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ORIGINAL PAPER

Functional characterization of the *Gentiana lutea* zeaxanthin epoxidase (*GlZEP*) promoter in transgenic tomato plants

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Abstract The accumulation of carotenoids in plants depends critically on the spatiotemporal expression profiles of the genes encoding enzymes in the carotenogenic pathway. We cloned and characterized the *Gentiana lutea* zeaxanthin epoxidase (*GlZEP*) promoter to determine its role in the regulation of carotenogenesis, because the native gene is expressed at high levels in petals, which contain abundant chromoplasts. We transformed tomato (*Solanum lycopersicum* cv. Micro-Tom) plants with the *gusA* gene encoding the reporter enzyme β-glucuronidase (GUS) under the control of the *GlZEP* promoter, and investigated the reporter expression profile at the mRNA and protein levels. We detected high levels of *gusA* expression and GUS activity in chromoplast-containing flowers and fruits, but minimal levels in immature fruits containing green chloroplasts, in sepals, leaves, stems and roots. *GlZEP-gusA* expression was strictly associated with fruit development and chromoplast differentiation, suggesting an evolutionarily-conserved link between ZEP and the differentiation of organelles that store carotenoid pigments. The impact of our results on current models for the regulation of carotenogenesis in plants is discussed.

Keywords *Gentiana lutea* · Zeaxanthin epoxidase · Promoter · β-Glucuronidase · Transgenic tomato · Carotenoid · Chromoplast

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Introduction

Carotenoids are abundant isoprenoid pigments produced by all photosynthetic organisms as well as certain non-photosynthetic bacteria and fungi (Goodwin 1980). In chloroplasts, carotenoids are accessory light-harvesting pigments that protect the photosynthetic apparatus from photo-oxidation (Frank and Cogdell 1996; Demmig-Adams and Adams 2002). They also act as precursors for the plant hormones abscisic acid (ABA) (Creelman and Zeevart 1984) and strigolactone (Gomez-Roldan et al. 2008; Umehara et al. 2008). Chromoplasts are specialized plastids

found in flowers and fruits that have adapted to store carotenoids. The accumulation of carotenoids confers a range of pigmentation in the yellow-orange-red spectrum that attract animals and therefore facilitate the dispersal of pollen and seeds (Bartley and Scolnik 1995).

There is significant interest in the regulation of carotenoid biosynthesis in plants because of their health-promoting antioxidant activity (Kloer and Schulz 2006) and the specific nutritional importance of carotenoids such as β-carotene (Von Lintig and Vogt 2004; Giuliano et al. 2008; Farre et al. 2010; Bai et al. 2011). However, this has shifted attention away from the key roles that carotenoids play in the continuation of the plant life cycle by attracting pollinating insects and herbivores that distribute seeds. Therefore, relatively little is known about the regulation of carotenoid biosynthesis in petals and fruits, and the link between carotenoid synthesis and chromoplast differentiation.

Gentiana lutea flowers contain large amounts of lutein, violaxanthin, antheraxanthin and β-carotene (Zhu et al. 2003). The chromoplasts in *G. lutea* petals originate either from pre-existing fully-developed chloroplasts or from immature proplastids (He et al. 2002). There is a strong temporal correlation during flower development between the accumulation of carotenoids and the formation of chromoplasts, which coincides with the induction of carotenogenic gene expression (Zhu et al. 2002, 2003). Zeaxanthin epoxidase (ZEP) catalyzes the conversion of zeaxanthin to violaxanthin via antheraxanthin, and is therefore the key enzyme responsible for the accumulation of antheraxanthin and violaxanthin in *G. lutea* petals (Zhu et al. 2003). ZEP is also the first committed enzyme in the ABA biosynthesis pathway (Marin et al. 1996; Seo and Koshiba 2002). Expression profiling in *G. lutea* has shown that *GlZEP* mRNA is abundant in fully-developed petals that contain mature chromoplasts but only minimal amounts are present in younger petals that still contain chloroplasts, and in leaves and stems (Zhu et al. 2003; and unpublished data). Steady-state *GlZEP* mRNA levels increase 1.8-fold between the hard bud stage (S1) and the fully-open flower stage (S5) (Zhu et al. 2003).

To gain insight into the regulation of *GlZEP* during petal development and chromoplast differentiation, we isolated the *GlZEP* promoter and evaluated different constructs for their activity in transgenic tomato plants by fusing them to the *gusA* reporter gene. Histochemical GUS assays revealed that a construct containing 677 bp of the *GlZEP* upstream promoter were sufficient to confer strong GUS activity in chromoplast-rich tissues but not in tissues containing chloroplasts, similar to the expression profile of the native gene in *G. lutea*. These data indicate that the 677-bp *GlZEP* promoter contains evolutionarily-conserved sequences that confer high level expression in chromoplast-rich tissues.

Materials and methods

Plant material

Gentiana lutea leaves, stems and flowers were obtained from the Hokkaido Experimental Institute of Health Science (Japan). The tissues were frozen in liquid nitrogen immediately after harvesting and then stored at −80°C.

Tomato (*Solanum lycopersicum* cv. Micro-Tom) plants were grown in the greenhouse at 25°C with a 16-h photoperiod. The leaves, stems, roots, flowers and fruits of wild-type and T4 homozygous transgenic plants were used for histochemical GUS assays immediately after harvesting, or were frozen in liquid nitrogen and stored at −80°C until required for GUS staining or *gusA* mRNA profiling. Fruits were harvested at four different stages: immature green (IG), mature green (MG), orange and ripe red.

Isolation of genomic DNA and RNA

Genomic DNA was extracted from 5 g of leaf tissue as described by Sambrook et al. (1989). Total RNA was extracted using TRIZOL® Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and DNA was digested with RNase-free DNase I (QIAGEN, Valencia, CA, USA). Total RNA was quantified using a NANODROP 1000 spectrophotometer (Thermo Scientific, Vernon Hills, Illinois, USA).

