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RESEARCH PAPER

Generation of transgenic maize with enhanced provitamin A content

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Abstract

Vitamin A deficiency (VAD) affects over 250 million people worldwide and is one of the most prevalent nutritional deficiencies in developing countries, resulting in significant socio-economic losses. Provitamin A carotenoids such as β-carotene, are derived from plant foods and are a major source of vitamin A for the majority of the world's population. Several years of intense research has resulted in the production of 'Golden Rice 2' which contains sufficiently high levels of provitamin A carotenoids to combat VAD. In this report, the focus is on the generation of transgenic maize with enhanced provitamin A content in their kernels. Overexpression of the bacterial genes *crtB* (for phytoene synthase) and *crtI* (for the four desaturation steps of the carotenoid pathway catalysed by phytoene desaturase and ζ-carotene desaturase in plants), 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. The levels attained approach those estimated to have a significant impact on the nutritional status of target populations in developing countries. The high β-carotene trait was found to be reproducible over at least four generations. Gene expression analyses suggest that increased accumulation of β-carotene is due to an up-regulation of the endogenous lycopene β-cylase. These experiments set the stage for the design of transgenic approaches to generate provitamin A-rich maize that will help alleviate VAD.

Key words: β-carotene, CRTB, CRTI, γ-zein promoter, lycopene β-cyclase, provitamin A carotenoids, provitamin A-rich maize, vitamin A deficiency (VAD).

Introduction

Carotenoids are C_{40} polyenes that are abundant in fruits, vegetables, and green plants (reviewed in Olson, 1989; Howitt and Pogson, 2006). 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 post-translationally (Fig. 1) (reviewed in Hirschberg, 2001; Cunningham, 2002; Fraser and Bramley, 2004; Howitt and Pogson, 2006). The key regulatory step of the pathway is mediated by phytoene synthase (PSY) and involves the condensation of two geranylgeranyl pyrophosphate (GGPP) to form 15-*cis*-phytoene, a colourless C_{40} compound. Phytoene is converted to all-*trans*-lycopene (a red pigment) by four desaturation reactions (mediated by phytoene desaturase, PDS, and ζ-carotene desaturase, ZDS) and by an isomerization reaction (mediated by CRTISO). Lycopene is cyclized by ε and/or β-cyclase to give rise to the yellow-orange pigments, β-carotene (with two β-ionone rings) and α-carotene (with one ε-ionone ring and one β-ionone ring). Alpha- and β-carotene are subsequently hydroxylated and modified to form the various xanthophylls.

Carotenoids function in plant tissues as accessory pigments in photosynthesis, as attractants for seed dispersal and pollination, as precursors of some scents and of the growth regulator ABA, and as antioxidants (reviewed

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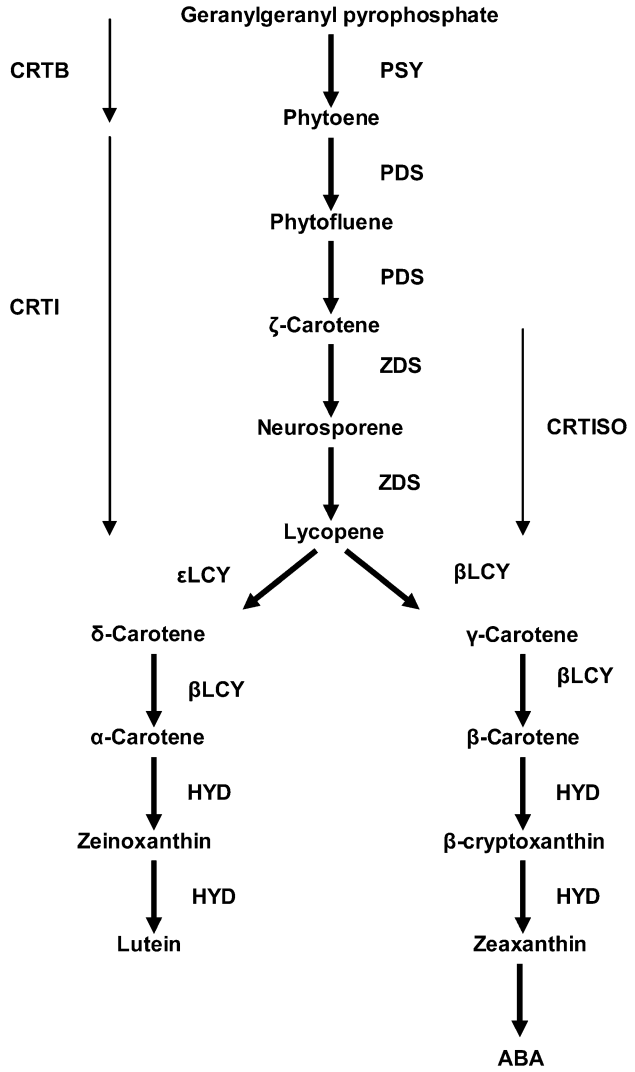

Fig. 1. Carotenoid biosynthetic pathway in maize. PSY, phytoene synthase; PDS, phytoene desaturase; ZDS, ζ-carotene desaturase; CRTISO, carotenoid isomerase; βLCY, β-cyclase; εLCY, ε-cyclase; HYD, carotene hydroxylases; CRTB, bacterial homologue of PSY; CRTI, bacterial homologue of PDS and ZDS.

in Hirschberg, 2001; Cunningham, 2002; Fraser and Bramley, 2004; Howitt and Pogson, 2006). Whereas carotenoid function is dependent on plastid and cell type, their role as antioxidants appears to be ubiquitous. This role is perhaps best understood in chloroplasts, where desaturated (coloured) carotenoids quench triplet chlorophyll and singlet oxygen (produced during photosynthetic light capture), preventing the formation of reactive oxygen species (ROS) and photo-oxidation of the contents of the organelle (reviewed in Niyogi, 1999). Mammals do not synthesize carotenoids *de novo* and thus they must be ingested in the diet. Of the ~700 carotenoids found in nature, 20–50 are common in the human diet and about 20 are found in human blood and tissues (Johnson, 2004). Dietary carotenoids have received considerable attention because they have been implicated in preventing various eye and cardiovascular diseases, as well as several types of cancer and other age-related diseases, probably via their role as antioxidants and/or as regulators of the immune system (reviewed in Fraser and Bramley, 2004; Johnson, 2004).

Carotenoids with unsubstituted β-ring end groups, such as α-carotene, β-carotene, and β-cryptoxanthin, have provitamin A activity. β-carotene has twice the activity of the others because it has two unsubstituted β-rings. Provitamin A carotenoids are cleaved in the intestinal lumen to produce retinal (vitamin A). The efficiency of bioconversion depends on a number of factors (e.g. the nature of the food matrix that is ingested), and bioefficacies are significantly lower in developing countries than in developed countries (West *et al*., 2002). Vitamin A is an essential micronutrient for human health, and the World Health Organization estimates that greater than 100 million children worldwide have vitamin A deficiency (VAD) (www.who.int/vaccines-diseases/en/vitamina/science/sci01.shtml). Nearly all of these cases are in developing countries whose populations rely on a single staple crop for their sustenance. It has been estimated that half of all VAD cases become severe and result in blindness and death.

