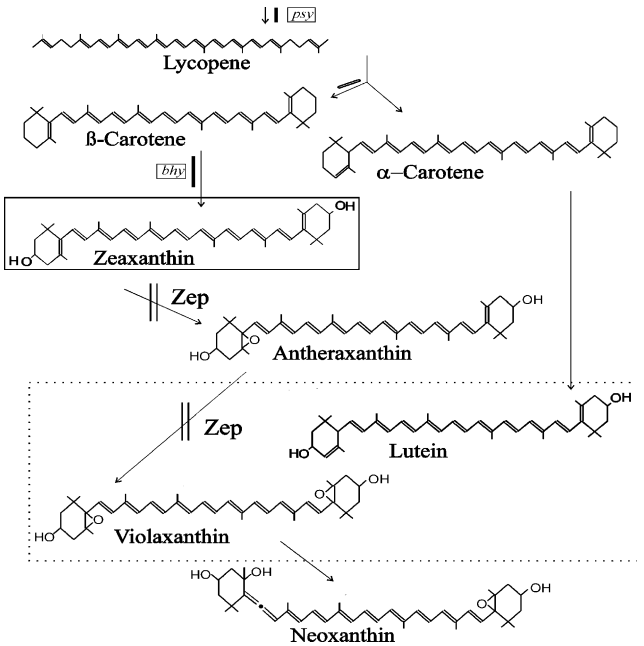

FIG. 5. Carotenoid pathway in potato tuber. Carotenoids in dotted box accumulate in the wild type, inactivation of zeaxanthin epoxidase (Zep) is indicated by double lines. Metabolic flow into all carotenoid branches is indicated by a black bar next to the arrow, specific flow into the β-branch by open bars. Boxed genes are up-regulated in transformants.

which more or less increases the pools of all subsequent carotenoids (Fig. 5, indicated by black bar).

In other lines, the zeaxanthin level is strongly enhanced at the expense of its epoxidation product violaxanthin, whereas the amount of lutein stays about constant. A causal relationship between the inactivation of zeaxanthin epoxidase and zeaxanthin accumulation is supported by a drastically increased zeaxanthin to violaxanthin ratio of up to 24 and the high portion of zeaxanthin reaching more than 60% of total carotenoids. In many lines, inactivation of epoxidation is superimposed by an up-regulation of the whole carotenoid metabolism. Among them, the antheraxanthin content remains either low (e.g. line 47-17, 50-24) or is also strongly increased (e.g. lines 47-18, 60-154). The increased flow into the pathway is preferentially channeled into the β-carotene branch of the pathway (Fig. 5, indicated by open bar). This may be explained by higher β-carotene hydroxylase transcript levels (Fig. 3). An enrichment of the corresponding enzyme may pull the metabolic flow away from α-carotene and lutein biosynthesis. When comparing the zeaxanthin content with the transgene insertions, no direct relationship was seen but the strength of reduction in zeaxanthin epoxidation products was more pronounced in co-suppressed lines with a high number of transgene insertions (Table 1, Fig. 4). Many carotenogenic enzymes like zeaxanthin epoxidase recognize only one-half of the substrate molecule catalyzing two subsequent modifications at both sides. Detailed enzymatic studies are available for carotene desaturases (Raisig *et al.*, 1996). For conversion of substrate and intermediates the affinities are quite similar. For 2-step carotenogenic enzymes, some authors discussed that dimerization is important for catalysis of the second reaction (Bartley *et al.*, 1990; Pecker *et al.*, 1992). When assuming this situation also for zeaxanthin epoxidase, low concentrations due to a high but not complete inactivation below a critical level may prevent the dimerization process. This would explain the relatively high accumulation of monoepoxy antheraxanthin versus violaxanthin in some lines like 47-18, 60-154. When the expression of the epoxidase is stronger repressed, the formation of antheraxanthin is also negatively affected (line 47-17). In our case, lines with co-suppressed *zep* genes showed a more efficient inhibition of the epoxidation reactions than the lines with an antisense construct. Quite often, the introduction of a homologous sense construct causes a strong inhibition of the transcript level of the endogenous gene in a portion of independent transformants (Meyer, 1995). A potent inactivation by co-suppression has been shown before for phytoene synthesis, the first committed step in carotenogenesis (Fray and Grierson, 1993). The negative effect on tomato fruit pigmentation was very similar to inhibition of the same reaction by antisense RNA (Bramley *et al.*, 1992).

