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ARTICLE

Expression of *phytoene synthase1* and Carotene Desaturase *crtI* Genes Result in an Increase in the Total Carotenoids Content in Transgenic Elite Wheat (*Triticum aestivum* L.)

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Dietary micronutrient deficiencies, such as the lack of vitamin A, are a major source of morbidity and mortality worldwide. Carotenoids in food can function as provitamin A in humans, while grains of Chinese elite wheat cultivars generally have low carotenoid contents. To increase the carotenoid contents in common wheat endosperm, transgenic wheat has been generated by expressing the maize *y1* gene encoding phytoene synthase driven by a endosperm-specific *1Dx5* promoter in the elite wheat (*Triticum aestivum* L.) variety EM12, together with the bacterial phytoene desaturase *crtI* gene from *Erwinia uredovora* under the constitutive *CaMV 35S* promoter control. A clear increase of the carotenoid content was detected in the endosperms of transgenic wheat that visually showed a light yellow color. The total carotenoids content was increased up to 10.8-fold as compared with the nontransgenic EM12 cultivar. To test whether the variability of total carotenoid content in different transgenic lines was due to differences in the transgene copy number or expression pattern, Southern hybridization and semiquantitative reverse transcriptase polymerase chain reaction analyses were curried out. The results showed that transgene copy numbers and transcript levels did not associate well with carotenoid contents. The expression patterns of endogenous carotenoid genes, such as the phytoene synthases and carotene desaturases, were also investigated in wild-type and transgenic wheat lines. No significant changes in expression levels of these genes were detected in the transgenic endosperms, indicating that the increase in carotenoid transgenic wheat endosperms resulted from the expression of transgenes.

KEYWORDS: Carotenoid; bacterial phytoene desaturase (CRTI); endosperm-specific *1Dx5* promoter; particle bombardment; phytoene synthase (PSY); wheat

INTRODUCTION

Carotenoids represent a diverse group of pigments widely distributed in nature. They contribute to red, orange, and yellow colors in flowers, fruits, and vegetables. Carotenoids are derived from isoprenoid precursors and are synthesized in plants as well as some bacteria, fungi, and algae. In plants, carotenoids fulfill a variety of critical functions. They participate in light harvesting in photosynthetic membranes and also protect the photosynthetic apparatus from photo-oxidation (*1*, *2*). Carotenoids act as precursors of the growth regulator abscisic acid (*3*). In addition, carotenoids function as attractants to pollinators and seed dispersal agents (*4*). Carotenoids are important not only to those organisms where they are synthesized but also to animals and humans (*5*). Carotenoids have long been recognized as essential nutrients and compounds beneficial to health, which have fundamental roles in human nutrition as antioxidants and vitamin A precursors, and their consumption is increasingly associated with protection from a range of diseases (*6*, *7*). Animals, including humans, are unable to synthesize carotenoids de novo, and they have to depend on diet for these essential products. Therefore, food biofortification with enhanced pro-vitamin A carotenoids offers a sustainable way to combat vitamin A deficiency and thus comes to be one of the major driving forces stimulating carotenoid research and metabolic engineering of carotenoids in food crops.

Significant progress has been made in our understanding of carotenoid biosynthesis and metabolism in plants (*8*). In higher plants, all of the steps of carotenoid biosynthesis occur in plastids by enzymes that are coded for by nuclear genes and imported into the organelle posttranslationally (**Figure 1**) (*3*, *5*, *9*, *10*). The first committed step in the carotenoid biosynthesis pathway is the head-to-head condensation of two geranylgeranyl pyrophosphate (GGPP) molecules to produce phytoene catalyzed by phytoene synthase (PSY). The phytoene is desaturated into red-colored lycopene by the action of two desaturases: phytoene

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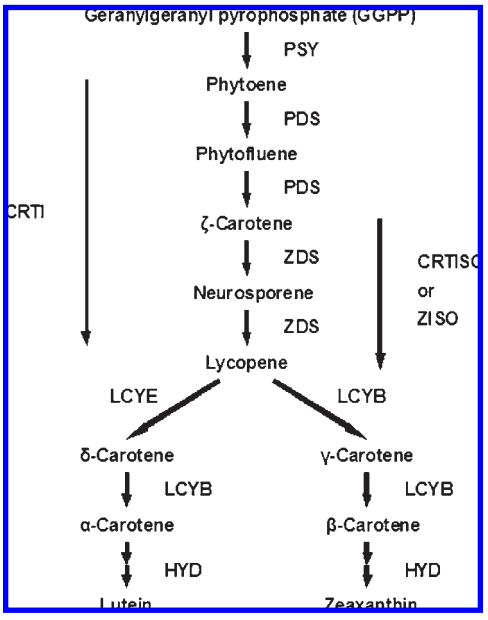

Figure 1. Carotenoid biosynthetic pathway in plants. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; ZISO, ζ-carotene isomerase; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; HYD, carotene hydroxylases; and CRTI, bacterial carotene desaturase.

desaturase (PDS) and ζ-carotene desaturase (ZDS). This pathway gives rise to poly-*cis* compounds that are converted to their all-*trans* forms through the action of the carotenoid isomerases: carotene isomerase (CRTISO) or ζ-carotene isomerase (ZISO). The existence of ZISO is supported by work in maize (*11*) but has not yet been demonstrated at the molecular level. A branching point in the plant carotenoid pathway is marked by the cyclization of lycopene, which is cyclized either to yield α-carotene (β, ε-carotene) by lycopene ε-cyclase (LCYE) and lycopene β-cyclase (LCYB) or to produce β-carotene by LCYB alone (*12*). Hydroxylation of β-carotene and α-carotene produces the well-known xanthophylls zeaxanthin and lutein, respectively.