Attempts to modify the carotenoid content of seeds have focused on seed-specific manipulation of various steps in the carotenoid pathway. Overexpression of the bacterial *crtB* (for PSY) in the oilseeds of canola led to ~50-fold increase in total carotenoids (Shewmaker *et al*., 1999). These increases occurred mainly in α- and β-carotene. Using an endogenous *PSY* gene, enhanced seed-specific accumulation of α- and β-carotene was also achieved in *Arabidopsis*, but unlike canola, there was less flux into α-carotene versus other carotenoids, primarily lutein and violaxanthin (Shewmaker *et al*., 1999). In rice endosperm, which lacks provitamin A, overexpression of the daffodil *PSY* led to the production of phytoene (Burkhardt *et al*.,1997), but when coupled with expression of the bacterial *crtI* gene (which mediates the four desaturation reactions) and/or the daffodil lycopene β-cyclase gene (*LCYB*), there was enhanced accumulation of lutein, β-carotene, and zeaxanthin (Ye *et al*., 2000). These lines served as the prototype for 'Golden Rice' (Al-Babili and Beyer, 2005). Whereas the β-cyclase gene appeared to be dispensable in these experiments, it was suggested that the source of PSY might be limiting, and thus different *PSY* genes were tested for their ability to be expressed in rice endosperm. These experiments led to the production of 'Golden Rice 2' (GR2), which expresses maize *PSY* and *crtI* (Paine *et al*., 2005). GR2 accumulates ~23-fold more provitamin A than the prototype plants. While efforts to enhance total and provitamin A carotenoids have been successful, they have raised important questions regarding the regulation of the carotenoid biosynthetic pathway.

The health benefits of yellow maize have long been recognized (Mangelsdorf and Fraps, 1931), and yellow maize varieties have been the target of breeding strategies for nearly a century (reviewed in Poneleit, 2001). Maize has two *PSY* genes, *Y1* and *PSY2*. *Y1* and *PSY2* are expressed in leaves, embryo, and endosperm. However, the role of *PSY2* in endosperm appears to be limited because yellow varieties have functional *Y1* genes, while white varieties such as Hi-II carry a loss-of-function *y1* allele (Buckner *et al*., 1996; Palaisa *et al*., 2003; Gallagher *et al*., 2004). It has been hypothesized that yellow maize arose from ancestral white varieties by gain-of-function events associated with insertion events in the promoter of *Y1* prior to domestication ~10,000 years ago (Palaisa *et al*., 2003).

In devising a strategy to enhance the provitamin A content of maize, one question that must be addressed is how much is needed to alleviate VAD. This is related to the question of bioavailability, or 'the fraction of an ingested nutrient that becomes available to the body for utilization in physiological functions or for storage' (Jackson, 1997; Fraser and Bramley, 2004). There are numerous factors that influence bioavailability, including nutrient status of the host, species of carotenoid, food matrix and amount of food consumed in the meal. Taking these factors into account, nutritionists have estimated a goal of 15 μg provitamin A g^{-1} dry weight of kernel (www.harvestplus.org). Assuming a child's reasonable portion to be 200 g dry maize d^{-1}, this provitamin A concentration in the kernel would enable a daily intake approximating 50% of the US Institute of Medicine Estimated Average Requirement (EAR) for vitamin A (http://www.iom.edu).

Maize is an important staple crop, with world production averaging over 600 million metric tons in 2003 (http://faostat.fao.org). However, the traditional yellow maize varieties have low amounts of provitamin A ranging from 0.25 μg to 2.5 μg g^{-1} dry weight. In Sub-Saharan Africa, maize is the most important food crop contributing an estimated 328 kilocalories per capita per day, and globally, Africa accounts for 25–35% of cases of child and maternal vitamin A deficiency (West, 2002). Therefore, the nutritional improvement of maize for provitamin A content would have a significant impact on the target populations.

Conventional breeding techniques over the years have led to the isolation of a few high β-carotene maize lines that approach the target of 15 μg g^{-1} of provitamin A g^{-1} dry weight kernel, with the highest β-carotene rich line having a maximum of 13.6 μg g^{-1} of provitamin A g^{-1} dry weight kernel (Kurilich and Juvik, 1999; Islam, 2004; Harjes *et al*., 2008). Such breeding techniques are useful in exploiting variation in natural populations. On the other hand, transgenic approaches can be used as tools to complement conventional breeding techniques in meeting the estimated levels of provitamin A in target countries. Moreover, transgenic approaches allow for a comprehensive understanding of the regulation of the carotenoid pathway, which in turn, might provide important information for further manipulation of the pathway by either conventional or transgenic approaches.

A transgenic approach to enhance the provitamin A content of maize kernels is reported here. To our knowledge, this is the first such attempt for metabolic engineering of carotenoids to enhance provitamin A content in maize. Our rationale was based on the successful experiments from the 'Golden Rice 2' project. However, it was not clear whether these attempts to manipulate the carotenoid pathway in maize would be successful because of the differences between rice and maize. Although rice and maize endosperm tissues have very similar physiological functions, rice seeds are much smaller than maize kernels, and there are significant differences between the transcriptomes of rice and maize endosperm (Lai *et al*., 2004). It is shown here that maize seeds can be metabolically engineered to produce high levels of provitamin A comparable to 50% EAR values by overexpressing the bacterial *crtB* and *crtI* genes in an endosperm-specific manner, using a modified and highly active γ-zein promoter.

Materials and methods

Construction of plasmids

Plasmid pRC4 contains the endosperm-specific 27 kDa γ-zein promoter, a tobacco etch virus 5′ untranslated region (TEV), an *Nco*1/*Sac*I site for cloning genes of interest and a soybean vegetative storage protein terminator (Tvsp) (Chikwamba *et al*., 2002). Plasmids pMON75555 and pMON75574 served as the source of the coding sequence(s) of the *Erwinia herbicola crtB* and *crtI* genes, respectively (kindly provided by the Monsanto Corporation). The *crtB* and *crtI* genes in these plasmids are fused to the transit peptide (TP) of the pea gene for the small subunit of Rubisco (*rbc*S). The *rbc*S TP-*crtB* gene was amplified from pMON75555 using primers CRTB-For: 5′-CCCACATGTCTTCTATGATATCC-TCTTCCG-3′ and CRTB-Rev: 5′-CAGCTATGACCATGATTAC-GC-3′. The 1219 bp PCR fragment was cloned into the *Nco*I/*Sac*I site of pRC4 to generate plasmid pRB. The 1753 bp *rbc*S TP-*crtI* coding sequence was amplified using the primers CRTI-For: 5′-CATGCCATGGCTTCTATGATATCCTCTTC-3′ and CRTI-Rev, and inserted into pRC4 to generate plasmid pRI. To generate the plasmids pRBS and pRIS, the γ-zein promoter was modified and cloned in a two-step process resulting in duplication of the -444/-174 region. First, a 692 bp fragment was amplified from pRC4 using primers pRC4-208C: 5′-TCACCATGGGCTATCGTTCG-3′ and zein-444N: 5′-CCCAAGCTTCCTCTCTGTGTGCAAAGAAA-3′. This fragment was cloned into the *Hin*dIII and *Xho*I sites of pRB and pRI followed by insertion of a 287 bp fragment amplified from pRC4 with primers zein-444N and zein-174C: 5′-CCCAAGCTTT-ACTTCTGCGTGGCTCAGTT-3′. This fragment was inserted into the *Hin*dIII site of the pRB and pRI plasmids. The resulting pRBS and pRIS plasmid constructs were sequenced to verify that the manipulations did not introduce errors.