Cloning of the *GlZEP* promoter

Gentiana lutea genomic DNA (20 μg) was completely digested with *Ban*II and ligated using 10 Weiss units of T4 DNA Ligase (Invitrogen, Carlsbad, CA) to generate circular molecules. These were used as templates for amplification of the *GlZEP* promoter

region by long accurate (LA) PCR with the Takara LA PCR kit (Takara, Shuzo, Japan), using forward primer FP1 (5′-CCC TAA ACC CTT CAA CAT CAC TGG TTT CAA GAT TCC-3′, positions +311 to +346 where position +1 is the first nucleotide of *ZEP* cDNA) and reverse primer RP1 (5′-GAA TGA GAG CCA ATC CAA GGA CAT GAA GCA GCA CCA-3′, positions +119 to +154) based on the GenBank *GlZEP* cDNA sequence (accession number EF203254). The product was transferred to vector PCR® II TOPO® (TA Cloning Kit, Invitrogen, Carlsbad, CA) for sequencing using the Big Dye Terminator v3.1 Cycle Sequencing Kit on a 3130 × 1 Genetic Analyzer (Applied Biosystems, Foster City, CA).

Promoter-GUS constructs

GlZEP promoter fragments were fused to the *gusA* gene in vector pBI101 (Clontech Laboratories, Mountain View, CA, USA) (Jefferson et al. 1987). The full-length *GlZEP* promoter region was amplified from *G. lutea* genomic DNA using forward primer 5′-GTC GAC CCT TAA TGG CGG TAA TTA TGT TCT GTT ATC-3′ (positions −2225 to −2194; *Sal*I restriction site underlined) and reverse primer 5′-GGA TCC TAA TCC AAT TAC AAA AGA GTG AAA AGA-3′ (positions −27 to −1; *Bam*HI restriction site underlined). The 2,225-bp amplified promoter fragment was transferred to the PCR® II TOPO® vector using the Invitrogen TA Cloning® kit, to generate plasmid pCR-GlZEPPro. Both plasmids (pCR-GlZEPPro and pBI101) were digested with *Sal*I and *Bam*HI, allowing the GlZEPPro fragment to be inserted upstream of *gusA* in the pBI101 vector, yielding the final construct pBI-GlZEPPro-GUS (*Zep-gusA*).

Three 5′ deletions of the *GlZEP* promoter region were also created by PCR. Primers 5′-GTC GAC CCT TAA TGG CGG TAA TTA TGT TCT GTT ATC-3′ (positions −2225 to −2195) and reverse primer 5′-GGA TCC TTC TTG CTT CAA TTT AGT TAC AAT TTG CTA G-3′) (positions 252–283) were used to amplify the full-length *GlZEP* promoter with 5′-UTR for pBI-GlZEPPro-5UTR-GUS (*Zep5utr-gusA*). Then forward primers D1 (5′-GTC GAC TTA TGA GTA CCG AGG TAT GCC TT-3′) (positions −1709 to −1684), D2 (5′-GTC GAC GAG TGC AGG TCT GTT ACA GTC AG-3′) (positions −1134 to −1109) and D3 (5′-GTC GAC GAT TCG AAT TGA GCG AAT AGT C-3′) (positions −677 to −655) were combined with reverse primer (5′-GGA TCC TAA TCC AAT TAC AAA AGA GTG AAA AGA-3′) (positions −27 to −1) to generate the three stepwise deletions, with *Sal*I and *Bam*HI restriction sites as discussed above. The amplified promoter was transferred to PCR®II TOPO® then pBI101 upstream of *gusA* using the strategy described above. The vectors were named pBI-GlZEPProD1-GUS (*D1709-gusA*), pBI-GlZEP-ProD2-GUS (*D1134-gusA*) and pBI-GlZEPProD3-GUS (*D677-gusA*). The integrity of all intermediate and final constructs was confirmed by sequencing.

Transient expression of promoter-GUS constructs in tomato

Plasmids pBI101, pBI121 (*35s-gusA*), pBI-GlZEPPro-GUS (*Zep-gusA*), pBI-GlZEPPro-5UTR-GUS (*Zep5utr-gusA*), pBI-GlZEPProD1-GUS (*D1709-gusA*), pBI-GlZEPProD2-GUS (*D1134-gusA*) and pBI-GlZEPProD3-GUS (*D677-gusA*) were transferred to *Agrobacterium tumefaciens* strain LBA 4404 by electroporation (Mattanovich et al. 1989). Individual colonies were seeded into 5-ml aliquots of YEM medium (0.5% beef extract, 0.1% yeast extract, 0.5% peptone, 0.5% sucrose, 2 mM $MgSO_4$, pH 7.2) containing 50 μg/ml kanamycin and 25 μg/ml rifampicin, and were shaken at 300 rpm, 28°C overnight. Each culture was then used to inoculate 50 ml induction medium (YEM medium supplemented with 20 μM acetosyringone, 10 mM MES, pH 5.6) containing 50 μg/ml kanamycin and 25 μg/ml rifampicin, incubated as above. Bacteria were recovered by centrifugation (2,700×*g*), resuspended in infiltration medium (10 mm $MgCl_2$, 10 mM MES, 200 μM acetosyringone, pH 5.6) to an OD_{600} of ∼1.0, and then incubated at room temperature with gentle agitation (20 rpm) for 3 h. Approximately 600 μl of the infiltration medium was then injected into fruits at the mature green (MG) stage (25–30 days after anthesis) through the stylar apex using a 1-ml syringe with needle (Orzaez et al. 2006). Injected fruits were left on the vine for 3 days, and then harvested and sectioned for histochemical staining.

Stable tomato transformation

Tomato stems from 1-month-old sterile plants were transformed using the procedure described by Pfitzner (1998). Briefly, 0.5–1 cm stems from plants growing

on sterile germination medium (MS salts containing 0.6% agar) were severed, placed on MSOZR medium (MS salts, MS Fe-EDTA, B5 vitamins and 30 g sucrose supplemented with 5 μM acetosyringone, 2 mg/l zeatin riboside and 0.6% agar) and pre-incubated for 24 h in a growth chamber (25°C, 16-h photoperiod). The stems were then dipped into the bacterial suspension in MSO medium, blotted on sterile paper and placed back on the same MSOZR plates. After 2 days in the growth chamber as above, the stems were transferred to plates containing selective shoot regeneration medium (MSOZR medium supplemented with 50 μg/ml kanamycin, 500 μg/ml carbenicillin and 0.6% agar) and incubated in the growth chamber as above for 2 weeks. The shoots were subcultured on fresh medium for another 2 weeks and then transferred to selective shoot regeneration medium (MSOZR medium supplemented with 50 μg/ml kanamycin, 250 μg/ml carbenicillin and 0.6% agar) to regenerate shoots from proliferating callus. Shoots up to 1 cm in length were excised from callus and transferred to 5 × 10 cm containers containing selective root medium (MSO medium supplemented with 1 mg/l zeatin riboside, 50 μg/ml kanamycin, 250 μg/ml carbenicillin and 0.6% agar). Plantlets with roots appeared after 2–3 weeks and were transferred to soil in the greenhouse (25°C, 16-h photoperiod). Transgenic tomato lines were advanced to the T_4 homozygous generation for promoter analysis.