The identification of the carotenoid biosynthetic genes in plants and other organisms has opened the door to the biotechnological overproduction of carotenoids of nutritional interest in crops. Some of the metabolic engineering approaches have been carried out to date in crop plants to increase the levels of nutritionally relevant carotenoids in many different species. Significant progress has been made recently in quantitative and qualitative manipulation of the carotenoid metabolic pathway in plants (*5*, *8*, *13*−*16*). A significant increase in total carotenoid levels by expression of plant *PSY* in a tissue-specific manner was achieved in tomato fruit (*17*). Overexpression of the bacterial *crtB* (for PSY) in the oilseeds of canola led to ∼50-fold increase in total carotenoids (*18*). Using an endogenous *PSY* gene, enhanced seed-specific accumulation of β-carotene and other downstream productions, primarily lutein and violaxanthin, was also achieved in *Arabidopsis* (*19*). However, "Golden Rice" represents one of the best-known successful examples for metabolic engineering of carotenoids for improving crop nutritional value. In rice endosperm, which lacks carotenoids, overexpression of the daffodil *PSY* led to the production of phytoene (*20*), but when coupled with expression of the bacterial *crtI* gene (which mediates the four desaturation reactions) and/or the daffodil LCYB gene, it yielded the yellow-colored grains containing β-carotene and xanthophlls in a total carotenoid content of 1.6 μg g^{-1} dry weight (*21*). In "Golden Rice 2", the expression of maize *PSY1* and the bacterial *crtI* gene result in the accumulation of β-carotene in rice endosperm up to 37 μg g^{-1} dry weight (*22*). In another study, tuber-specific overexpression of three genes (*crtB*, *crtI*, and *crtY*) necessary for tuber carotenoid biosynthetic pathway leads to the production of "golden" potato with a profound increase of β-carotene to a level of 47 μg g^{-1} dry weight (*23*). In maize, the overexpression of the bacterial *crtB* and *crtI* genes under the control of a "super γ-zein promoter" for endosperm-specific expression resulted in an increase of total carotenoids of up to 34-fold with a preferential accumulation of β-carotene in the maize endosperm (*24*).

Wheat is a major cereal crop in many parts of the world and as such is a primary target for improvement of agronomic characteristics by genetic engineering. Carotenoids are the main components of grain yellow pigment, which determines the seeds and products color. In the grains of durum wheat (*Triticum turgidum* L. *durum*), the main carotenoid pigment is lutein (*25*), which confers a natural yellow color to pasta product. Adom et al. (*26*) also found that lutein and its stereoisomer zeaxanthin were the major carotenoids of yellow-colored kernels, and there were significantly different contents in different cultivars. In contrast, white flour varieties are usually selected in common wheat (*T. aestivum*) with a low carotenoid content in the grains. Over the years, the wheat carotenoid biosynthesis studies always focus on detecting the molecular marker associated with the difference of carotenoid content. Quantitative trait loci (QTL) for endosperm yellow color accounting for a large proportion of the genetic variation have been mapped on chromosome arms 7AL and 7BL (*27*, *28*). Smaller QTLs were also detected on chromosomes 2A, 3A, 4A, 4B, 5A, and 5B (*29*−*31*). The biosynthesis pathway of lutein and zeaxanthin, the main components of wheat carotenoid, involves more than 10 enzymatic steps (*16*). However, very few genes responsible for the differences in carotenoid accumulation in the wheat grain have been identified.

According to the past studies, in many plants, PSY was thought to be a rate-limiting step, and multiple PSYs share the regulation of carotenoid metabolic flux (*19*). The maize *y1* gene (*PSY1*) had been found to be associated with the carotenoid accumulation in endosperm, thus demonstrating the significance of the gene in the grain carotenoid biosynthetic pathway (*32*−*34*). To improve the nutritional value of Golden Rice, a maize *y1* gene has been used to substitute for the daffodil *PSY* (*22*). In addition, the *Erwinia* genes were successfully used to enhance or modify the carotenoid content of several crops like rice (*21*, *22*), tomato (*35*), canola (*36*), and potato (*37*). From a biotechnological point of view, the employment of *Erwinia* phytoene desaturase CRTI appears very advantageous, because it could substitute for the three plant enzymes, phytoene desaturase, ζ-carotene desaturase, and carotene *cis*−*trans*-isomerase (CRTISO).

The transgenic approaches can be a useful tool to explore and to improve the nutritional value of wheat through an increase in the carotenoid content. A transgenic approach to enhance the total carotenoids content of wheat endosperm is reported here. To our knowledge, this is the first such attempt for metabolic engineering of carotenoids to enhance the carotenoid contents in hexaploid wheat. On the basis of the successful experiments for metabolic engineering of carotenoids in some crop plants, such as rice (Golden Rice) and maize, we introduced a maize *y1* gene and a bacterial *crtI* gene into the endosperm of wheat (*Triticum aestivum* L.) variety EM12. It is shown here that wheat seeds can be metabolically engineered to produce high levels of carotenoids by overexpressing the heterogeneous maize *y1* gene in an endosperm-specific manner, using a modified and highly active high molecular weight (HMW) glutenin subunit *1Dx5* promoter.

Table 1. Primer Sequences Used in This Study[a]

name	accession no.	primer sequences	product size (bp)
Y1-upper	AY773475	ACGCCGTCGAC**ATG**GCCATCATACTCGT	1240
Y1-lower	AY773475	CGCGGATCC**CTA**GGTCTGGCCATTTCTC	
Y1-F	AY773475	AGTGTACGTATCACCGTTTGTGTGAAC	497
Y1-R	AY773475	ATCGCCGCTTTGGACATACCATCCGTA	
CRTI-F	D90087	GATATCCTCTTCCGCTGTGAC	623
CRTI-R	D90087	AGACCTTCCCTGACTCCCAA	
PSY1-F	EF600063	CTCAAGAAGGCGAGGTACAAGA	428
PSY1-R	EF600063	AGGATCTGCCGGTACAAC	
PSY2-F	BE604139	AAGGCCGCCCGTATGATA	351
PSY2-R	BE604139	GTGCCAGCTCGTCCAGTG	
PDS-F	FJ517553	GCACGAGGGCTGGTCTATCAA	680
PDS-R	FJ517553	GGACCTCACCACCCAAAGACTGAA	
ZDS-F	FJ169496	CAGCAATCTTTTCCGCCTCA	524
ZDS-R	FJ169496	TACCACCCCTGTCTGTTATG	

[a] Primer sequences are indicated in the 5′-to-3′ direction. Underlined nucleotides are recognition sites of restriction endonuclease. The start codon and termination codon (complementary sequences) of genes are indicated as bold letters.