Transformation and regeneration of maize

Immature zygotic embryos of the Hi-II germplasm were transformed with plasmids pRB, pRI, pRBS, and pRIS in two different combinations, using a standard protocol for biolistic transformation at the Iowa State University Plant Transformation Facility (Frame *et al*., 2000). Plasmid pBAR184 carries the *Streptomyces hygroscopicus* phosphinothricin acetyltransferase gene (*bar*) under the control of the maize ubiquitin promoter and allows for selection of bialophos-resistant transformation events (Frame *et al*., 2000). The two different plasmid combinations were: pBAR184+pRB+pRI and pBAR184+pRBS+pRIS. In brief, plasmid DNA was introduced into immature embryos via microprojectile bombardment using the PDS 1000/He biolistic gun (Bio-Rad, Hercules, CA). Transformants were selected based on their resistance to the herbicide, bialaphos. Genomic DNA was isolated from transformed calli as previously described (Chikwamba *et al*., 2002), and transgene integration was confirmed for all bialaphos-resistant callus lines by PCR using gene-specific primers.

Transgenic plants were regenerated from approximately 20 of the positive events for each transformation, and the regenerated plants (T_0) were crossed with Hi-II to generate T_1 seeds. To generate T_2, T_3, and T_4 seeds, the most intensely coloured, yellow/orange seeds from T_1, T_2, and T_3 transgenic lines were selected and planted. The mature plants were then self-pollinated.

Analysis of carotenoid composition by HPLC

Carotenoids were extracted from dried kernels at 40 DAP (days after self-pollination) using a modification of the method of Granado *et al*. (2001) as previously described by Li *et al*. (2007). Approximately 20 kernels were ground to a fine powder. A 1 g aliquot of the powder was incubated with 6 ml of methanol containing 0.1 g butylated hydroxytoluene (BHT) l^{-1} while stirring for 15 min at 50 °C. After cooling, an equal volume of tetrahydrofuran (THF) was added and the carotenoids were extracted by vigorous mixing. A 0.5 ml aliquot of the methanol/THF extract was then saponified by adding 1.0 ml of 40% potassium hydroxide in methanol containing 0.1 M pyrogallol followed by vigorous mixing for 3 min. The potassium hydroxide was removed by washing with 2 ml of water. After addition of an internal standard, β-apo-8′-carotenal (Sigma-Aldrich, St Louis, MO) in methanol, the carotenoids were partitioned into 5 ml of hexane/methylene chloride (5:1 v/v containing 0.1 g l^{-1} BHT). The upper phase was dried under vacuum. The dried extract was reconstituted with 100 µl methyl-*tert*-butyl ether (MTBE) followed by 300 µl of methanol. A 100 µl aliquot was injected into the HPLC system. The components included a 717 Plus autosampler with temperature control set at 5 °C, two 510 solvent-delivery systems, and the 996 photodiode array detector (Waters Corporation, Milford, MA). The system operated with Empower Pro Software Version 1 (Waters Corporation). Carotenoids were separated on a 5 µm C30 Carotenoid Column (4.6×250 mm; Waters Corporation) eluted by a mobile-phase gradient from 100% methanol (containing 1 g ammonium acetate l^{-1}) to 100% MTBE over 60 min. The flow rate was 1.0 ml min^{-1}. Solvents were HPLC grade; the THF was stabilized with BHT. Calibration standards for α-carotene, β-cryptoxanthin, δ-carotene, lutein, lycopene, phytoene, zeaxanthin, and α-cryptoxanthin/zeinoxanthin were purchased from Carotenature (Lupsingen, Switzerland); β-carotene was purchased from Fluka Chemical Corp (Milwaukee, WI) These standards, and the β-apo-8′-carotenal internal standard, were used to generate internal standard calibration curves. Due to the low isomeric purity of commercially available standards for phytofluene, the phytofluene concentrations in the maize kernels were extrapolated from the phytoene calibration curve, as previously described (Barr *et al*., 2004).

Determination of transgene copy number

Genomic DNA was isolated from frozen leaves of T_2 and T_4 plants following previously described methods (Dietrich *et al*., 2002). Genomic DNAs from maize germplasms B73 and Hi-II were used as negative controls. Approximately 10 µg of genomic DNA was digested with *Xho*I to determine copy number of the transgene(s). The digested DNA fragments were electrophoretically resolved on a 0.8% agarose gel and immobilized onto a nylon membrane (Perkin-Elmer Life Sciences, Inc). DNA fragments of *crtB* (0.8 kb) and *crtI* (1 kb) from plasmids pRB and pRI were used as probes. Blotting, hybridization, washes, and detection were performed as previously described by Wetzel *et al*. (1994).

RNA extraction and RT-PCR analysis

Total cell RNA was isolated from developing transgenic T_4 maize seeds at 23 DAP according to the method described in Wetzel and Rodermel (1998). The isolated RNA was treated with RNase-free DNase (Life Technologies, Rockville, MD, USA) to remove genomic DNA contamination and RNA quality was assessed by gel electrophoresis. Following first-strand cDNA synthesis (Superscript ® first-strand cDNA synthesis kit; Life Technologies), PCR amplification was performed using gene-specific primers. Primers were designed using the Primer3 software (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). The PCR primers used to amplify maize carotenoid biosynthesis genes are as follows: *PSY* (gil1098664 Forward 5′-TGGGCCAAACGCCAACTACATT-3′, Reverse 5′-ACAACAACAGGGAAGCCCAT-3′); *PDS* (gil2707976; Forward 5′-TGCCAAACAAGCCAGGAGAA-3′, Reverse 5′-AGCAAAG-ACCAACTCCAGCA-3′); *ZDS* (gil56462565; Forward 5′-ATGC-AGTTGCTCTTGCCCTT-3′, Reverse 5′- AACCGCCCTTTGCA-GTTGTCTT-3′); *LCYB* (gil27728514; Forward 5′-CTCTACGC-CATGCCCTTCT-3′, Reverse 5′-CCAGTAGTGTGGCTCCAGGT-3′); *HYD1* (gil61393909, Forward 5′-CATCTCCCTCCTCGCC-TAC-3′, Reverse 5′-CTCCACAGACCATCCGAAAT-3′); *HYD3* (gil61393917, Forward 5′-ACGTGTTCGCCATCGTCAA-3′, Reverse 5′-AACTCATTTGGCACACTCTGGC-3’).