Histochemical and fluorimetric GUS assays

Histochemical GUS assays were carried out according to Jefferson et al. (1987) with minor modifications. Leaves, flowers, hand-cut stem and root segments, and sectioned fruits at different developmental stages were incubated at 37°C overnight (12 h) in the dark in 1 mM X-Gluc (5-bromo-4-chloro-3-indolyl-β-D-glucuronide) in 100 mM sodium phosphate (pH 7.0), 10 mM EDTA, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 0.3% (v/v) Triton X-100 and 20% (v/v) methanol to eliminate endogenous GUS activity (Kosugi et al. 1990). After 12 h staining, tissues were destained in an ethanol series (50, 70, 80 and 95%) to remove chlorophyll, and then stored in 70% (v/v) ethanol, and photographed with a digital camera.

Fluorometric GUS assays were carried out as described by Jefferson et al. (1987) with minor modifications. Plant tissues (100 mg) were ground to powder under liquid nitrogen, dispersed in 0.8 ml extraction buffer (50 mM Na_2HPO_4 pH 7.0, 10 mM EDTA, 0.1% (v/v) sodium dodecanoyl(methyl)aminoacetate, 10 mM 2-mercaptoethanol and 0.1% (v/v) Triton X-100) and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant (250 μl) was mixed with 250 μl 2 mM 4-methylumbelliferyl-β-D-glucuronide (MUG) on ice and 250 μl was transferred immediately to a fresh tube containing 2 ml of GUS stop buffer (0.2 M Na_2CO_3) to serve as a control. GUS assays were performed at 37°C for 1 h before the reaction was stopped by adding 2 ml of GUS stop buffer. The released fluorescent product, 4-methylumbelliferone (MU), was measured on an FP-750 spectrofluorometer (JASCO, Germany) with excitation at 365 nm and emission at 455 nm. The protein content of extracts was determined as described by Bradford (1976). GUS enzyme activity was expressed in pmoles MU/h·μg of soluble protein. Each assay was carried out twice.

DNA blot analysis

Leaf genomic DNA (20 μg) was digested with *Eco*RI, fractionated by 0.8% (w/v) agarose gel electrophoresis and transferred to a positively-charged nylon membrane (Roche, Mannheim, Germany) according to the manufacturer's instructions. Nucleic acids were fixed by UV crosslinking and hybridized with a digoxigenin-labeled 512-bp *gusA* probe at 42°C overnight using DIG Easy Hyb buffer (Roche Diagnostics GmbH, Mannheim, Germany). The probe was synthesized by PCR using the PCR-DIG Probe Synthesis Kit (Roche, Mannheim, Germany), forward primer 5′-CCT GTA GAA ACC CCA ACC CGT GA-3′, reverse primer 5′-ACG CTG CGA TGG ATT CCG GCA TA-3′ and pBI121 as the template. The membrane was washed twice for 5 min in 2× SSC, 0.1% (w/v) SDS at room temperature, twice for 20 min in 0.2× SSC, 0.1% (w/v) SDS at 68°C, and then twice for 10 min in 0.1× SSC, 0.1% (w/v) SDS at 68°C. After immunological detection with anti-DIG-AP (Fab-Fragments Diagnostics GmbH, Germany) chemoluminescence generated by chloro-5-substituted adamantyl-1,2-dioxetane phosphate (CSPD) (Roche, Mannheim, Germany) was detected on Kodak BioMax light film (Sigma–Aldrich, St. Louis, USA).

Quantitative real-time PCR

First strand cDNA was synthesized from 2 μg total RNA using Ominiscript Reverse Transcriptase in a 20-μl total reaction volume following the manufacturer's recommendations (QIAGEN, Valencia, CA, USA). Quantitative real-time PCR was performed on a BIO-RAD CFX96^{TM} system using a 25-μl mixture containing 10 ng cDNA, 1× iQ SYBR Green Supermix (BIO-RAD) and 0.2 μM of each primer. For the amplification of *gusA* (GenBank accession no. U12639; Jefferson et al. 1987) we used forward primer 5′-CGT GGT GAT GTG GAG TAT TGC-3′ and reverse primer 5′-ATG GTA TCG GTG TGA GCG TC-3′. For the internal tomato *β*-actin control (GenBank accession no. U60482; Agarwal et al. 2009) we used forward primer 5′-GCT GGA TTT GCT GGA GAT GAT GC-3′ and reverse primer 5′-TCC ATG TCA TCC CAA TTG CTA AC-3′. To calculate relative expression levels, serial dilutions (0.2–125 ng) were used to produce standard curves for each gene. PCRs were performed in triplicate using 96-well optical reaction plates, comprising a heating step for 3 min at 95°C followed by 40 cycles of 95°C for 10 s, 57°C for 30 s and 72°C for 20 s. Amplification specificity was confirmed by melt curve analysis of the final PCR products in the temperature range 50–90°C with fluorescence acquired after each 0.5°C increment. The fluorescence threshold value and gene expression data were calculated using the CFX96^{TM} system software.

