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ORIGINAL PAPER

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Surrogate biochemistry: use of *Escherichia coli* to identify plant cDNAs that impact metabolic engineering of carotenoid accumulation

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Abstract Carotenoids synthesized in plants but not animals are essential for human nutrition. Therefore, ongoing efforts to metabolically engineer plants for improved carotenoid content benefit from the identification of genes that affect carotenoid accumulation, possibly highlighting potential challenges when pyramiding traits represented by multiple biosynthetic pathways. We employed a heterologous bacterial system to screen for maize cDNAs encoding products that alter carotenoid accumulation either positively or negatively. Genes encoding carotenoid biosynthetic enzymes from the bacterium *Erwinia uredovora* were introduced into *Escherichia coli* cells that were subsequently transfected with a maize endosperm cDNA expression library; and these doubly transformed cells were then screened for altered carotenoid accumulation. DNA sequencing and characterization of one cDNA class conferring increased carotenoid content led to the identification of maize cDNAs encoding isopentenyl diphosphate isomerase. A cDNA that caused a reduced carotenoid content in *E. coli* was also identified. Based on DNA sequence analysis, DNA hybridization, and further functional testing, this latter cDNA was found to encode the small subunit of ADP-glucose pyrophosphorylase, a rate-controlling enzyme in starch biosynthesis that has been of interest for enhancing plant starch content.

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Introduction

Carotenoids constitute a diverse class of C_{40} terpenoids found in plants, algae, bacteria, and fungi, with multiple physiological and nutritional roles. In plants, carotenoids are synthesized and accumulated in plastids and are associated with light-harvesting in photosynthetic complexes. They provide protection against photooxidative damage, serve as precursors to the plant hormone abscisic acid and provide dramatic color to fruits and flowers (Hirschberg 2001). In humans and animals, various carotenoids derived from plant sources act as antioxidants and protect against certain diseases (van den Berg et al. 2000), while other carotenoids are precursors to vitamin A and to retinoid compounds involved in development (Kiefer et al. 2001). Brightly colored carotenoids also enhance the eye-appeal of foods for human consumption (Hornero-Mendez et al. 2000; Mann et al. 2000; van de Berg et al. 2000).

The endosperm tissues of cereal crops, such as maize, wheat, and rice, serve as major food staples world-wide, although they are deficient in adequate levels of nutritionally essential carotenoids. For example, rice endosperm lacks carotenoids (Burkhardt et al. 1997), while maize endosperm has relatively low levels (5–11 μg/g fresh weight; Yu 1999), as compared with tomato fruits at 2.4 mg/g dry weight (Römer et al. 2000), marigold flowers at 5.7 mg/g (Mann et al. 2000), and red pepper fruits at 7–8 mg/g dry weight (Hornero-Mendez et al. 2000). This wide range in carotenoid content indicates the potential for metabolic engineering of cereals to accomplish increased carotenoid content and improved carotenoid composition, directed at specific nutritional or horticultural goals. In fact, the use of genes encoding the carotenoid biosynthetic enzymes combined with use of tissue-specific promoters has led to metabolic engineering for the enhanced accumulation of specific carotenoids in rice, canola, tomato, and tobacco (Shewmaker et al. 1999; Mann et al. 2000; Römer et al. 2000). Future objectives will likely include metabolic pyramiding, to manipulate multiple biosynthetic pathways that have been identified

as targets for plant improvement. For example, in addition to improvement of carotenoid accumulation, enhancement of starch accumulation might be pursued (Stark et al. 1992). Efforts to modify multiple pathways will certainly require integrated knowledge of pathway interactions and an understanding of the regulation of individual enzymatic steps.