The PCR reactions were carried out using approximately 1 µg of total RNA, 10 µM of each primer, 200 µM dNTPs, and 2 units of *Taq* polymerase (Invitrogen). After an initial denaturation step for 2 min, the PCR reactions were performed for 31 cycles including 1 min of denaturation at 94 °C, followed by 1 min of annealing at 58 °C and finally 1 min of extension at 72 °C. RT-PCR experiments were repeated twice with different RNA extractions from 3–5 separate T_4 seed samples. 18S *rRNA* was used as control.

Results

Maize transformation

To assess the effects of *crtB* and *crtI* overexpression on carotenoid accumulation in Hi-II maize, four different plasmid constructs were generated (schematically represented in Fig. 2). Plasmids pRB and pRI contain the bacterial *crtB* and *crtI* genes, respectively, fused to the N-terminal transit peptide of the pea gene for the small subunit of Rubisco (*rbc*S). The transit peptide specifies plastid targeting (in this case, to endosperm amyloplasts); it is removed from the precursor during or shortly after import into the organelle. The coding sequences were placed under the control of the endosperm-specific 27 kDa γ-zein promoter (Marks *et al*., 1985; Yang *et al*., 2002).

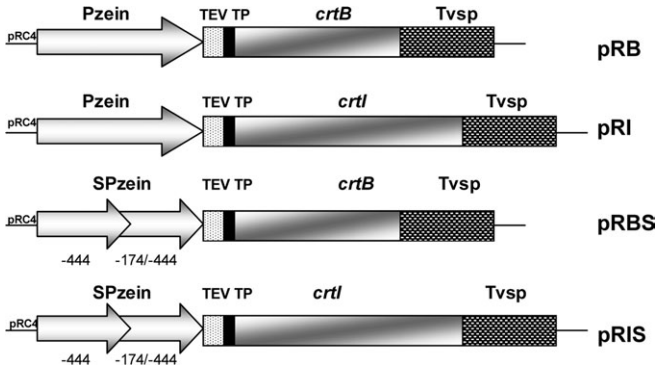

Fig. 2. Constructs used in the maize transformations. Four constructs were generated: pRB, pRI, pRBS, and pRIS. The core plasmid was pRC4 (see Materials and methods). Pzein, 27 kDa γ-zein promoter; SPzein, 'super 27 kDa γ-zein promoter' obtained by repeating the -444/-174 region; TEV, tobacco etch virus 5′ untranslated region; TP, transit peptide from pea Rubisco small subunit (*rbc*S); *crtB*, *Erwinia herbicola* phytoene synthase; *crtI*, *E. herbicola* phytoene desaturase; Tvsp, soybean vegetative storage protein terminator.

Plasmids pRBS and pRIS contain a 'super γ-zein promoter', which was obtained by duplication of the -444/-174 region of the 27 kDa γ-zein promoter. This duplication results in enhanced promoter activity in transient expression assays using maize endosperm tissue (Marzabal *et al*., 1998), and there was a need to test whether this promoter functions *in planta*.

For transformation, immature zygotic Hi-II embryos were particle-bombarded with the pRB, pRI, pRBS, and pRIS constructs in two different combinations: pRI+pRB and pRIS+pRBS. Hi-II was chosen because it is a white kernel germplasm amenable for transformation, and it was hoped that successful transformation events could easily be detected by a change in colour of white Hi-II kernels to yellow/orange.

Transformed callus lines were selected based on their resistance to the herbicide, bialaphos (conferred by the *bar* gene); prior to analysis, individual clones were subcultured several times. Chromosomal DNAs were isolated from the resistant lines and screened for the presence of the transgene(s) by PCR analysis. Of 660 embryos that were co-transformed with pRB and pRI, 41 calli were resistant to bialaphos and 20 of these were positive for the presence of both *crtB* and *crtI*. To obtain pRBS and pRIS co-transformed callus lines, 870 embryos were bombarded and 58 bialaphos-resistant events were identified; 30 of these were PCR-positive for the *crtB* and *crtI* sequences.

Plantlets were regenerated from 20 independently transformed callus lines for each set of constructs, and the plants were grown to maturity in the greenhouse. Because of poor synchronization of male and female flowers from transformed T_0 plants, T_1 progeny were obtained by backcrossing the T_0 plants (as females) with pollen from Hi-II (white maize).

Analysis of T_1 kernels

The hypothesis was that successful transformations would give rise to yellow kernels on transgenic Hi-II ears. Therefore, as a first approach to assess the effect of individual *crtB* and *crtI* gene expression in the maize endosperm, the phenotypes of ears from 31 regenerated plantlets that had been backcrossed with Hi-II were observed. These ears were derived from 13 different callus lines (five of pRB+pRI and eight of pRBS+pRIS). The T_1 progeny from ears of all the regenerated (T_0) plants from the 'super γ-zein promoter' transformants (pRBS+pRIS) had yellow and white seeds (Fig. 3A). However, the T_1 progeny from ears of the pRB+pRI transformed maize lines resembled Hi-II, and only one of these lines (line 15) had pale yellow and white seeds (data not shown). Chi-square analysis (P=0.05) showed that the yellow and white seeds from four of the pRBS+pRIS lines (lines 24, 25, 37, and 49) segregated in a 1:1 ratio, suggesting the presence of at least one functional copy of each gene in the genome. Seeds from lines 27 and 48 segregated in 3:1 ratio for white and yellow, respectively.

To examine carotenoid contents of the T_1 seeds in greater detail, the progeny of six of the eight pRBS+pRIS transformed lines (lines 24, 25, 27, 37, 48, and 49) with the most intensely yellow/orange seeds were analysed. It should be noted that the transgenic T_0 ears had fewer T_1 kernels than the Hi-II parent line, with the number of kernels ranging from 40–150 per ear (Fig. 3B). Approximately 40–70% of these seeds were white while the rest were yellow/orange. Carotenoid contents were not examined for T_1 seeds from ears of the pRB+pRI callus lines because, with one exception (line 15), they were white. The ears from line 15, however, had very few seeds; these were used for planting and generating T_2 seeds. Carotenoid analyses on the T_2 progeny from this line are described below.

Detailed HPLC analyses were performed to determine carotenoid content and composition of kernels from individual maize lines. Approximately 20 yellow/orange transgenic maize seeds per ear at 40 DAP were needed for extraction of carotenoids and HPLC analysis. Because of the lack of adequate amounts of seed material, it was not possible to perform replicate HPLC analyses on individual transgenic maize lines. The different carotenoids measured/identified by HPLC are as follows: lutein, zeaxanthin, zeinoxanthin, lycopene, δ-carotene, α-carotene, β-cryptoxanthin, 13-*cis*-β-carotene, 15-*cis*-β-carotene, *trans* β-carotene, 9-*cis*-β-carotene, phytoene, and phytofluene.