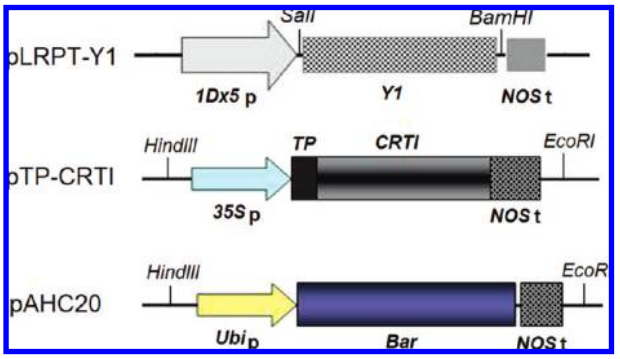

Figure 2. Constructs used in the wheat transformation. Three constructs were generated as follows: pLRPT-Y1, pTP-CRTI, and pAHC20. *1Dx5* p, wheat endosperm-specific HMW glutenin subunit *1Dx5* promoter; *35S* p, constitutive cauliflower mosaic virus (*CaMV*) *35S* promoter; *Ubi* p, constitutive maize *Ubiquitin-1* promoter; *Y1*, maize phytoene synthase (PSY1) gene; *TP*, transit peptide from pea Rubisco small subunit (*rbcS*); *CRTI*, *E. uredovora* phytoene desaturase gene; *Bar*, Bialaphos resistance gene; and *NOS* t, *Agrobacterium tumefaciens* nopaline synthase (*NOS*) terminator.

MATERIALS AND METHODS

Constructs of Plasmid. The phytoene synthase gene coding sequence of maize was obtained by RT-polymerase chain reaction (PCR) without any untranslated regions derived from leaves of *Zea mays* cv. B73 seedlings. The primers were designed according the reported maize *y1* gene (GenBank AY773475, also named *y1* gene): Y1-upper and Y1-lower (see in **Table 1**.). Using primers, a *Sal*I site was added at the 5′-end adjacent to the start codon and a *Bam*HI site at the 3′-end (restriction sites were shown as underlined). The 1240 bp coding sequence of *y1* gene was amplified as a *Sal*I-*Bam*HI fragment and inserted into the plasmid pLRPT (*38*), which contains an endosperm-specific *1Dx5* promoter, multiple cloning sites for cloning genes of interest, and a *NOS* terminator, to generate plasmid pLRPT-Y1. The resulting pLRPT-Y1 plasmid constructs were sequenced to verify that the manipulations did not introduce errors.

Plasmid pTP-CRTI carrying an *Erwinia uredovora* phytoene desaturase *crtI* gene was generated from pYPIET4 (*39*). In plasmid pYPIET4, a pea Rubisco small subunit transit peptide (TP) was fused to the 5′-region of *crtI* gene, to target the *crtI* gene to the plastid. The fusional *TP-crtI* gene was driven by a constitutive *CaMV 35S* promoter and *NOS* terminator. According to the map, plasmid pYPIET4 was digested with *Eco*RI and *Hin*dIII, and a 3.1 kb *Eco*RI-*Hin*dIII fragment carrying the minimal expression cassettes (Promoter-Gene open reading frame-Terminator) of *TP-crtI* gene was isolated. The desired 3.1 kb fragment was ligated into the *Eco*RI-*Hin*dIII site of a 2.7 kb fragment removing from the vector pUC18 (purchased from TaKaRa Biotechnology Co. Ltd., Japan), to generate plasmid pTP-CRTI.

Another plasmid used in transformation approach was pAHC20 carrying a selectable *Bar* gene, which confers resistance to the herbicide phosphinothricin. The *Bar* gene was under the control of the constitutive maize *ubiquitin* (*Ubi-1*) promoter and *NOS* terminator. Thus, the desired plasmids pLRPT-Y1, pTP-CRTI, and pAHC20 were all shown in **Figure 2**.

Plant Material. EM12 is a commercial wheat cultivar from the central area of China, a hexaploid bread wheat species, with the genome constitution AABBDD. This wheat variety produces flour with a white color, due to the low content of the carotenoids in the endosperm. Experimental wheat lines were sown in the field, and spikes were harvested 14−16 days postanthesis. Scutella from immature embryos were used as the experimental targets for bombardment.

Transformation Procedure. The transformation procedure was performed based on the bombardment method developed by Barceló and Lazzeri (*40*) and fully described by Sparks and Jones (*41*). Scutella were aseptically isolated from the immature embryos as the targets, which were to be bombarded. For each bombardment, 30−50 scutella (~0.5−1.0 mm in diameter) were placed in the center of a plate of MS-based induction medium containing 2 mg L^{-1} picloram (MSSP2). Explants were cultured in darkness at 26 °C for 1 day prior to bombardment. Two extra plates of samples were used as controls, one bombarded with only gold particles and the other one not bombarded. Plasmid constructs were precipitated onto the gold particles (Bio-Rad Laboratories, Inc., United States) to a total amount of 5 μg before bombardment. The amounts of DNA added were calculated such that equimolar quantities were used in a 2:2:1 ratio of plasmids of pLRPT-Y1:pTP-CRTI:pAHC20. Bombardments were performed using a PDS 1000/He particle gun (Bio-Rad). After the bombardment, scutella were spread over the surface of the medium in groups of 10−15 and cultured at 26 °C in darkness for 3 weeks to induce embryogenesis. The callus induction stage was followed by regeneration of embryogenic calli in the light. Embryogenic calli were subsequently transferred to regeneration medium (R) containing 0.1 mg L^{-1} 2,4-D and 5 mg L^{-1} zeatin (R_{DZ} medium) and then cultured for 3 weeks prior to transfer onto hormone-free R medium containing 3−5 mg L^{-1} L-PPT. The regeneration stage was performed in four rounds of 3 weeks each, and the selection of putative transformants was done on the last three rounds of regeneration until all control plantlets had died. Surviving plants were transferred to soil and grown to maturity under greenhouse conditions. The seeds (T_1) were then collected from each independent T_0 progeny to produce the T_1 plant lines.

Carotenoid Extraction and Analysis. Total carotenoids were extracted from single half grains or flour samples with a modificative extraction approach, which has been described by Panfili et al. (*42*). This method was made according to AACC method 14-50 (*43*) with slight modifications. One gram of wheat flour sample was extracted with 5 mL of water-saturated *n*-butyl alcohol (WSB) on a horizontal shaker for 60 min at 150 oscillations per minute and overnight at 90 oscillations per minute. The extract was then filtered by means of a Whatman #1 paper, and the residue was washed two times with 5 mL of WSB until a colorless extract was obtained. Finally, the solvent was first evaporated under vacuum at 40 °C and then dried under a stream of nitrogen. The dried extract was reconstituted with 20 μL of WSB. The absorbance was measured at 450 nm on a spectrophotometer (Hitachi High-Technologies Corp., Japan), and a standard solution of β-carotene was used for the calculation. Results are expressed as micrograms of dry weight. All samples were analyzed in low light or darkness and on the ice where possible.