The total carotenoid and zeaxanthin concentrations in the tuber depend on their development (Fig. 2). For all transgenic lines, maximum zeaxanthin levels were reached after about 8 weeks of tuber growth. In the case of antisense plants, the zeaxanthin content in full-size developed tubers was the same as in the mini tubers. However, in the co-suppressed plants, normal size tuber accumulated substantially less zeaxanthin (Table 1). The pattern of zeaxanthin formation at different developmental stages is influenced by the differential regulation of *psy* and *bhy*. The transcript levels of the first gene is significantly higher after 6 weeks, the latter after 8 weeks (Fig. 3). This is reflected by maximum total carotenoid values for most of the transformants after 6 weeks of development but enrichment of zeaxanthin during prolonged tuber growth.

Among all vegetables, yellow and orange pepper (*Capsicum annuum*) varieties contain the highest zeaxanthin levels. From the values presented in different publications (Davies *et al.*, 1970; Matus *et al.*, 1991; Minguez-Mosquera and Hornero-Mendez, 1994), zeaxanthin concentrations of 200–600 μg/g dry weight can be estimated in special varieties like Agridulce. In maize grains of several inbred lines, the amount of zeaxanthin which is not the predominant carotenoid ranged from 0.6 to 27.4 μg/dw

Metabolic Engineering **4,** 263–272 (2002)
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(Quackenbush *et al*., 1963) and most cultivars had a similar zeaxanthin content as our wild-type Baltica potato line. The zeaxanthin value for the Agridulce pepper variety is about an order of magnitude higher than in the mini tubers of our best potato line 47-18 (Table 1) and also in the transgenic tomatoes engineered for xanthophyll production (Dharmapuri *et al*., 2002). *Escherichia coli* with the deoxyxylulose 5-phosphate pathway for prenyl pyrophosphate synthesis is a good model system of a plastid-type early terpenoid metabolism. In *E. coli* transformed with carotenogenic genes, an increase of carotenoids through enhanced precursor supply might be achieved by metabolic engineering of several early reactions leading to the formation and conversion of prenyl pyrophosphates. This includes over-expression of the genes encoding geranylgeranyl pyrophosphate synthase and isopentenyl pyrophosphate isomerase (Albrecht *et al*., 1999; Wang *et al*., 1999). A similar approach to overcome limitations of carotenoid biosynthesis by provision of prenyl pyrophosphate precursors can be envisioned to increase the zeaxanthin yield in our potato transformants.

Deregulation of the carotenoid biosynthetic pathway upon transfer and expression of carotenogenic genes is a well-known but not well-understood phenomenon (Sandmann, 2001b). For example, over-expression of a phytoene desaturase gene resulted in an enhanced cyclization reaction (Römer *et al*., 2000) in tomato fruit, and the transfer of a phytoene synthase in combination with a phytoene desaturase gene led to an induction of cyclase and hydroxylase activity in rice grains (Ye *et al*., 2000). In the analyzed transformants with sense and antisense blocking of carotenoid epoxidation, we could demonstrate that enhanced transcript levels of the phytoene synthase gene at least at a certain stage of tuber development is the decisive factor for carotenoid overproduction (Fig. 3). A contribution by enhanced expression of Dxs which is a limiting enzyme for terpenoid synthesis in plant leaves (Estevez *et al*., 2001), is negligible in the tubers of our transformants. At the moment it is still obscure what determines the upregulation of *psy* expression, since in some lines such as 47-36 and 47-52 an increased formation of all individual carotenoids to similar extents can be found although epoxidation is not inhibited. Obviously, the enhanced carotenogenesis in these transformants is independent of the molecular genetic inhibition of violaxanthin accumulation as one of the end products. In Baltica transformants with integrated T-DNA from the binary vector containing only the GBSS promoter together with the kanamycin resistance gene, the total carotenoid content remained unchanged compared to non-transformed plants.