Results

Cloning of the *GlZEP* promoter

The *GlZEP* promoter was cloned by inverse PCR using cleaved and circularized *G. lutea* genomic DNA as the template and outward-facing primers based on the *GlZEP* cDNA sequence (GenBank accession number EF203254). After sequencing the resulting product, a 2,637-bp fragment was isolated directly from genomic DNA using gene-specific primers based on the new template. This fragment (Genbank accession number: EF203262) comprised 2,225 bp of the upstream promoter and 412 bp of the *GlZEP* cDNA. The 2,225 bp promoter fragment was designated the full-length *GlZEP* promoter, and position +1 was assigned to the first nucleotide of the *GlZEP* cDNA (Zhu et al. 2003). The PlantCARE database (Lescot et al. 2002, http://www.dna.affrc.go.jp/PLACE/signalscan.html) was used to identify putative *cis*-acting regulatory elements, revealing two potential TATA boxes at positions −72 and −84 as well as six CAAT boxes, which are known to play an important role in enhancing eukaryotic promoter efficiency (Table 1). We identified several elements that respond to light, including a GT1 motif, two box I motifs, three G-boxes, a GAG motif, four box 4 motifs (which form part of a conserved DNA module involved in light responsiveness) and a chs-CMA2a motif (Table 1). The multitude of light response elements is likely to regulate *GlZEP* expression according to day length and other cues involved in the control of flower development. We also identified several hormone/stress response elements including one ethylene response element (ERE), two CGTCA motifs (methyl jasmonate sensitive), two MYB binding sites involved in drought stress, a heat shock element, three Box-W1 motifs that responds to fungal elicitors, and a circadian control element (Table 1).

Transient expression of *GlZEP* promoter in tomato fruits using different promoter constructs

Tomato fruits at the mature green (MG) stage were injected with bacterial cultures carrying the vectors pBI101 (promoterless-*gusA*), pBI121 (*35s-gusA*) and *Zep-gusA*. Fruits were harvested 3 days later and transverse sections were stained for GUS activity. As expected, fruits expressing *gusA* controlled by the CaMV35S promoter (*35s-gusA*) or the full-length *GlZEP* promoter (*Zep-gusA*) showed substantial GUS activity (Fig. 1) whereas no GUS activity was detected in fruits containing the promoterless control vector pBI101 (Fig. 1).

The promoter was characterized in more detail by generating a series of 5′ stepwise truncations containing 1,709, 1,134 and 677 bp of upstream sequences respectively and inserting these fragments upstream of the *gusA* gene into vector pBI101. We also created a construct containing the full-length *GlZEP* promoter plus the 5′-UTR (*Zep5utr-gusA*). We evaluated the four new constructs by transient expression in tomato fruits as above, using the *35s-gusA* vector as a control. Histochemical GUS assay showed that all four constructs behaved in a similar manner in terms of expression patterns to the full-length *GlZEP* promoter (Fig. 1). The GUS activity (identified as blue color) of

Table 1 Putative *cis*-acting regulatory elements identified in the 2,225 bp *GlZEP* promoter region using the PlantCARE database (Lescot et al. 2002)

Function	*Cis*-element	Sequence	Position from cDNA	Origin of isolated promoter
Common *cis*-acting element in promoter and enhancer regions	CAAT-box	CAAT	−1957, −349, −221	*Hordeum vulgare*
		CAAAT	−2157, −1369	*Brassica rapa*
		CAATT	−1496	*Glycine max*
Cis-acting regulatory element involved in light responsiveness	Box I	TTTCA	−2160	*Pisum sativum*
		AA	−275	
	Box 4	ATTAAT	−599, −262, −237, −215	*Petroselinum crispum*
	chs-CMA2a	TCACT	−1567	*P. crispum*
		TGA		
	GAG-motif	AGAGAGT	−1068	*Arabidopsis thaliana*
	G-box	CACATGG	−1613	*Solanum tuberosum*
		CACGTC	−1585, −1355	*Zea mays*
	GT-1-motif	GCGGTA	−2217	*Oryza sativa*
		ATT		
Ethylene-responsive element	ERE	ATTTCAAA	−276	*Dianthus caryophyllus*
Cis-acting regulatory element involved in MeJA-responsiveness	CGTCA-motif	CGTCA	−2064, −1527	*H. vulgare*
MYB biding site involved in drought-inducibility	MBS	CAACTG	−1191, −997	*A. thaliana*
Cis-acting element involved in heat stress responsiveness	HSE	CNNGAANNTT	−2123	*Lycopersicon esculentum*
		CNNG		
Fungal elicitor responsive element	Box-W1	TTGACC	−2082, −746, −100	*P. crispum*
Cis-acting regulatory element involved in circadian control	Circadian	CAANNNNNATC	−822	*L. esculentum*

Zep5utr-gusA construct was slightly higher than that of the full-length *GlZEP* promoter (*Zep-gusA* construct) indicating that the 5′-untranslated region contains sequences necessary for high levels of expression. The GUS activities of *D1709-gusA* and *D1134-gusA* constructs were at a similar level compared to that of the full-length *GlZEP* promoter (*Zep-gusA*). The *D667-gusA* construct exhibited a slightly reduced GUS activity compared to that of the full-length *GlZEP*, *D1709-gusA* and *D1134-gusA* constructs (Fig. 1). Nevertheless the GUS expression pattern of the *D667-gusA* construct assessed histochemically exhibited similar expression to that of the full-length promoter indicating that all *cis*-acting elements necessary to confer high-level GUS activity in tomato fruits are contained within the proximal 677 bp of the *GlZEP* promoter sequence.

Histochemical analysis of GUS activity in stably transformed tomato plants

Tomato plants were stably transformed with two of the constructs described above: the full-length *GlZEP* promoter-GUS fusion (*Zep-gusA*) and the positive control pBI121 (*35s-gusA*). Histochemical GUS assays were carried out on 12 primary transformants expressing *Zep-gusA* and eight expressing *35s-gusA*. In the *Zep-gusA* plants, GUS activity was detected in fruits from the mature green stage onwards, but not in leaves, sepals, petals or immature green (IG) fruits. The distribution of GUS activity was the same in all 12 independent lines, although the intensity differed significantly. In contrast, GUS activity was detected in all the tissues of all eight *35s-gusA* plants.