Figure 3C illustrates the total carotenoid (sum of all carotenoids measured) and provitamin A (sum of α-carotene, β-cryptoxanthin, 13-*cis*-β-carotene, 15-*cis*-β-carotene, *trans* β-carotene, 9-*cis*-β-carotene) contents of T_1 seeds from the six pRBS+pRIS lines. Hi-II and B73 (yellow maize inbred) are shown as controls. The provitamin A contents of the T_1 seeds were variable, ranging

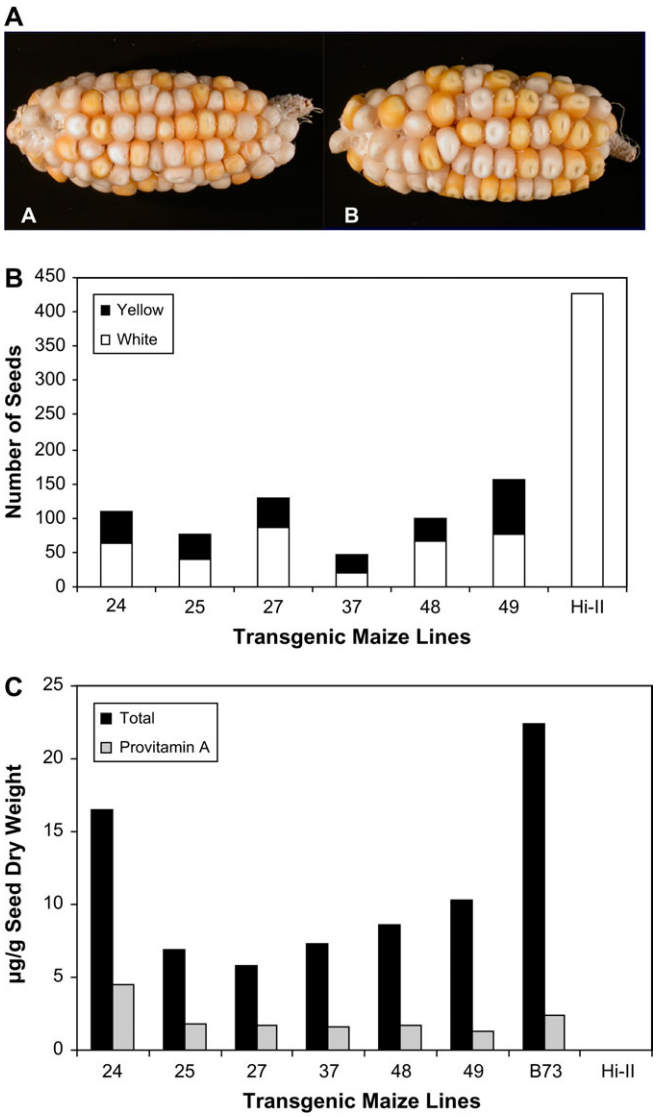

Fig. 3. Analysis of T_1 kernels. (A) Representative ears from transformed plants with enhanced provitamin A content. The *crtB*/*crtI* cotransformants were regenerated from callus and crossed with Hi-II (white maize). There is segregation of white and yellow in the T_1 kernels. The lines are 48 (A) and 49 (B). (B) Total number of T_1 seeds on ears from six individual transgenic maize lines.The number of yellow/orange and white seeds on each ear are shown. (C) Total carotenoid and provitamin A content of T_1 seeds from transformed plants. HPLC analysis was performed on bulked samples of 20 T_1 seeds from each T_0 ear. The parental plants were the pRBS/pRIS regenerants from the transformed callus lines crossed with Hi-II. The amount of total carotenoid (in µg g^{-1} dry weight of the seed) is equal to the sum of all carotenoids, and the provitamin A carotenoids were calculated as the sum of α-carotene, β-cryptoxanthin, 13-*cis*-β-carotene, 15-*cis*-β-carotene, *trans* β-carotene, 9-*cis*-β-carotene contents present in the extracted sample.

from 1–4.5 µg g^{-1} seed dry weight. Compared with Hi-II that had an undetectable level of coloured carotenoids (as expected), the greatest increase in total carotenoids was observed in line 24, which had over half the amount of total carotenoids as B73. Yet, line 24 had a higher provitamin A content than B73 (approximately 4.5 µg g^{-1} versus 2 µg g^{-1}), indicating an enhanced flux into the provitamin A pool in the transformants (~30% of the total carotenoid pool is provitamin A in line 24 versus 10% in B73). This difference in partitioning was evident in the other five lines, as well.

In summary, analyses of T_1 seeds show that overexpression of bacterial *crtB* and *crtI* under the control of the 'super γ-zein promoter', but not the γ-zein promoter, results in significantly higher levels of provitamin A content in maize endosperm when compared to the parent Hi-II as well as the yellow maize inbred, B73.

Variability in the provitamin A content of transgenic maize seed

To generate T_2 seeds, 5–6 yellow/orange T_1 seeds were planted from each of lines 24, 25, 27, 4, and 49 (Fig. 3B), resulting in T_1 plants 24-1, 24-3, 24-4, etc. Each of these T_1 plants was then self-pollinated. T_1 seeds from line 37 were not used because of the paucity in number of seeds available for further analyses. Approximately 20 yellow seeds per individual line from the resulting T_2 ears were then bulked and detailed HPLC analysis was performed for carotenoid content and composition. Great variability was observed in carotenoid content among the T_2 kernels from individual ears of each line (Fig. 4A). However, in all pooled samples (e.g. 24-1 + 24-3 +…+ 24-6), the mean carotenoid contents were similar to or higher than in the T_1 seeds in Fig. 3C; the variation in provitamin A content between lines ranged from 2 µg g^{-1} to 13.8 µg g^{-1} kernel dry weight.

HPLC analyses were also conducted on T_2 progeny seeds from one of the pRB+pRI lines (the pale-yellow line, line 15). The T_2 progeny from this line contained segregating white and yellow kernels, and the kernels had a greater variability in colour than the T_1 seeds on the parent plant. These experiments revealed that total provitamin A levels ranged from 2–4 µg g^{-1} seed dry weight of bulked T_2 seeds (data not shown).