For our goal to engineer a zeaxanthin-rich potato, the up-regulation of carotenogenesis which was observed in some of the transgenic lines was an unexpected but highly advantageous effect. When some of the generated Baltica lines in Table 1 with a dark yellow to orange tuber coloration (Fig. 1) due to a highly elevated zeaxanthin content will be developed, a single average-sized potato can contribute about 0.5–0.6 mg of zeaxanthin to our diet. With these lines, we provide a staple crop rich in zeaxanthin. Its consumption directly as a vegetable or after processing to different potato products may offer a good possibility to lower the risk of age-related macular degeneration according to the results of the Eye Disease Case Control Study Group (Seddon *et al*., 1994).

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REFERENCES

Albrecht, M., Misawa, N., and Sandmann, G. (1999). Metabolic engineering of the terpenoid biosynthetic pathway of *Escherichia coli* for production of the carotenoids β-carotene and zeaxanthin. *Biotechn. Lett*. **21**, 791–795.

Bartley, G. E., Schmidhauser, T. J., Yanofsky, C., and Scolnik., P. A. (1990). Carotenoid desaturases from *Rhodobacter capsulatus* and *Neurospora crassa* are structurally and functionally conserved and contain domains homologous for flavoprotein disulfide oxidoreductases. *J. Biol. Chem*. **265**, 16020–16024.

Bramley, P. M, Teulieres, C., Blain, I., Bird, C., and Schuch, W. (1992). Biochemical characterization of transgenic tomato plants in which carotenoid synthesis has been inhibited through the expression of antisense RNA to pTOM5. *Plant J*. **2**, 343–349.

Bevan, M. (1984). Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res*. **12**, 8711–8721.

Conn, P. F., Lambert, C., Land, E. J., Schalch, W., and Truscott, T. G. (1992). Carotene–oxygen radical interactions. *Free Rad. Res. Comm*. **16**, 401–408.

Davies, H. V. (1996). Recent developments in our knowledge of potato transgenic biology. *Potato Res*. **39**, 411–427.

Davies, B. H., Mathews, S., and Kirk, J. T. O. (1970). The nature and biosynthesis of carotenoids of different colour varieties of *Capsicum annuum*. *Phytochemistry* **9**, 797–805.

Dharmapuri, S., Rosati, C., Pallari, P., Aquilani, R., Bouvier, F., Camara, B., and Giuliano, G. (2002). Metabolic engineering of xanthophyll content in tomato fruits. *FEBS Lett*. **519**, 30–34.

Dietze, J., Blau, A., and Willmitzer, L. (1995). *Agrobacterium*-mediated transformation of potato (*Solanum tuberosum*). *In* "Gene Transfer to Plants XXII" (I. Potrykus and G. Spangenberg, Eds.), pp. 24–29, Springer-Verlag, Berlin.

Eppler, K. S., Sander, L. C., Ziegler, R. G., Wise, S. A., and Craft, N. E. (1992). Evaluation of reversed-phase liquid-chromatographic columns for recovery and selectivity of selected carotenoids. *J. Chromatogr*. **20**, 89–101.

Estevez, J. M., Cantero, A., Reindl, A., Reichler, S., and Leon, P. (2001). 1-Deoxy-D-xylulose-5-phosphate synthase, a limiting enzyme for plastidic isoprenoid biosynthesis in plants. *J. Biol. Chem.* **276**, 22901–22909.

Fraser, P. D., Pinto, M. E. S., Holloway, D. E., and Bramley, P. M. (2000). Application of high performance liquid chromatography with photodiode array detection to the metabolic profiling of plant isoprenoids. *Plant J.* **24**, 551–558.

Fray, R. G., and Grierson, D. (1993). Identification and genetic analysis of normal and mutant phytoene synthase genes of tomato by sequencing, complementation and co-suppression. *Plant Mol. Biol.* **22**, 589–602.