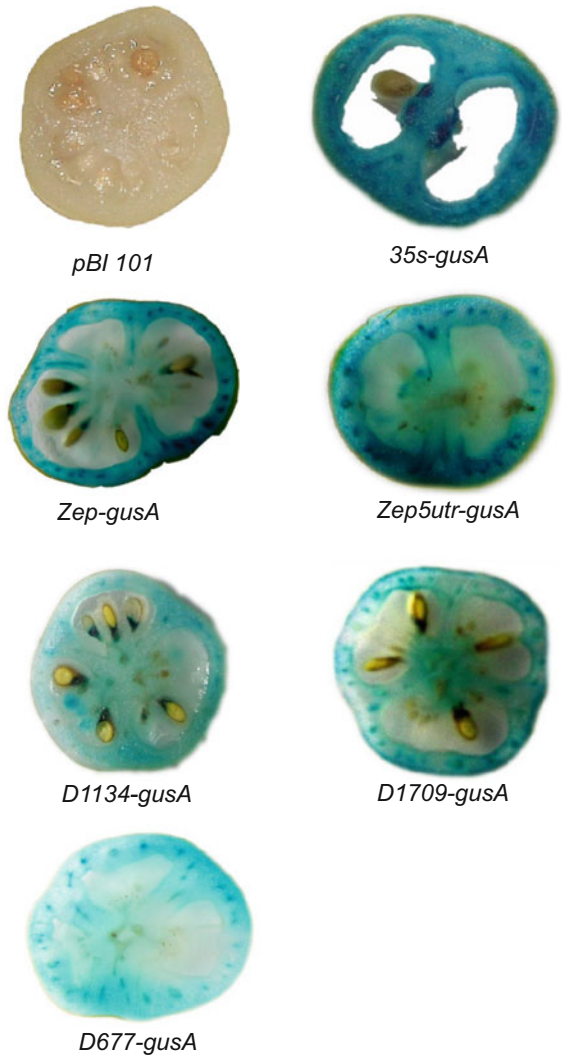

Fig. 1 GUS activity in tomato fruits transiently expressing promoterless-*gusA* (*pBI101*), *35s-gusA*, *Zep-gusA*, *Zep5utr-gusA*, *D1709-gusA*, *D1134-gusA*, *D667-gusA*, respectively

Four representative independent *Zep-gusA* lines exhibiting 3:1 segregation of the *gusA* gene and little variation in GUS activity were analyzed by DNA blot using *gusA* as the probe (data not shown). All four lines contained one or two transgene copies in the tomato genome. One *Zep-gusA* line (no. 4) and one *35S-gusA* line also showing 3:1 segregation were selected to produce T4 homozygous lines for further analysis. Plants from these lines were grown to maturity and GUS activity was assessed in different tissues.

In the *Zep-gusA* plants, GUS activity in young and mature leaves, stems and roots was below the threshold for histochemical detection (Fig. 2). GUS activity was also undetectable in sepals and petals, but could be detected in the ovary and pistils (Fig. 3). Low GUS activity was detected in the central column and placenta tissues of immature green fruits, increased in mature green fruits, peaked in orange fruits and decreased slightly in red ripe fruits (Fig. 2c). A distinct spatiotemporal pattern of GUS activity was observed in the pericarp, with no activity in immature green fruits but increasing activity later in development, peaking in orange and ripe red fruits (Fig. 2c). Pericarp cells in young immature green fruits contain a large number of regular-sized chloroplasts, but these differentiate progressively into chromoplasts and completely replace the chloroplasts in the pericarp cells of ripe red fruits (Forth and Pyke 2006; Egea et al. 2010). The *Zep-gusA* reporter gene is therefore developmentally regulated in close association with chromoplast differentiation. In contrast to the above, high levels of GUS activity were observed in all the tissues of the *35s-gusA* plants and throughout the pericarp during all ripening stages (Figs. 2, 3).

Quantitative analysis of GUS activity in stably transformed tomato plants

The quantitative analysis of GUS activity in *Zep-gusA* transgenic plants indicated that only low levels of GUS were present in the leaves, but higher levels were present in flower tissues such as stamens, pistils and petals (Fig. 4). GUS activity was low in immature green fruits, but increased five-fold during development peaking in orange fruits. The quantitative and histochemical GUS assays were concordant (Figs. 2, 3, 4).

Quantitative analysis of *gusA* gene expression in stably transformed tomato plants

The transcriptional activity of the *GlZEP* promoter was analyzed in more detail by measuring *gusA* mRNA levels in different tissues of the *Zep-gusA* tomato plants by quantitative real-time PCR (Fig. 5). These measurements in separate extracts from leaves, sepals, petals, stamens and pistils revealed relatively high *gusA* levels in stamens and the lowest levels in leaves. Expression studies during fruit development showed very low steady state *gusA* mRNA levels in immature green fruits, an up to sixfold increase in