The HPLC analyses in Fig. 4A revealed that transgenic lines derived from lines 24 and 25 had the highest provitamin A content amongst all of the T_2 ears examined (details are provided in Supplementary Table S1 at *JXB* online). Therefore, the focus was on these lines for analysis and further experimentation. Figure 4B shows the average carotenoid contents of the five individual lines from lines 24 and 25 (Fig. 4A) for each of the major carotenoid species. These analyses show that, in comparison to Hi-II, transgenic lines 24 and 25 have increased levels of all carotenoid species. While lutein, zeaxanthin, and zeinoxanthin are the major carotenoids in B73, the provitamin A carotenoids such as β-cryptoxanthin, α-carotene, and β-carotene form a minor proportion of the total. In the transgenic lines 24 and 25, lutein, zeaxanthin, and zeinoxanthin are also the major carotenoids, however, there is a 2–3-fold reduction in the proportion of lutein. This appears to be compensated for by increased levels of phytoene and β-carotene in both

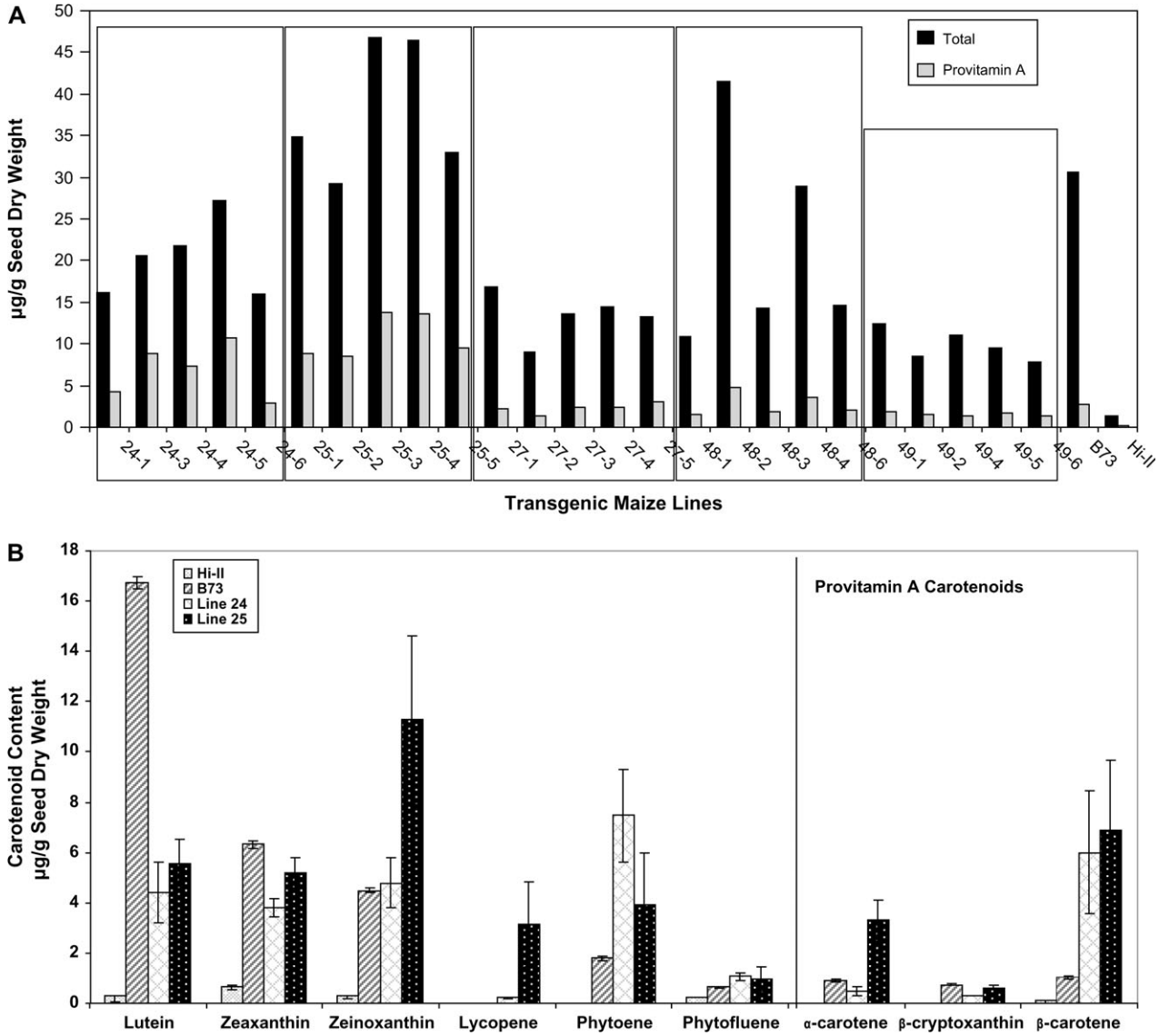

Fig. 4. Analysis of T_2 kernels. (A) Total carotenoid and provitamin A contents of transgenic T_2 maize lines. The transgenic T_1 plants in Fig. 3B were self-pollinated, and HPLC analysis was conducted on pooled samples of 20 T_2 seeds from the resulting ears. The amount of total carotenoid is equal to the sum of all carotenoids and the provitamin A carotenoids were calculated as the sum of α-carotene, β-cryptoxanthin, 13-*cis*-β-carotene, 15-*cis*-β-carotene, *trans* β-carotene, 9-*cis*-β-carotene contents present in the extracted sample. (B) Carotenoid composition of T_2 seeds from transgenic lines derived from lines 24 and 25. Data represent the average carotenoid content (±SD) of seeds from five individual ears per line.

lines. The increase in β-carotene is approximately 7–13-fold when compared to the yellow maize, B73. However, there are some differences in carotenoid compositions between lines 24 and 25. In particular, line 25 appears to accumulate enhanced levels of zeinoxanthin, lycopene, and α-carotene versus line 24.

To determine whether the high β-carotene trait could be stably transmitted, the T_3 and T_4 generation seeds were evaluated by planting 5–6 yellow/orange T_2 and T_3 seeds from transgenic lines 24 and 25 that showed the highest β-carotene accumulation in the T_2 seeds (24-3, 24-5, 25-3, and 25-4 from Fig. 4A). The T_2 and T_3 plants were self-pollinated and yellow/orange seeds from the resulting ears were used for detailed HPLC analyses. Figure 5 shows the average provitamin A contents of T_3 and T_4 seeds derived from lines 24-3, 24-5, 25-3, and 25-4. As in the T_2 generation, variability was observed between individual lines and between generations with provitamin A contents ranging from 4–7 µg g^{-1} seed dry weight of bulked T_3 seeds in individual ears from line 24, and 1–2 µg g^{-1} seed dry weight in line 25. Provitamin A levels in T_4 seeds from parent line 24 were found to be more variable and ranged from 2.5–10 µg g^{-1} seed dry weight of bulked T_4 seeds whereas for T_4 seeds from line 25, the provitamin A content ranged from 5–7 µg g^{-1} seed dry weight of bulked T_4 seeds.

In summary, the data show that the high β-carotene trait, resulting from the overexpression of *crtB* and *crtI,* can be transmitted in transgenic maize, but that there is a great degree of variability amongst individual lines and between different generations.

Increase in lycopene β-cyclase expression correlates with increase in β-carotene content in transgenic maize

To test whether the variability in carotenoid content was due to differences in transgene copy number, Southern blot analysis of genomic DNA was performed on low and high β-carotene lines from T_2 as well as T_4 generation plants from lines 24 and 25. The *crtI* and *crtB* gene sequences from plasmids pRB and pRI were used as probes. The probes did not hybridize to Hi-II DNA but produced several detectable bands from lanes containing transgenic maize lines. As expected for plants transformed via particle bombardment, Southern blot analysis of T_2 and T_4 plants showed that the transgenic lines had a variable transgene copy number, ranging from 8–12 copies for both *crtB* and *crtI*. In this study, copy numbers did not correlate well with carotenoid content (Table 1). For instance, transgenic line 24-5-9-1 contains at least eight copies of *crtB* and 10 copies of *crtI* with a pro-vitamin A content of 9.8 μg g^{-1} seed dry weight, whereas line 25-3-5-1 contains at least 12 copies each of *crtB* and *crtI* and has 2.1 μg g^{-1} seed dry weight provitamin A content.