Fray, R. G., Wallace, A., Fraser, P. D., Valero, D., Hedden, P., Bramley, P. M., and Grierson, D. (1995). Constitutive expression of a fruit phytoene synthase gene in transgenic tomatoes causes dwarfism by redirecting metabolites from the gibberellin pathway. *Plant J.* **8**, 693–701.

Gilmore, A. M., and Yamamoto, H.Y. (1991). Resolution of lutein and zeaxanthin using a non-endcapped, lightly carbon-loaded C_{18} high-performance liquid chromatographic column. *J. Chromatogr.* **543**, 137–145.

Giuliana, G., Aquiliani, R., and Dharmapuri, S. (2000). Metabolic engineering of plant carotenoids. *Trends Plant Sci.* **5**, 406–409.

Hanson, A. D., and Shanks, J. V. (2002). Plant metabolic engineering—Entering the S curve. *Metab. Eng.* **4**, 1–2.

Iwanzik, W., Tevini, M., Stute, R., and Hilbert, R. (1983). Carotinoidgehalt und -zusammensetzung verschiedener deutscher Kartoffelsorten und deren Bedeutung für die Fleischfarbe der Knolle. *Potato Res.* **26**, 149–162.

Khachik, F., Bernstein, P. S., and Garland, D. L. (1997). Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. *Invest. Ophthalmol. Vis. Sci.* **38**, 1802–1811.

Khurana, S. C., and McLaren, J. S. (1982). The influence of leaf area, light interception and season on potato growth and yield. *Potato Res.* **25**, 329–342.

Krinsky, N. I. (1994). The biological properties of carotenoids. *Pure Appl. Chem.* **66**, 1003–1010.

Kuntz, M., Römer, S., Suire, C., Hugueney, P., Weil, J. H., Schantz, R., and Camara, B. (1992). Identification of a cDNA for the plastid-located geranylgeranyl pyrophosphate synthase from *Capsicum anuum*: Correlative increase in enzyme activity and transcript level during fruit ripening. *Plant J.* **21**, 25–34.

Landrum, J. T., and Bone, R. A. (2001). Carotenoids in the retina and lens: Possible acute and chronic effects on human visible performance. *Arch. Biochem. Biophys.* **385**, 28–40.

Marin, E., Nussaume, L., Quesada, A., Gonneau, M., Sotta, B., Hugueney, P., Frey, A., and Marion-Poll, A. (1996). Molecular identification of zeaxanthin epoxidase of *Nicotiana plumbaginifolia*, a gene involved in abscisic acid biosynthesis and corresponding to the ABA locus of *Arabidopsis thaliana*. *EMBO J.* **15**, 2331–2342.

Matus, Z., Deli, J., and Szaboles, J. (1991). Carotenoid composition of yellow pepper during ripening: Isolation of β-cryptoxanthin 5,6-epoxide. *J. Agric. Food Chem.* **39**, 1907–1914.

Meyer, P. (1995). Understanding and controlling transgene expression. *TIBTECH* **13**, 332–337.

Minguez-Mosquera, M. I., and Hornero-Mendez, D. (1994). Comparative study of the effect of paprika processing on the carotenoids in peppers (*Capsicum annuum*) of the Bola and Agridulce varieties. *J. Agric. Food Chem.* **42**, 1555–1560.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* **15**, 473–497.

Pecker, I., Chamovitz, D., Linden, H., Sandmann, G., and Hirschberg, J. (1992). A single polypeptide catalysing the conversion of phytoene to ζ-carotene is transcriptionally regulated during tomato fruit ripening. *Proc. Natl. Acad. Sci. USA* **89,** 4962–4966.

Quackenbush, F. W., Firch, J. G., Brunson, A. M., and House, L. R. (1963). Carotenoid, oil, and tocopherol content of corn inbreds. *Cereal Chem.* **40**, 250–253.