Genomic DNA Extraction and Southern Hybridization. Total genomic DNA was isolated from frozen leaves of primary transformants and their progeny using the cetyl trimethyl ammonium bromide (CTAB) method (*44*). The presence of transgene was examined by PCR. Primers used to the *y1* gene amplification were Y1-F and Y1-R (see **Table 1**) at an annealing temperature of 62 °C, releasing an expected amplified product of 497 bp. The *crtI* gene was amplified with CRTI-F and CRTI-R primers (see **Table 1**) at 64 °C annealing temperature giving a PCR product of 623 bp. Genomic DNA from unbombarded wheat cultivar EM12 was

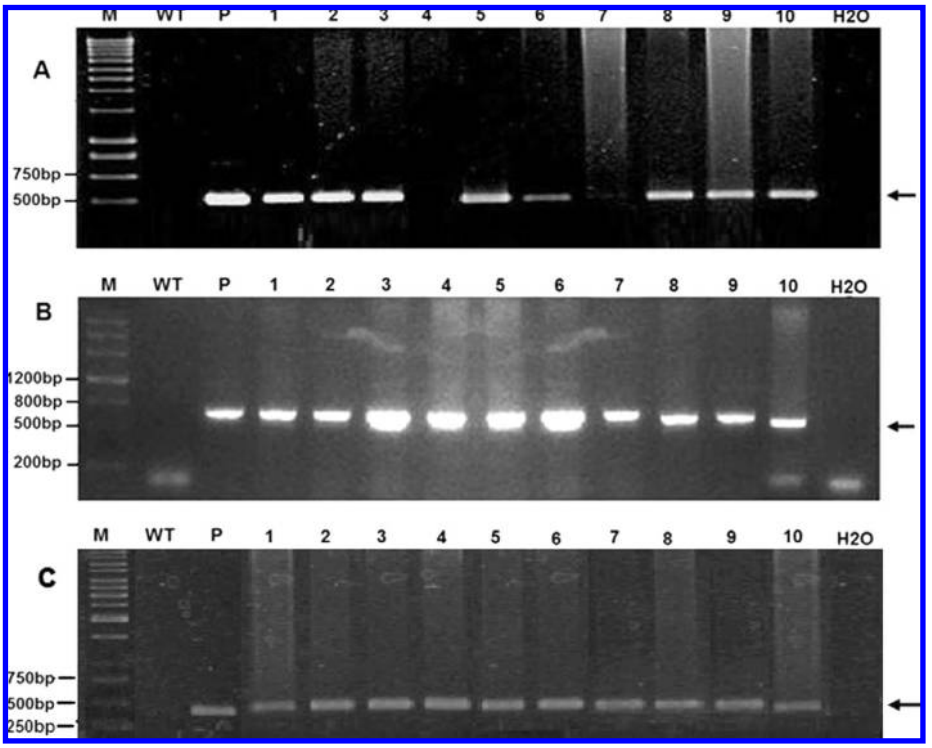

Figure 3. PCR analysis of genomic DNA extracted from primary generations for the transgenes. (**A**) *Y1* gene PCR products (497 bp), (**B**) *TP-Crtl* gene PCR products (623 bp), and (**C**) *Bar* gene PCR products (403 bp). M, DNA molecular weight marker; P, positive control, plasmid pLRPT-Y1 (**A**) or pTP-CRTI (**B**) or pAHC20 (**C**); WT, wild-type EM12 cultivar; H_2O, water control; and 1−12, transgenic lines (1) B1-1-3, (2) B1-2-1, (3) B1-5-3, (4) B1-6-1, (5) B3-2-6, (6) B3-3-1, (7) B3-4-5, (8) B4-2-1, (9) B4-2-3, and (10) B4-5-3.

used as a negative control. Integration of the *y1* and *crtI* genes was subsequently confirmed by Southern blot analysis of genomic DNA. Approximately 15 μg of genomic DNA was digested with *Bam*HI, *Eco*RI, *Kpn*I, and *Sac*I, separately, to determine copy number of the transgenes. The DNA fragments of *y1* (497 bp) and *crtI* (623 bp) from plasmids pLRPT-Y1 and pTP-CRTI were used as probes. Blotting, hybridization, membrane washing, and detection were performed with DIG High Prime DNA Labeling and Detection Starter Kit II (F. Hoffmann-La Roche, Ltd., Switzerland).

RNA Extraction and RT-PCR Analysis. Total RNAs from tissues (root, leaf, inflorescence, and endosperm) of the T_0 and T_1 generation of transformants were isolated using the Plant RNA Mini Kits (Watson Biotechnologies, Inc., China) following the manufacturer's instructions. The first-strand cDNA was synthesized by M-MLV Reverse Transcriptase (Promega, America) using 1 μg of total RNA as the template. The RT-PCR reactions of *y1* and *crtI* gene were performed as previously described condition in PCR analysis of genomics DNA. The semiquantitative RT-PCR analysis was also used to examine the expression pattern of endogenetic carotenogenes. The primers used in RT-PCR reactions were designed according to the reported cDNA sequences encoding carotenoid genes, and the GenBank accession numbers of these genes were seen in **Table 1**. The relative amounts of each PCR product were readily quantified by direct scanning with a densitometer of ethidium-stained 1.5% TAE-agarose gels with a Molecular Imager Gel Doc 2000 System (Bio-Rad) equipped with the Quantity One 1-D analysis software. To normalize for equal amounts of total RNA and efficiency of cDNA synthesis from various samples, the intensities of the band were normalized with the average intensity of the endogenetic *PSY* product in leaves across the samples investigated. The reproducibility of the transcriptional patterns revealed by semiquantitative RT-PCR was tested by at least three independent assays, and each percentage value is the mean of the three independent experiments.