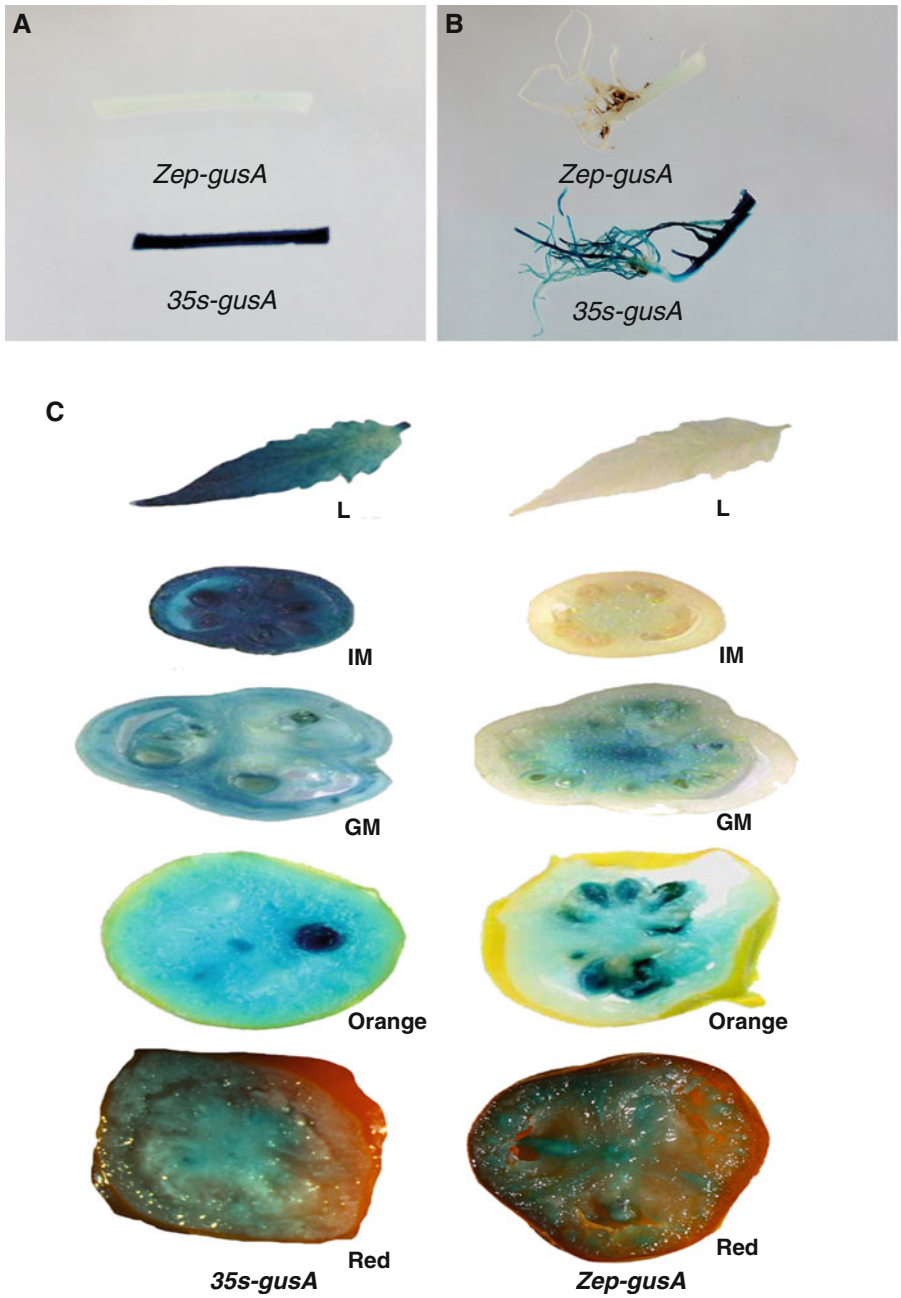

Fig. 2 Histochemical GUS staining of typical transgenic tomato plant carrying *Zep-gusA* and *35S-gusA* constructs, respectively. **a** stems; **b** roots; **c** leaves and fruits. *L* leaf, *IM* immature green fruit, *MG* mature green fruit, *Orange* orange fruit, *Red* red ripen fruit; *Zep-gusA*, pBI-GlZEPPro-GUS; *35s-gusA*, pBI121. All *four lines* tested exhibited very similar staining patterns

orange fruits and a slight decrease in red ripe fruits (Fig. 5). These data were in full agreement with the levels of GUS activity determined in the histochemical and fluorometric assays (Figs. 2, 3, 4).

Discussion

Carotenoid biosynthesis is differentially regulated in tissues containing chloroplasts and chromoplasts,

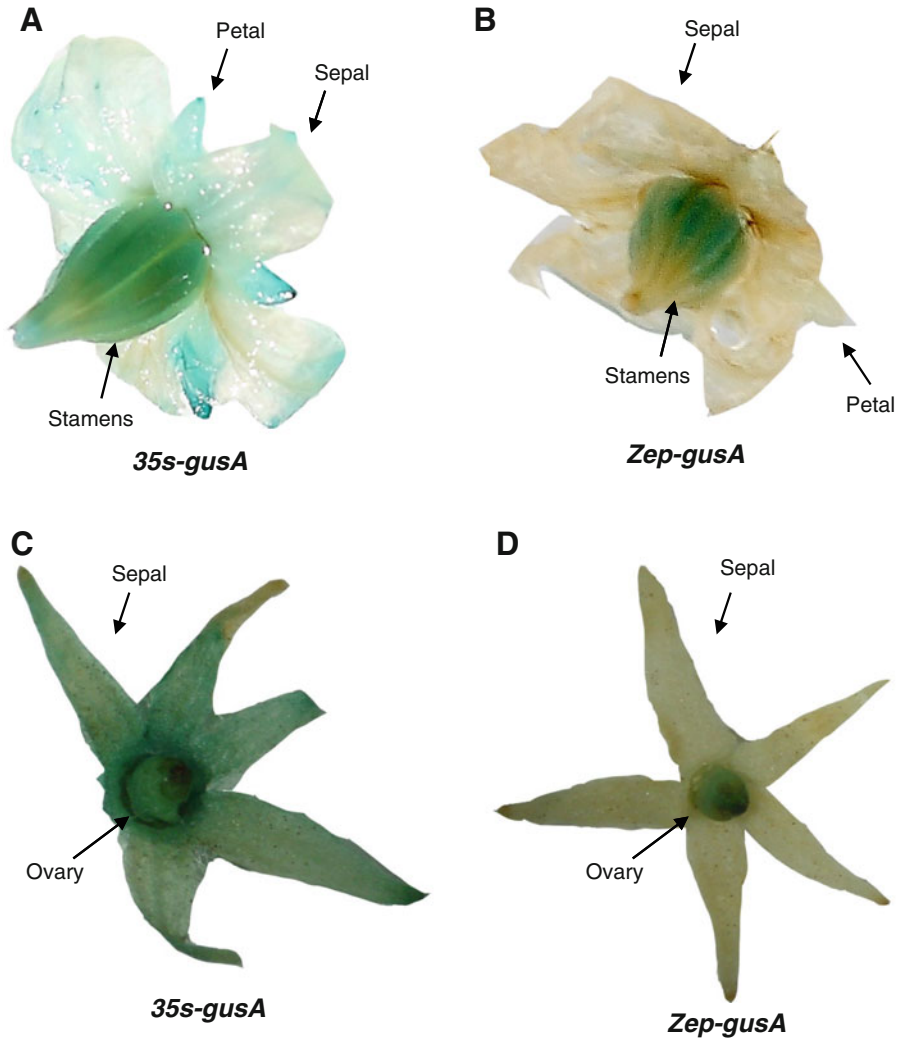

Fig. 3 Histochemical GUS staining in flowers of transgenic tomato plants carrying *Zep-gusA* and *35s-gusA* constructs, respectively. All *four lines* tested exhibited very similar staining patterns