The use of transgenic plants to investigate the regulation of biosynthetic pathway flux is tedious and time-intensive; alternatively, some questions can be addressed by preliminary studies in bacterial systems having biochemical environments comparable with the subcellular milieu of a plant cell. Since the *Escherichia coli* cytoplasm is biochemically similar to the plastid location of the carotenoid biosynthetic pathway, this organism is suitable for testing strategies for pathway manipulation (Matthews and Wurtzel 2000), identifying the function of gene products (Li et al. 1996), or characterizing the products of altered biosynthetic enzymes (Schmidt-Dannert 2000). The bacterial system also has potential for the isolation of genes that might impact either positively or negatively on carotenoid accumulation in plants. Genes encoding carotenoid biosynthetic enzymes from plants or from carotenogenic bacteria are introduced into *E. coli* to condition carotenoid biosynthesis and accumulation in an organism that otherwise does not synthesize carotenoids (Lotan and Hirschberg 1995). Transformed cells appear colored due to the accumulation of specific carotenoids, providing an easily screenable phenotype. By looking for changes in colony pigmentation, this heterologous system may be used to identify plant gene products that affect carotenoid accumulation, both positively and negatively.

In this study, *E. coli* cells were transformed with one of two gene clusters, conferring the accumulation of lycopene or betacarotene, followed by transfection with a maize endosperm cDNA library. We report on two classes of isolated cDNAs that were found to confer changes in carotenoid accumulation in *E. coli* and we discuss the potential implications for pathway manipulation in plants.

Materials and methods

Bacterial strains and plasmids

E. coli strain TOP10 F' (Stratagene, San Diego, Calif.) was used for screening maize cDNAs encoding products that affect the carotenoid content of bacteria engineered for carotenoid biosynthesis; and a *glgC*$^{-}$ mutant (AC70RI-504) lacking ADP-glucose pyrophosphorylase (ADPG; Greene and Hannah 1998) was used for the functional testing of a putative ADPG subunit encoded by one maize cDNA identified in the above-mentioned carotenoid screening. Plasmids pAC-BETA-04 (Sun et al. 1996) and pACCRT-EIB Cunningham Jr. et al. 1993) conferred accumulation in *E. coli* of betacarotene and lycopene, respectively; and carotenoid identities were confirmed by high performance liquid chromatography (HPLC) analysis (data not shown). Although we used different gene cassettes for screening, the carotenoid pathway endproduct was unimportant and functioned only as a reporter of pathway flux. The plasmids pMONcSh2-377.1 (pSh2), encoding Shrunken2 (Sh2), the maize large subunit of ADP-glucose pyrophosphorylase, and pMONcBt2–375.6 (pBt2), encoding Brittle2 (Bt2), the maize small subunit of ADP-glucose pyrophosphorylase, were used for functional testing of the pAGP cDNA characterized in this study. To construct deletion control pΔSh2, a 1.2-kb *Hin*dIII fragment containing the *Sh2* cDNA was removed from pMONcSh2-377.1. The deletion control pΔBt2 was constructed by removal of a 1.7-kb *Hin*cII *Bt2* cDNA from pMONcBt2-375.6.

Construction of a cDNA expression library from maize endosperm

Poly(A)$^{+}$ RNA extracted from maize B73 endosperm at 14 days after pollination (Belanger et al. 1986) was used as a substrate for the synthesis of a unidirectional cDNA expression library in Uni-ZAP XR (Stratagene, San Diego, Calif.).The library was excised to produce pBluescript II SK(–) containing cDNA inserts ranging over 0.8–1.5 kb and cloned into restriction sites *Eco*RI and *Xho*I (Sambrook et al. 1989).

Identification of cDNAs that alter carotenoid accumulation in *E. coli*

The maize endosperm cDNA library was used to transfect *E. coli* TOP10 F' cells containing pACCRT-EIB or pAC-BETA-04. The double-transformants, selected by the addition of appropriate antibiotics, were plated at a density of about 100 colony-forming units/plate (100-mm Petri dishes) and incubated overnight at 37 °C, followed by 5 days incubation at room temperature to screen for colonies having relatively increased or decreased pigmentation. Altered pigment phenotype was confirmed by retransformation of isolated maize cDNAs. Colony images were collected by scanning on a Hewlett Packard Scan Jet 6100C scanner with Desk Scan II software and were processed with Adobe Photoshop 4.0.1.