RESULTS

To assess the effects of maize *y1* and bacterial *crtI* overexpression on carotenoid accumulation in common wheat, three different plasmid constructs were generated (schematically represented in **Figure 2**). Plasmids pLRPT-Y1, pTP-CRTI, and pAHC20 contain a maize *y1* gene, a bacterial *crtI* gene, and a selectable *Bar* gene, respectively. The *crtI* gene was fused to the N-terminal transit peptide (TP) of the pea gene for the small subunit of Rubisco (*rbcS*), which specifies plastid targeting (in this case, to endosperm amyloplasts) and is removed from the precursor during or shortly after import into the organelle. The fusion *TP-crtI* gene was under the control of constitutive *CaMV 35S* promoter and *NOS* terminator. In pLRPT-Y1 construct, the *y1* gene without any untranslated regions from *Z. mays* was obtained using RT-PCR amplification and driven by the wheat endosperm-specific *1Dx5* promoter (*38*). For selection, the *Bar* gene was carried in plasmid pAHC20 and driven by another constitutive maize *Ubi-1* promoter.

For transformation, a large number of translucent scutella were isolated from immature EM12 embryos and particle-bombarded with the pLRPT-Y1, pTP-CRTI, and pAHC20 constructs (**Figure 2**). EM12 was chosen because it had a low carotenoid content in endosperm, and it was hoped that successful transformation events could easily be detected by a change in color of white endosperm to yellow/orange.

The transgenic wheat plants were regenerated from the immature scutella transformed with multiple gene plasmids via particle bombardment. Transformed calli were selected based on their resistance to the herbicide phosphinthricin (conferred by the *Bar* gene). The leaf genomic DNAs extracted from transgenic plants surviving after the selection for the *Bar* gene were used for PCR analysis for the presence of transgenes. To obtain the three plasmids cotransformed calli, 982 scutella isolated from wheat variety EM12 were bombarded, and 10 herbicide-resistant plantlets were identified (**Figure 3C**); eight of these were PCR-positive for the *y1* (**Figure 3A**) and *crtI* (**Figure 3B**) sequences. The results indicated that eight independently regenerated plant lines had actually been integrated with the *y1* and *crtI* gene.

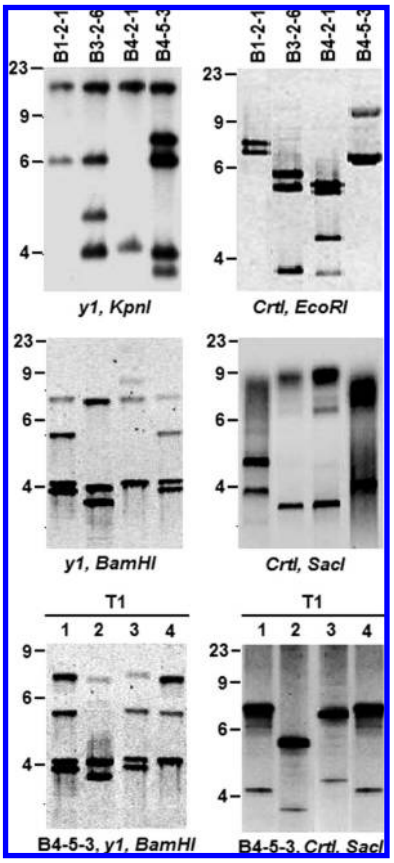

Figure 4. Southern blot analysis of transgenic plants carrying transgenes, using leaf genomic DNA digested with the single cutters *Bam*HI (*y1*) or *Sac*I (*crtI*), plus the noncutter *Kpn*I (*y1*) or *Eco*RI (*crtI*). The lines named B1-2-1, B3-2-6, B4-2-1, and B4-5-3 represent independent T_0 lines regenerated with cotransformants. The numbers between T_1 and each blot correspond to different T_1 descendants of a T_0 line named B4-5-3.

To investigate the patterns of integration of transgenes into the host wheat genome, further Southern blot analysis of genomic DNA from the T_0 and T_1 regenerated plants was performed. Transgene integration patterns of four regenerated lines (lines B1-2-1, B3-2-6, B4-2-1, and B4-5-3) were analyzed. Southern blot analyses were performed following restriction digestion with endonucleases that cut at a single site or noncutter site in the transgene sequences. The PCR fragment of *y1* and *crtI* gene from plasmid pLRPT-Y1 and pTP-CRTI were used as probes. For the analysis of *y1* gene, two transgenic lines (B1-2-1 and B4-2-1) had 2−4 hybridization signals, and the other two lines (B3-2-6 and B4-5-3) had more than four hybridization signals, none of the transgenic lines had a single hybridization signal (**Figure 4**). In contrast to the relatively complex integration patterns obtained for the *y1* gene, Southern blots for the *crtI* gene displayed a lower integration and simpler integration patterns. Following restriction digest with *Eco*RI or *Sac*I and hybridization with the *crtI* probe, the detected lines showed 2−3 hybridization signal (**Figure 4**). It is concluded that most of the integration events of transgene involved the low copy number transgene insertions.

By the next generation, the T_1 plants from an independent T_0 transformant B4-5-3 were chosen for Southern blot analysis following digestion using a single cutter. Analysis of the individual T_1 progeny from B4-5-3 allowed the patterns of inheritance of the transgenes *y1* and *crtI* to be determined (**Figure 4**). Southern blot analysis of *y1* gene indicated that the number of hybridizing band was three in progeny 2 and 4, while four bands were present in progeny 1 and 3. The different number of hybridization signals suggested that there was a segregation of the *y1* gene in T_1 progeny. It is concluded that the maize *y1* gene was inserted at multilocus in line B4-5-3. In addition, Southern blot analyses of *crtI* gene in four T_1 plants from T_0 line B4-5-3 showed no segregation of the *crtI* bands (**Figure 4**). It is concluded that the bacterial *crtI* gene was inserted at a single locus in line B4-5-3.

Mature seeds (T_1) from T_0 transgenic lines and control plants were air-dried and dehusked, and to observe the endosperm, they were cut horizontally with a scalpel. In most cases, the T_1 transformed seeds show a deeper yellow color (**Figure 5A**) than that of the wild-type seeds (**Figure 5B**). For endosperms, the T_1-transformed endosperms displayed a light yellow color (**Figure 5C**), indicating carotenoid formation, whereas the control endosperms were bright white (**Figure 5D**).