reflecting important functional differences between these tissues and the different roles carotenoids fulfill in each setting (reviewed by Zhu et al. 2010). To ensure that green tissues and fruits/flowers can independently accumulate different carotenoids, many carotenogenic enzymes exist as multiple isoforms encoded by separate genes. The tomato genome, for example, encodes two isoforms of *GGPPS* (geranylgeranyl diphosphate synthase), *PSY* (phytoene synthase), *LYCB* (lycopene β-cyclase) and *BCH* (β-carotene hydroxylase), one set expressed preferentially in green tissues and the other expressed preferentially in flowers and fruits (Ronen et al. 2000; Galpaz et al. 2006). Chromoplast-specific isoforms of lycopene β-cyclase have also been identified in *Citrus* (Alquezar et al. 2009; Dalal et al. 2010; Mendes et al. 2011), kiwifruit (Ampomah-Dwamena et al. 2009), saffron (Ahrazem et al. 2010) and papaya (Blas et al. 2010; Devitt et al. 2010).

There has been great interest in the investigation of carotenoid biosynthesis and its regulation in plants, primarily because of the dietary benefits of carotenoids and the drive to develop crops with higher levels of β-carotene. However, this has drawn attention away from the natural role of carotenoids in plants, i.e. the promotion of pollination and seed dispersal, which can only be investigated by looking at the expression profiles of carotenogenic genes in homologous and

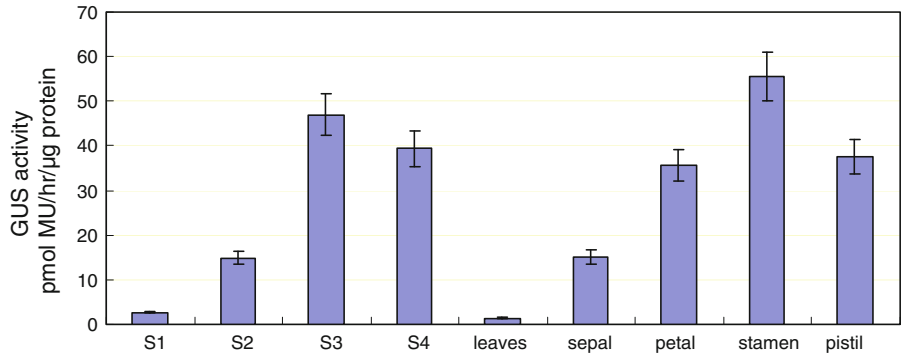

Fig. 4 GUS expression in different tissues of transgenic tomato plants carrying *Zep-gusA*. GUS activity was determined in triplicate measurements in four independent biological replicates (independent transgenic plants). *Columns* represent GUS activity expressed in pmoles MU/h·µg of soluble protein in fruits at different stages of maturity (S1–S4) and leaves, sepals, petals, stamens and pistils. Error bars represent standard error of the mean. S1, fruits at immature green stage; S2, fruits at mature green stage; S3, fruits at orange fruit stage; S4, fruits at red ripen fruit stage

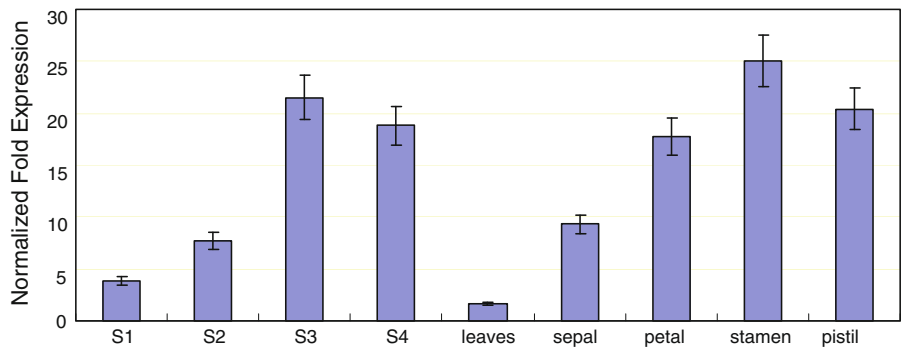

Fig. 5 Expression of *gusA* gene in different tissues of transgenic tomato plants carrying *Zep-gusA*. Quantitative real-time PCR was performed with cDNA prepared from leaves, sepals, petals, stamens, pistils and different stage fruits. Relative expression was determined in triplicate measurements in four independent biological replicates. *Columns* represent the relative *gusA* expression levels normalized against *β*-actin gene with standard errors. Error bars represent standard error of the mean. S1, fruits at immature green stage; S2, fruits at mature green stage; S3, fruit at orange fruit stage; S4, fruit at red ripen fruit stage

heterologous genetic backgrounds and linking the expression profiles of different enzymes to the carotenoids that accumulate in different tissues (Zhu et al. 2002, 2003; Li et al. 2010).

We have previously shown that the *G. lutea* zeaxanthin epoxidase gene (*GlZEP*) is expressed strongly in chromoplast-rich mature petals of *G. lutea* plants, but only minimally in chloroplast-containing younger petals and leaves (Zhu et al. 2003), suggesting the promoter may be active in tissues containing chromoplasts and repressed in tissues lacking them. In agreement with this, no GUS activity was detected in tobacco plants (whose petals and fruits lack chromoplasts) expressing a *GlZEP-gusA* transgene (data not shown) so we sought to carry out similar analysis in tomato plants, which contain abundant chromoplasts in mature fruits and flowers. Chromoplasts in tomato fruits begin differentiating at the breaker stage, and full conversion of chloroplasts into chromoplasts occurs when the fruits are completely ripe (reviewed by Egea et al. 2010). Chloroplast to chromoplast differentiation can be conveniently assessed using the pericarp pigmentation during tomato fruit development. We selected the miniature dwarf tomato cultivar Micro-Tom (Scott and Harbaugh 1989) as a model to investigate *GlZEP* promoter activity in chromoplast-containing tissues because of its small size and short life cycle (70–90 days from sowing to fruit ripening) (Meissner et al. 1997). This cultivar has previously been used for the analysis of metabolic and

developmental pathways (Haroldsen et al. 2011; Carvalho et al. 2011).