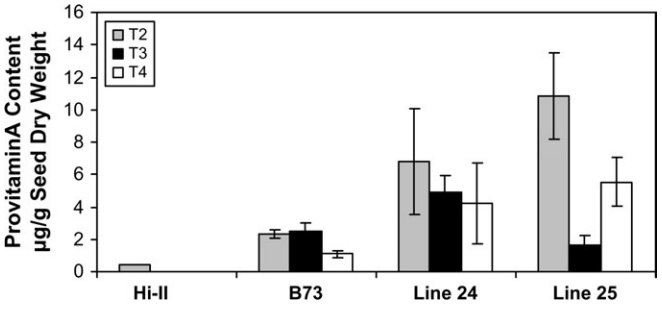

Fig. 5. Provitamin A content of T_2, T_3, and T_4 seeds from transgenic maize lines 24 and 25. HPLC analyses were conducted on pooled samples of 20 T_2, T_3, and T_4 seeds of individual maize lines, respectively. Data shown represent the average provitamin A content (±SD) of seeds from 3–8 ears for each line.

To understand the variability in the maize lines further and to determine the basis for the increased accumulation of β-carotene in these lines, the expression of *crtB* and *crtI*, and representative carotenoid biosynthesis genes in T_4 seeds of transgenic maize lines 24 and 25, were analysed by semi-quantitative RT-PCR. These included genes that encode enzymes known to mediate regulatory steps of the pathway, as well as the poorly characterized β-carotene hydroxylases *HYD1* and *HYD3*.

crtB and *crtI* expression was detected in most of the transgenic lines analysed. Relative differences were also observed in *crtB* and *crtI* expression between individual lines; similar to the Southern blot analyses, these differences in expression did not correlate well with carotenoid content (Table 1). As expected, expression of *PSY* was undetectable in Hi-II as well as in all the transgenic lines since they have the Hi-II background (Fig. 6). Over-expression of *crtB* and *crtI* did not appear to alter the expression of the endogenous *PDS* gene when compared to the parent Hi-II. *HYD1* mRNA levels remained unaltered in lines 24 and 25. There appeared to be a slight reduction in the expression of *ZDS* and β-carotene hydroxylase (*HYD3*), but the expression of the

Table 1. *Transgene copy number, expression, and carotenoid content in transgenic lines 24 and 25*

Transgenic line (T_4 generation)	Transgene copy numbers[a]		Relative transcript levels[b]		Total carotenoids (μg g^{-1} dry seed weight)[c]	Provitamin A (μg g^{-1} dry seed weight)[c]
	crtB	*crtI*	*crtB*	*crtI*		
Hi-II[d]	0	0	0	0	1.01	0.39
24-3-10-2	>8	>10	2.35	1.06	11.0	3.1
24-3-10-3	>8	>10	1.77	2.31	9.2	2.7
24-3-10-4	>8	>10	0.67	0.9	9.6	2.3
24-3-10-5	>8	>10	1.8	0.9	17.4	5.2
24-5-9-1	>8	>10	0.72	1.59	33.6	9.8
24-5-9-3	>12	>12	NA[e]	NA	13.3	3.5
24-5-9-5	>12	>12	NA	NA	17.1	4.0
25-4-9-2	>12	>12	0.25	0.56	26.4	7.0
25-4-9-9	>12	>12	NA	NA	16.9	5.6
25-3-5-1	>12	>12	0.5	0.35	6.6	2.1

[a] Transgene copy numbers were determined by Southern blot hybridization.
[b] Relative transcript levels (normalized to 18S rRNA) were determined by reverse transcriptase (RT)-PCR.
[c] Total carotenoids (sum of all carotenoid contents measured) and provitamin A contents (sum of α-carotene, β-cryptoxanthin, 13-*cis*-β-carotene, 15-*cis*-β-carotene, *trans* β-carotene, 9-*cis*-β-carotene) were determined by HPLC analysis.
[d] Hi-II is shown as a control.
[e] NA, data not available.

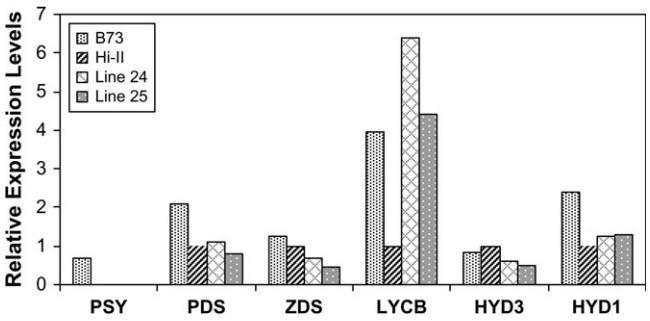

Fig. 6. Maize carotenoid gene expression. Total RNA was isolated from T_4 kernels of lines 24 and 25. Transcript levels were measured by RT-PCR using gene-specific primers and the data were normalized using 18S rRNA accumulation as a control. Histogram represents average expression levels of determinations from two separate experiments conducted with pooled 3–4 seeds for each line.

endogenous lycopene β-cylcase (*LCYB*) was dramatically increased in both lines compared with Hi-II.

Discussion

The 'super γ-zein' promoter enhances provitamin A content in transgenic maize

Experiments in rice and maize revealed that overexpression of bacterial *crtB* and/or plant *PSY* genes results in the accumulation of phytoene (Burkhardt *et al*., 1997; G Barry, unpublished data). To test whether accumulated phytoene can serve as the source of enhanced levels of provitamin A in maize, the strategy was to overexpress the *E. herbicola crtI* gene, as well as with the *crtB* gene, in an endosperm-specific manner. This strategy was used in the prototype 'Golden Rice' experiments (Ye *et al*., 2000; Al-Babili and Beyer, 2005). Our studies show that overexpression of both *crtB* and *crtI* is necessary to enhance provitamin A levels in maize. Endosperm-specific overexpression of *crtB* alone did not result in enhanced provitamin A content (data not shown).

The effect of two different endosperm-specific promoters (γ-zein and 'super γ-zein' promoter) in increasing the provitamin A carotenoid content in maize was also evaluated. Most of the T_1 lines (4/5) obtained after transformation with *crtB* and *crtI* under the control of the γ-zein promoter had white kernels with undetectable levels of carotenoids. On the other hand, all of the individual T_1 lines containing *crtB* and *crtI* genes under the control of the 'super γ-zein promoter' had segregating white and yellow/orange seeds with significantly higher carotenoid contents when compared to the parent Hi-II. In addition, T_1 transgenic lines containing pRBS and pRIS constructs had increased total as well as provitamin A contents when compared to the parent Hi-II (Fig. 3C).Thus, our studies corroborate previous transient expression studies in maize (Marzabal *et al*., 1998) and provide critical *in planta* data to show that the modified 'super γ-zein promoter' is sufficient for metabolic engineering of carotenoids in maize. However, this experimental design is not appropriate to assess whether the 'super γ-zein promoter' is a stronger promoter than the γ-zein promoter, as multiple transgene copies and position effects might play a role in the levels of expression observed in the stable transformants. Transgenic maize lines with single copy insertions of promoter-reporter gene constructs might be better suited to evaluate the relative strength of this promoter.