Raisig, A., Bartley, G., Scolnik, P., and Sandmann, G. (1996). Purification in an active state and properties of the 3-step phytoene desaturase from *Rhodobacter capsulatus* after overexpression in *Escherichia coli*. *J. Biochem.* **119**, 559–564.

Rocha-Sosa, M., Sonnewald, U., Frommer, W., Stratmann, M., Schell, J., and Willmitzer, L. (1989). Both developmental and metabolic signals activate the promoter of the class I patatin gene. *EMBO J.* **8**, 23–29.

Römer, S., Fraser, P. D., Kiano, J. W., Shipton, C. A., Misawa, N., Schuch, W., and Bramley, P. (2000). Elevation of the provitamin A content of transgenic tomato plants. *Nat. Biotechnol.* **18**, 666–669.

Rosati, C., Aquilano, R., Dharmapuri, S., Pallara, P., Marusic, C., Tavazza, R., Bouvier, F., Camara, B., and Giuliano, G. (2000). Metabolic engineering of beta-carotene and lycopene content in tomato fruit. *Plant J.* **24**, 413–419.

Ruban, A. V., Young, A. J., and Horton, P. (1996). The effects of illumination on the xanthophyll composition of the photosystem II light-harvesting complexes of spinach thylakoid membranes. *Biochemistry* **35**, 674–678.

Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989). "Molecular Cloning: a Laboratory Manual," Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Sandmann, G. (2001a). Carotenoid biosynthesis and biotechnological application. *Arch. Biochem. Biophys.* **385**, 4–12.

Sandmann, G. (2001b). Genetic manipulation of carotenoid biosynthesis: Strategies, problems and achievements. *Trends Plant Sci.* **6**, 14–17.

Seddon, J. M., Ajani, U. A., Sperduto, R. D., Hiller, R., Blair, N., Burton, T. C., Farber, M. D., Gragoudas, E. S., Haller, J., Miller, D. T., Yannuzzi, L. A., and Willett, W. (1994). Dietary carotenoids. Vitamins A, C and E and age-related macular degeneration. *J. Am. Diet. Assoc.* **273**, 1413–1420.

Seo, M., and Koshiba, T. (2002). Complex regulation of ABA biosynthesis in plants. *Trends Plant Sci.* **7**, 41–48.

Shewmaker, C. K., Sheehy, J. A., Daley, M., Colburn, S., and Ke, D. Y. (1999). Seed-specific overexpression of phytoene synthase: Increase in carotenoids and other metabolic effects. *Plant J.* **20**, 401–412.

Simkin, A. J., Breitenbach, J., Kuntz, M., and 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.

Steiger, S., Schäfer, L., and Sandmann, G. (1999). High-light upregulation of carotenoids and their antioxidative properties in the cyanobacterium *Synechocystis* PCC 6803. *J. Photochem. Photobiol.* **B 521**, 14–18.

van den Berg, H., Faulks, R., Fernando Granado, H., Hirschberg, J., Olmedilla, B., Sandmann, G., Southon, S., and Stahl, W. (2000). The potential for the improvement of carotenoid levels in foods and the likely systemic effects. *J. Sci. Food Agric.* **80**, 880–912.

van der Steege, G., Nieboer, M., Swanvin, J., and Tempelaar, M. J. (1992). Potato granule-bound starch synthase promoter-controlled GUS expression: Regulation of expression after transient and stable transformation. *Plant Mol. Biol.* **20**, 19–30.

Wang, C.-W., Oh, M.-K., and Liao, J. C. (1999). Engineered isoprenoid pathway enhances astaxanthin production in *Escherichia coli*. *Biotechnol. Bioeng.* **62**, 235–241.

Weiler, E. W. (1979). Radioimmunoassay for the determination of free and conjugated abscisic acid. *Planta* **144**, 255–263.

Ye, X., Al-Babili, S., Klöti, A., Zhang, J., Lucca, P., Beyer, P., and Potrykus, I. (2000). Engineering the provitamin A (β-carotene) biosynthetic pathway into carotenoid-free rice endosperm. *Science* **287**, 303–305.