The T_1 seeds from individual lines (approximately 2−5 seeds per individual lines) were analyzed for carotenoids by photometric analyses. **Table 2** showed the total carotenoid contents of the T_1 seeds from T_0-transformed lines, and results for the wild-type EM12 were shown as controls. The carotenoid contents of transgenic seeds were variable, ranging from 2.31 to 4.96 $\mu g\ g^{-1}$ seed dry weight. As compared with nontransgenic EM12 cultivar that had a lower carotenoid content of 0.46 $\mu g\ g^{-1}$ seed dry weight (as expected), the greatest increase (~10.8-fold) in total carotenoid was observed in line B4-5-3 (4.96 $\mu g\ g^{-1}$ seed dry weight).

To test whether the variability in total carotenoid content was due to differences in transgene copy number, Southern blot analysis of genomic DNA was performed on all of the T_0 transgenic lines. The labeled probes of *y1* and *crtI* genes did not hybridize to wild-type EM12 genomic DNA but displayed several detected hybridization signals from transgenic wheat lines. As expected for plants transformed via particle bombardment, Southern blot analysis of T_0 generations showed that the transgenic lines had a variable transgene copy number, ranging from two to five copies for both *y1* and *crtI*. However, copy numbers did not associate well with carotenoid contents (**Table 2**). For instance, transgenic line B1-5-3 contained at least four copies of *y1* gene and five copies of *crtI* gene with a total carotenoid content of 2.31 $\mu g\ g^{-1}$ seed dry weight, whereas line B4-5-3 contained at least five copies of *y1* gene and two copies of *crtI* gene with a total carotenoid content of 4.96 $\mu g\ g^{-1}$ seed dry weight.

To understand the variability in the wheat lines further and to determine the basis for the increased accumulation of carotenoids in these lines, the expression pattern of *y1* and *crtI*, and representative carotenoid biosynthesis genes in T_0 seeds of all the transgenic wheat lines, were determined. These genes encode enzymes known to mediate regulatory steps of the biosynthesis pathway, as well as two phytoene synthases (PSY1 and PSY2), PDS, and ZDS. Transgenic and wild-type plants were sown in a randomized plot in the greenhouse, and total RNA was extracted from immature milky stage endosperm. Leaf material was harvested from the seedlings. The semiquantitative RT-PCR was used to characterize mRNA transcript levels in the wild-type or to monitor potential changes in wheat endogenous mRNA transcript levels in the transgenic samples.

Figure 6 shows the results obtained with the wild-type materials. Almost all carotenoid biosynthetic genes investigated were found to be expressed in wheat endosperm, albeit at very low levels. *PSY1* mRNA was the least abundant, about 50-fold lower than that in leaves. The mRNAs for *PSY2*, *PDS*, and *ZDS* displayed similar expression patterns, approximately 5-fold lower than in leaves. The low levels of *PSY1* mRNA transcript found in endosperm could have cause the low carotenoid content accumulation in endosperm. In addition, the detectable amount of *PSY2* mRNA found in the endosperm explains that *PSY2* displayed a weak association with the carotenoid accumulation in wheat endosperm, as seen in maize and rice (*33*, *34*).

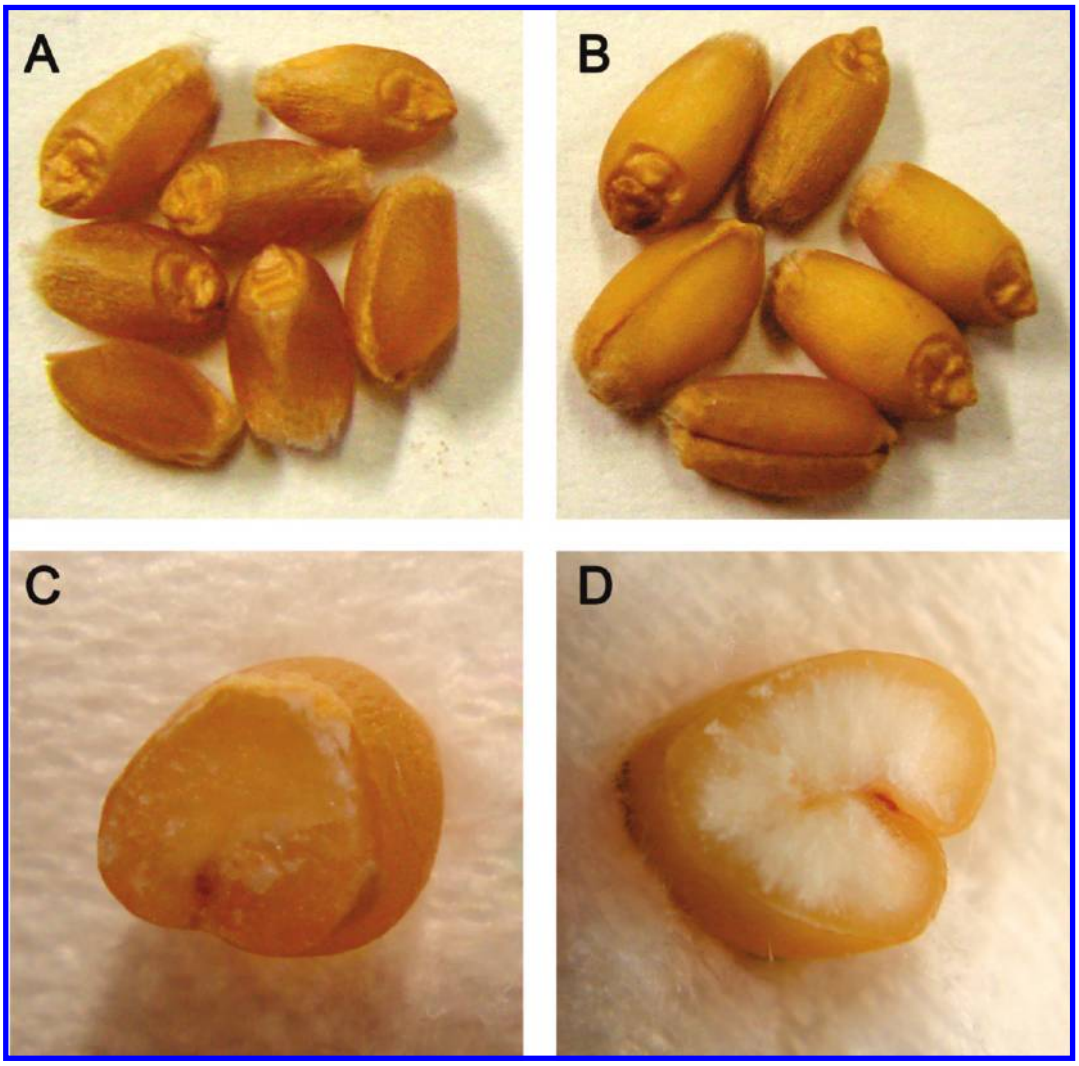

Figure 5. Morphological comparison between transgenic and nontransgenic seeds. (**A**) Seeds from cotransformed plants; (**B**) untransformed control, seeds from nontransgenic EM12 cultivar plants; (**C**) the endosperm of transgenic line; and (**D**) the endosperm of nontransgenic plant.