Variation in carotenoid content of transgenic maize lines

Previous studies revealed that T_1 seeds from individual transgenic lines in the 'Golden Rice' project varied greatly in their carotenoid content and composition (Hoa *et al*., 2003; Paine *et al*., 2005). In addition, some of the T_2 generation plants had higher carotenoid levels than the T_1 parents. As in rice, a significant degree of variability in carotenoid content was observed between individually transformed T_2, T_3, and T_4 lines. This variability was not due to differences in the number of transgenes, as both high and low β-carotene lines had similar numbers of *crtB* and *crtI* genes (Table 1). In the T_2 generation seeds, the variability ranged from 2–13.5 μg g^{-1} seed dry weight (Fig. 4A). T_3 seeds from lines 24 and 25 had surprisingly low levels of provitamin A (Fig. 5), although the provitamin A levels rebounded in T_4 to levels somewhat comparable (10 μg g^{-1} seed dry weight for line 24 and 7 μg g^{-1} seed dry weight for line 25) to those found in T_2 seeds from the same parent transgenic lines. The fact that bulked seeds were used for these analyses indicates that provitamin A levels were higher than the 7–10 μg g^{-1} seed dry weight in some of the kernels. These results suggest that variability is manifested not only in different generations but also in individual seeds of maize from a single ear. Some of this variability could be due to the germplasm used for transformation and/or to epigenetic effects. Other researchers have reported similar variation in transgenic maize and proceeded with the selection of lines that showed the highest levels of expression for subsequent generations (Chikwamba *et al*., 2002). Nevertheless, our studies indicate that the high β-carotene trait is heritable and can be maintained through generations.

Increase in lycopene β-cyclase transcript levels correlate with enhanced provitamin A content in transgenic maize

CRTI has been shown to be capable of desaturating large amounts of phytoene in transgenic rice (Paine *et al*., 2005). However, the enhanced accumulation of phytoene (4–5-fold, Fig. 4B) in transgenic maize suggests that bacterial CRTI is rate-limiting and/or CRTI activity is insufficient to desaturate all of the phytoene accumulating as a result of CRTB overexpression. These results also suggest that the carotenoid pathway can be further manipulated at the level

of PDS and ZDS in these transgenic lines. On the other hand, it is possible that sufficient levels of CRTI are present in the transgenic plants, but that additional phytoene is not accessible to CRTI and thus regulation through metabolite chanelling might limit the conversion of phytoene to lycopene. Such metabolite compartmentation of phytoene has been observed in other transgenic plant species (Fraser *et al*., 1999).

HPLC analyses also revealed that there is a 3-fold reduction in total lutein content, and an approximately 10-fold increase in the β-carotene content in both transgenic maize lines 24 and 25, with a preferential accumulation of *trans* β-carotene (see Supplementary Table S1 at *JXB* online). Our findings are consistent with previous observations in rice, tobacco, tomato, and canola showing that there is preferential accumulation of β-carotene when *PSY* (or *crtB*) and *crtI* are overexpressed (Misawa *et al*., 1993; Romer *et al*., 2000; Ye *et al*., 2000; Lindgren *et al*., 2003; Paine *et al*., 2005). In the case of *crtI*, the preferential formation of β, β carotenoids is due to the isomerization state of lycopene (all-*trans* for *crtI* versus poly-*cis* for the *PDS* and *ZDS*-catalysed steps in plants) (Park *et al*., 2002). It is suggested that the same holds true for *crtI* expression in maize.

It is worth noting that the increases in β-carotene in our transgenics were not matched by corresponding increases in the xanthophylls. An exception to this is the enhanced accumulation of zeinoxanthin in line 25 (Fig. 4B). This means that the proportion of β-carotene increased in the transgenic plants relative to xanthophylls in the β, β branch. This hypothesis was verified by RT-PCR analysis of select transgenic lines (Fig. 6). The results suggest that, in transgenic lines overexpressing the bacterial *crtB* and *crtI* genes, the endogenous maize carotenoid pathway is regulated at the level of lycopene β-cyclase and perhaps, at the level of β-hydroxylase leading to higher levels of β-carotene and lower levels of xanthophylls in the transgenic lines. These results are consistent with those in transgenic tomato plants overexpressing CRTI (Romer *et al*., 2000), but not with studies from 'Golden Rice' where they showed that the yellow colour of rice was not due to the up-regulation of endogenous carotenoid genes (Schaub *et al*., 2005). In addition, the reason(s) for varied levels of different carotenoids between individual lines of 24 and 25 (for example, enhanced accumulation of lycopene in line 25 versus line 24; Fig. 4B) are not obvious as both of these lines appear to have similar increases in *LCYB* expression. Further protein and metabolite flux analyses will be necessary to explain this phenomenon satisfactorily.

Recent studies with high β-carotene lines isolated via conventional breeding approaches showed that enhanced accumulation of β-carotene in these lines was due to a high degree of natural variability in the lycopene epsilon-cyclase (*LCYE*) gene, which alters the flux through the β, ε branch of the pathway and, thus the accumulation of β-carotene and lutein in the maize kernels (Harjes *et al*., 2008). Although the expression of all endogenous maize carotenoid biosynthesis genes have not been analysed, the fact that α-carotene and lutein are detected in the maize transgenic lines suggests that endogenous *LCYE* is active, but perhaps not to the extent that *LCYB* is active.

Many different approaches have been taken to increase provitamin A content in crop plants, such as tomato, potato, and rice, by manipulating various genes of the carotenoid pathway (Romer *et al*., 2002; Ducreux *et al*., 2005; Diretto *et al*., 2006; Sandmann *et al*., 2006). However, 'Golden Rice 2' is the only monocot that has been shown to accumulate substantial amounts of provitamin A to meet the daily requirements to overcome VAD. In 'Golden Rice 2' (Paine *et al*., 2005), modification of the *PSY* step by plant genes led to a significant enhancement in the provitamin A content in transgenic rice. Based on our studies, modification of *crtI* as well as *PSY* seem to be promising next steps to boost kernel provitamin A content in maize. In conclusion, the present results represent an important first step in the generation of high provitamin A maize to combat VAD in developing countries using metabolic engineering approaches. Coupled with classical breeding, the transgenic approach should be a powerful tool to combat VAD.

Supplementary data

Supplementary data can be found at *JXB* online.

Table S1. Carotenoid content and composition of T_2 seeds from transgenic maize lines 24 and 25 as determined by HPLC analysis.

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