DNA sequencing and analysis

DNA sequences were determined by primer walking for both strands, using the automated DNA Sequencing Facility at the University of Chicago Cancer Research Center. Sequence analysis was performed using Vector NTI Suite, ver 5.5 (InforMax, North Bethesda, Md.), and BLAST 2.1 (Altschul et al. 1997). GenBank accession numbers are: AF330034 for pIPPI0101 and AF330035 for pAGP.

Functional complementation of ADPG activity in *E. coli*

E. coli glgC^{-} cells containing plasmid combinations of interest were plated on LB plates, with appropriate antibiotics and 0.2% glucose, and were incubated at 37 °C for 14 h. To stain accumulated glycogen, plates were opened for 15 min in a glass chamber saturated with iodine vapors and scanned as above.

Results

Isolation of maize cDNAs conferring increased carotenoid accumulation in *E. coli*

To identify maize cDNAs that encode gene products enhancing carotenoid accumulation, *E. coli* TOP10 F' cells were transformed with pACCRT-EIB to condition lycopene accumulation followed by transformation with a maize endosperm cDNA expression library. Thirteen colonies appearing darker red were found to contain cDNAs that were size variants of the same sequence; and some of these colonies are seen in Fig. 1 (left panel, B–F)

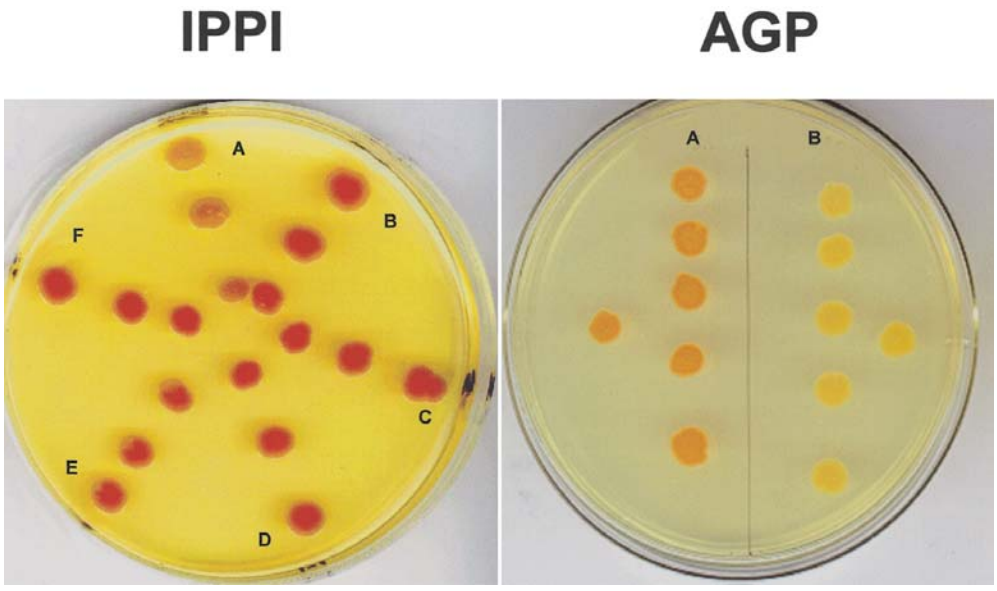

Fig. 1 Screening for cDNAs that impact carotenoid accumulation in *Escherichia coli*. *Left* Lycopene accumulation enhanced by cDNAs encoding isopentenyl diphosphate isomerase (*IPPI*). *E. coli* TOP10 F' cells were transformed with pACCRT-EIB and: *A* pBluescript II SK (–), *B* pIPPI0105, *C* pIPPI0102, *D* pIPPI0101, *E* pIPPI0106, or *F* pIPPI0108. *Right* Accumulation of betacarotene reduced by expression of AGP cDNA. *E. coli* TOP10 F' cells were co-transformed with pAC-BETA-04 and: *A* pBluescript or *B* pAGP