Table 2. Transgene Copy Number, Expression, and Carotenoid Content in the Transgenic Lines

	transgene copy numbers[a]		relative transcript levels[b]			
transgenic line (T_0)	*y1* gene	*crtI* gene	*y1* gene	*crtI* gene	average dry weight per seed (g)	average total carotenoids (±SD[c]) (μg g^{-1} dry weight)[d]
EM12[e]	0	0	0	0	0.035	0.81 (±0.125)
B1-1-3	≥3	≥4	0.28	NA	0.023	NA
B1-2-1	≥4	≥2	0.33	0.12	0.021	4.25 (±0.134)
B1-5-3	≥4	≥5	0.17	0.08	0.024	2.31 (±0.157)
B3-2-6	≥4	≥4	0.30	0.19	0.020	4.51 (±0.201)
B3-3-1	NA[f]	≥4	0.24	0.17	0.023	3.12 (±0.114)
B4-2-1	≥3	≥4	0.25	0.11	0.022	NA
B4-2-3	≥2	NA	NA	NA	0.020	3.89 (±0.120)
B4-5-3	≥5	≥2	0.27	0.15	0.027	4.96 (±0.148)

[a] Transgene copy numbers were determined by Southern blot hybridization. [b] Relative transcript levels were determined by RT-PCR. [c] Standard deviation. [d] Total carotenoids were determined by WSB method. [e] The wild-type EM12 variety is shown as the control. [f] NA, data not available.

To investigate whether carotenoid accumulation in transgenic wheat endosperm is due to a modification in this expression pattern of the wheat carotenoid biosynthetic genes, a similar set of experiments was carried out using the transgenic lines. The expression patterns of *y1* and *crtI* gene were detected in most of the transgenic lines and wild-type plants. Relative differences were also observed in *y1* and *crtI* expression between individual lines. Similar to the Southern blot analysis, these differences in expression did not correlate well with carotenoid content (**Table 2**). As expected, expression of endogenous *PSY1* was detectable at very low levels in wild-type endosperms as well as all of the transgenic lines (**Figure 7**). The expression of transgenic maize *y1* gene was only detectable in the endosperm of transformed lines, which confirmed the tissue specificity of the *1Dx5* promoter. Overexpression of *y1* and *crtI* did not appear to alter the expression of the endogenous carotenoid genes *PSY1*, *PSY2*, *PDS*, and *ZDS* when compared to the nontransgenic lines.

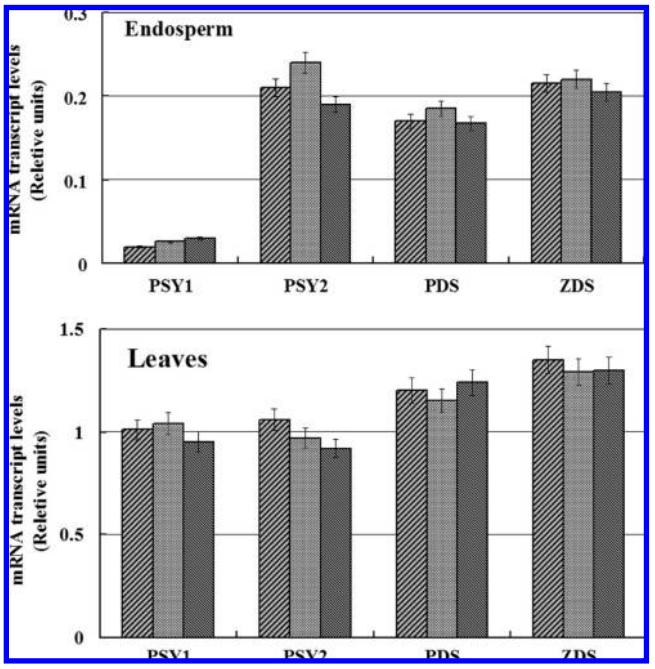

Figure 6. Semiquantitative RT-PCR analysis of the expression pattern of wheat genes involved in carotenoid biosynthesis in wild-type wheat plants. The expression patterns of PSY1, PSY2, PDS, and ZDS were assayed using RNA preparations from immature endosperm and from leaves. Differently shaded bars each represent RNA of different individual plants by three independent assays.

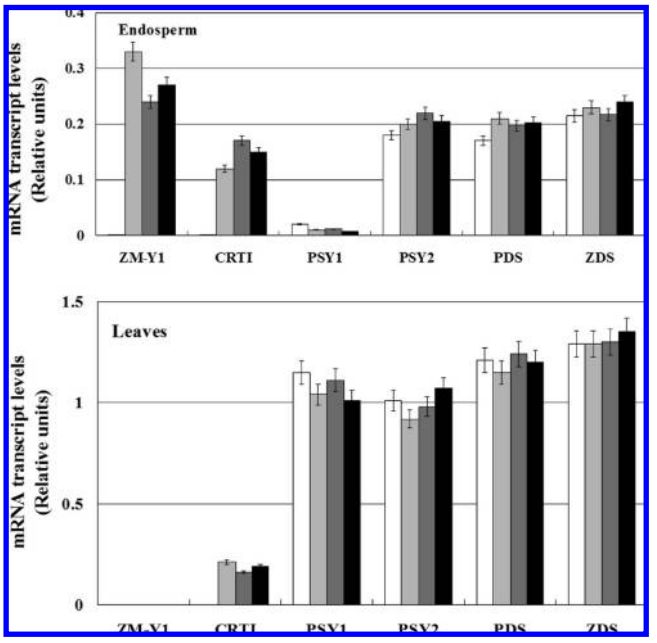

Figure 7. Semiquantitative RT-PCR analysis of the expression pattern of wheat genes involved in carotenoid biosynthesis in transgenic wheat plants. The expression patterns of PSY1, PSY2, PDS, and ZDS, even maize *y1* (ZM-Y1) and bacterial *crtI* gene, were all assayed using RNA preparations from immature endosperm and leaves. White bars, wild-type wheat line; light gray bars, transgenic line B1-2-1; dark gray bars, transgenic line B3-3-1; and black bars, transgenic line B4-5-3. The histogram represents the average expression levels of determinations from three independent experiments.