We investigated *GlZEP* promoter activity by transient expression and stable transformation in tomato plants transformed with a range of *GlZEP-gusA* reporter constructs. We recovered 12 independent transgenic plant lines expressing the *GlZEP-gusA* construct, all of which demonstrated the same profile of GUS activity albeit with varying staining intensity, so we selected four representative lines for further analysis. We investigated the activity of full-length and truncated promoter constructs in leaves, stems, roots, flowers and fruits at different developmental stages. GUS activity in young and mature leaves, stems and roots was below the threshold for histochemical detection (Fig. 2). GUS activity was also undetectable in sepals and petals, but could be detected in the ovary and pistils (Fig. 3). Similar observation of GUS staining was observed in the flowers of transgenic tomato expressing tomato *PDS* promoter- or tomato *CYC-B* promoter-driven *gusA* reporter gene (Corona et al. 1996; Dalal et al. 2010). In this case there is no apparent link to chromoplast differentiation, possibly reflecting the conserved function of carotenoids in flower tissues. The full-length *GlZEP* promoter (2,225 bp upstream of the transcriptional start site) was functional in the heterologous tomato environment and the expression profile of the reporter gene driven by the full-length promoter was identical to that observed in its homologous background (Zhu et al. 2003). High levels of GUS activity were observed in chromoplast-containing flowers and fruits, but there was only minimal expression in other tissues (fruits, sepals, leaves, stems and roots). Reporter gene activity was strictly correlated with fruit development and chromoplast differentiation, with minimal activity in immature green fruit but increasing activity in ripening orange fruit before falling off towards the end of the ripening process. The shortest *GlZEP* deletion construct (*D677-gusA*) contained 677 bp of upstream sequence but nevertheless resulted in only slightly lower levels of GUS activity compared to the full-length promoter (Fig. 1), suggesting that all *cis*-acting elements required for high-level GUS activity in chromoplast-rich tissues are contained within the proximal 677 bp of the promoter.

All promoters contain *cis*-acting elements that confer spatiotemporal specificity and responsiveness to external stimuli (Peremarti et al. 2010). We characterized the *GlZEP* promoter fragment in more detail by searching the sequence for relevant *cis*-acting elements using the PlantCARE database (Lescot et al. 2002). We identified TATA and CAAT boxes that are typical in eukaryotic promoters, as well as multiple light response elements (GT1, box I, G-boxes,GAG motif, Box 4 and chs-CMA2a) that are likely to link carotenoid biosynthesis to flower development by integrating day-length cues and other stimuli (Table 1). Carotenoid biosynthesis is regulated by light (Bartley and Scolnik 1993; Von Lintig et al. 1997; Simkin et al. 2003; Li et al. 2008; Welsch et al. 2008). A putative circadian responsive element was also found in the *GlZEP* promoter, which supports the diurnal rhythm in *ZEP* gene expression that has been reported in tobacco and tomato leaves (Audran et al. 1998; Thompson et al. 2000; Facella et al. 2008).

The *GlZEP* promoter also contains *cis*-acting elements involved in hormone biosynthesis and stress responses (Table 1). The presence of a drought-inducible MBS element agrees with previous reports that the expression of endogenous *ZEP* in tobacco and tomato roots is induced by drought stress (Audran et al. 1998; Thompson et al. 2000). Two ATCTA motifs are present as tandem repeats in the *GlZEP* promoter. Similar pairs have previously been identified in the *Arabidopsis thaliana PSY* promoter (mediating high-level basal transcription independent of light quality), and in the promoters of several genes related to photosynthesis (Welsch et al. 2003). Single copies of the ATCTA motif are found in other carotenogenic promoters such as Arabidopsis *DXS* (deoxy-xylulose-phosphate synthase) and *PDS* (Welsch et al. 2003), tomato and maize *PDS* (Welsch et al. 2003), and tomato *CYC-B* (Dalal et al. 2010). This motif is also present in several promoters involved in tocopherol biosynthesis (Welsch et al. 2003). In Arabidopsis, paired ATCTA motifs are recognized by AtRAP2.2, a member of the APETALA2/ERE-binding protein transcription factor family (Welsch et al. 2007). We engineered one of our constructs deliberately to eliminate one of the ATCTA motifs (*D1709-gusA*), and also generated two more substantially truncated constructs lacking both copies (*D1134-gusA* and *D677-gusA*). All three constructs performed similarly to the full-length promoter (*Zep-gusA*) suggesting that neither motif contributes significantly to the basal activity of the full-length *GlZEP* promoter. In contrast, deletion of the RAP2.2

transcription factor binding site in the *ShCYC-B* full-length promoter resulted in a considerable loss of promoter activity (Dalal et al. 2010). However, it is possible that the loss of adjacent sequences rather than the RAP2.2 element might be responsible for the fall in promoter activity and the only way to confirm the role of this element directly is to modify it by site-directed or linker-scanning mutagenesis.

The promoters of coexpressed genes often share common regulatory motifs and are potentially regulated by a common set of transcription factors. Therefore, the identification of relevant *cis*-acting regulatory elements in the promoter regions of important metabolic genes can provide leads that help uncover new mechanisms of transcriptional regulation (Liu et al. 2005; Nilsson et al. 2010). The expression profile of *Zep-gusA* in transgenic tomato plants is strikingly similar to that of tomato *PDS* (Corona et al. 1996) and *CYC-B* (Dalal et al. 2010), which have also been evaluated as reporter gene fusions in transgenic tomato plants, suggesting all three may be regulated by a common mechanism. It is also important to emphasize that the *GlZEP* promoter is correctly regulated in a heterologous background, indicating strong conservation of the regulatory mechanisms across species. Common motifs in the three promoters include the CAAT box, Box 4 and RAP2.2 (Corona et al. 1996; Welsch et al. 2007; Dalal et al. 2010) but further analysis and comparisons are required to identify additional known and unknown motifs that are shared between co-regulated promoters and that may help us to unravel further underlying regulatory mechanisms.

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