in comparison with control colonies that were secondarily transformed with an empty vector (Fig. 1, left panel, A). One plasmid, pIPPI0101, contained the longest cDNA insert of 1,113 nucleotides (nt); and the open reading frame (ORF; at nt 94–807), starting from the codon specifying the first methionine, was predicted to encode a polypeptide of 237 residues and 27.2 kDa. The sequence of this cDNA showed 70.2% nt homology with a partial cDNA from *Oryza sativa* (GenBank AF188065), which had been previously shown to encode a functional isopentenyl diphosphate isomerase (IPPI; Cunningham Jr. and Gantt 2000). The maize cDNA characterized here is likely to be full-length, as there was an upstream stop codon at nt 70–72, which appeared not to interfere with translation of the polypeptide and did not appear to be a sequencing error, as was also found in other maize IPPI-homologous expressed sequence tags (GenBank AI770794, AI782964, BE344624, AI770647). Neither the maize nor rice IPPI gene products were predicted to have a transit peptide sequence, according to the ChloroP 1.1 program (Emanuelsson et al. 1999). Genomic DNA hybridization indicated *IPPI* to be single copy (data not shown). These results suggest that, if there is a plastid IPPI in maize, it is either the product of an alternative transcript and/or translation product from the one detected gene or it may be encoded by another gene of sufficient nt variation as to elude detection by DNA hybridization. A single maize gene with putative differential expression conforms to predictions based on prior alignments of *IPPI* cDNA sequences from a range of plant species (Cunningham Jr. and Gantt 2000).

Isolation of maize cDNAs conferring decreased carotenoid accumulation in *E. coli*

To identify maize cDNAs that encode gene products interfering with carotenoid accumulation, an *E. coli* strain was engineered for betacarotene accumulation, followed by transformation with the maize endosperm cDNA expression library. A maize cDNA-containing plasmid named pAGP was isolated from the one colony (among 52,000 colonies screened) showing reduced pigmentation. As seen in Fig. 1 (right panel), cells containing pAC-BETA-04 and pAGP showed consistently lighter pigmentation (Fig. 1, right panel, B) compared with control colonies containing pAC-BETA-04 and empty vector (Fig. 1, right panel, A).

Sequence analysis of the pAGP cDNA and its putative gene product

The sequenced cDNA insert of pAGP was 1,754 nt with an ORF over nt 6–1,430, corresponding to a predicted polypeptide of 475 residues and a mass of 52.2 kDa. By comparing the full nt sequence of the insert from pAGP against other maize DNA sequences in GenBank, nt homology was found only for about 400 nt at its 3' end (nt 1,057–1,452) with a partial maize cDNA (GenBank S72425) for *Bt2*, whose gene product encodes the small subunit of ADPG (α-D-glucose-1-phosphate adenylyltransferase, E.C. 2.7.7.27). ADPG, a heterotetrameric plant enzyme, consists of two partially similar subunits, which are both required for the enzymatic catalysis of a rate-limiting step in starch biosynthesis. In maize, the ADPG small subunit is encoded by *Bt2*, while the large subunit is encoded by *Sh2* (Prioul et al. 1994; Greene and Hannah 1998). Both large and small ADPG subunits are known to share some degree of homology and, in