DISCUSSION

Experiments in rice revealed that overexpression of exogenous *PSY* gene results in the accumulation of phytoene (*20*). In the Golden Rice and provitamin A-rich maize experiments, the *Erwinia crtI* gene was expressed coupled with phytoene synthase gene (bacterial *crtB* or plant *PSY*) to enhance carotenoid level in seeds (*21*, *22*, *24*, *45*). This strategy was also used in our studies, which express the maize PSY1 (*y1*) gene in endosperm-specific manner, as well as with the *E. uredovora crtI* gene in the constitutive manner. Our studies show that overexpression of both *y1* and *crtI* is necessary to enhance carotenoid levels in wheat. Endosperm-specific overexpression of maize *y1* gene alone did not result in enhanced carotenoid content (data not shown).

The maize *y1* gene was introduced into wheat with an endosperm-specific *1Dx5* promoter in all experiments. This promoter had been employed previously in modifications of gluten quality though expressing several glutenin proteins (*38*, *46*). The effect of endosperm-specific *1Dx5* promoter in increasing the carotenoid content in wheat was also evaluated here. Most of the individual transgenic lines containing maize *y1* gene under the control of the *1Dx5* promoter had generated yellow seeds with significantly higher carotenoid contents when compared to the wild-type EM12 variety (**Figure 5** and **Table 2**). However, this experimental design is not appropriate to assess whether the *1Dx5* promoter is a stronger promoter, as multiple transgene copies and positional effects might play a role in the levels of expression observed in the stable transformants. Transgenic wheat lines with single copy insertions of promoter−reporter gene constructs might be better suited to evaluate the relative strength of this promoter. The stable inheritances of the maize *y1* gene in wheat and its spatial and temporal expression in the seeds during the maturation process (**Figure 7**) have led to a 10.8-fold increase in endosperm carotenoid contents (**Table 2**). The small amount of variation within individual line ($< 10\%$) suggests that increases above 5-fold are genuine elevations and not a consequence of biological variation. In comparison with high carotenoid-containing wheat varieties from conventional breeding programs, most of the maize *y1* lines had a higher total carotenoid content than common wheat varieties (ranging from 2.9 to 3.4 $\mu g\ g^{-1}$ seed dry weight), which have been analyzed (*47*).

We have investigated the expression pattern of *y1* and *crtI* in transgenic wheat lines and found that the bacterial *crtI* gene driven by the constitutive *CaMV 35S* promoter expressed in endosperms and leaves of transgenic plants, albeit at the lower levels than *y1* gene (**Figure 7**). The low expression levels observed with *crtI* gene are most likely caused by the employment of the constitutive *CaMV 35S* promoter, which may be less active in the endosperm than the endosperm-specific *1Dx5* promoter. Alternatively, inefficient translation may be considered to be caused by differences in codon usage between *Erwinia* and plants. This hypothesis has been verified in rice endosperm, where the codon optimization of a bacterial *crtI* resulted in a significant increase of the corresponding transcript level (*48*). Similarly, the codon adaption of a bacterial (1,3-1,4)-β-glucanase dramatically increased the corresponding protein level in barley (*49*). However, the enhanced carotenoid content indicated that the *Erwinia* enzyme CRTI is active in wheat endosperm.

The effects of introduced maize PSY and bacterial CRTI on the expression of critical endogenous carotenoid biosynthesis genes in wheat leaves and endosperms have also been investigated. The barely detectable alteration of the expression patterns of the endogenous carotenoid genes (*PSY1*, *PSY2*, *PDS*, and *ZDS*) had been found in transgenic wheat lines (**Figure 7**). The results suggest that, in transgenic lines, the yellow color of wheat is not due to the up-regulation of the endogenous wheat carotenoid biosynthetic pathway. These results are consistent with studies in

Golden Rice (*50*) but not with those overexpressing CRTI in tomato (*35*) and maize (*24*).

Some early research results confirmed that the overexpression of transgenic carotenoid genes under the control of constitutive promoter could lead to different changes in morphological traits and leaves carotenoid content. For example, the constitutive overexpression of PSY in tomato causes a dwarf phenotype (*51*), and in potato, a clear negative correlation was observed between tuber and leaf carotenoid levels when *CrtI* was under *CaMV 35S* promoter control (*23*). Even in rice, the constitutive overexpression of *CrtI* also led to the decrease in lutein in leaves (*50*). However, changes caused by constitutive expression of *E. uredovora CrtI* gene were not seen here (data not shown).

Many different approaches have been taken to increase carotenoids content in crop plants, such as canola, tomato, potato, rice, and maize by manipulating various genes of the carotenoid pathway (*16*, *18*, *23*, *24*, *35*, *37*). This high transformation efficiency will facilitate metabolic engineering in important food crops. The enhancement of carotenoid synthesis in common wheat represents a promising example for successfully engineering a nutritionally important biochemical pathway in wheat quality improvement. The results of the present research provide valuable reference for improving wheat nutritional quality through genetic engineering.

ABBREVIATIONS USED

CRTB, phytoene synthase (bacterial); CRTI, phytoene desaturase (bacterial); CRTISO, carotenoid isomerase; CTAB, cetyl trimethyl ammonium bromide; GGPP, geranylgeranyl pyrophosphate; LCYB, lycopene β-cyclase; LCYE, lycopene ε-cyclase; PCR, polymerase chain reaction; PDS, phytoene desaturase (plant); PSY, phytoene synthase (plant); QTL, quantitative trait loci; WSB, water-saturated *n*-butyl alcohol; ZDS, ζ-carotene desaturase; ZISO, ζ-carotene isomerase.

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