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Maize AGP                (51)GGGAGTRLYPLTKKRAKPAV  (140) WFQGTADAV  (252) ASMGIYVFSKD
Maize Bt2 (endosperm)    (51)GGGAGTRLYPLTKKRAKPAV  (140) WFQGTADAV  (252) ASMGIYVFSKD
Maize Bt2-like (leaf)    (95)GGGAGTRLYPLTKKRAKPAV  (182) WFQGTADAV  (295) ASMGIYVFSKD
Maize Sh2 (endosperm)    (90)GGGTGSQLFPLTSTRATPAV  (179) WFQGTADSI  (295) ASMGIYVFKKD
Maize Sh2-like (embryo)  (93)GGGTGTQLFPLTSTRATPAV  (182) WFQGTADAV  (299) ASMGVYVFKRD
Rice Bt2-like            (55)GGGAGTRLYPLTKKRAKPAV  (144) WFQGTADAV  (256) ASMGIYVISKN
Consensus                    GGGTGTQLFPLTSTRATPAV      WFQGTADAV      ASMGIYVFKKD
                             Signature 1               Signature 2       Signature 3
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Fig. 2 PROSITE signature patterns of ADP-glucose pyrophosphorylase (ADPG): *AGP*, *Bt2* from maize endosperm (GenBank AF334959), *Bt2*-like small subunit from maize leaf (GenBank AF334960), *Sh2* from maize endosperm (GenBank P55241), *Sh2*-like from maize embryo (GenBank CAA86227) and a *Rice Bt2*-like small subunit of ADPG from *Oryza sativa* (GenBank AAA33891). Regions of consensus *are shaded*

particular, have three shared signature sequence domains identified by the PROSITE database of protein families and domains (PDOC00638; Hofmann et al. 1999). The deduced AGP protein sequence was found to have all three signature sequences of ADPG (Fig. 2). However, while nt homology over a limited region indicated *AGP* to encode the small subunit of ADPG, a search of only maize sequences revealed amino acid homology over the entire coding sequence between AGP and a homologue of maize Sh2, the large ADPG subunit (GenBank AAB52952). A region encompassing almost the entire coding sequence of pAGP (residues 45–475) and Sh2 (residues 84–516) shared 48% identity and 68% similarity. Upon later database-searching, newly deposited *Bt2* (GenBank AF334959) and *Bt2*-like genes (GenBank AF334960) were found. While the AGP cDNA was almost identical to GenBank AF334959, AGP had an additional 102 nt at the 3' end (nt 1,653–1,754); and these additional nt were present in the sequence of the *Bt2*-like gene (GenBank AF334960) in a region (nt 6,800–6,907) that showed 83.3% homology. By comparison with the published genomic sequences, the *AGP* sequence reported here is full-length.

Southern hybridization

To further show similarity between *AGP* and *Bt2*, the corresponding cDNAs were used as hybridization probes of maize genomic DNA digested with four different restriction enzymes. The *AGP* cDNA and the *Bt2* cDNA each hybridized to a similar set of bands; and this pattern was unlike that obtained when a *Sh2* cDNA was used as a hybridization probe (data not shown). These hybridization results further support the similarity between *AGP* and *Bt2*.

Functional identification of AGP as a Bt2 homologue

To unambiguously demonstrate that AGP was a homologue of Bt2 and not Sh2, we tested AGP function by complementation of ADPG-defective *E. coli* cells. This bacterium produces glycogen via a homotetrameric ADPG encoded by the *glgC* locus; and mutants can be used to functionally demonstrate activity of the plant enzyme by introducing cDNAs encoding each of the two subunits to complement the missing activity (Greene and Hannah 1998). Since the plant ADPG requires both large and small subunits, by combining maize AGP with either Sh2 or Bt2, it could be demonstrated whether AGP behaved as a Bt2 or a Sh2 subunit, since only the holoenzyme would lead to the accumulation of iodine-stained glycogen in *glgC*$^-$ colonies. As expected, iodine-stained colonies were detected only when both Bt2 and Sh2 were expressed after transformation of *glgC*$^-$ cells with the corresponding cDNAs (Fig. 3A), but not when Sh2 alone was expressed (Fig. 3B), or when colonies lacked subunit expression (Fig. 3F). When AGP was co-expressed with Bt2 (Fig. 3C), no staining was detected, as found when no subunits were expressed (Fig. 3D).

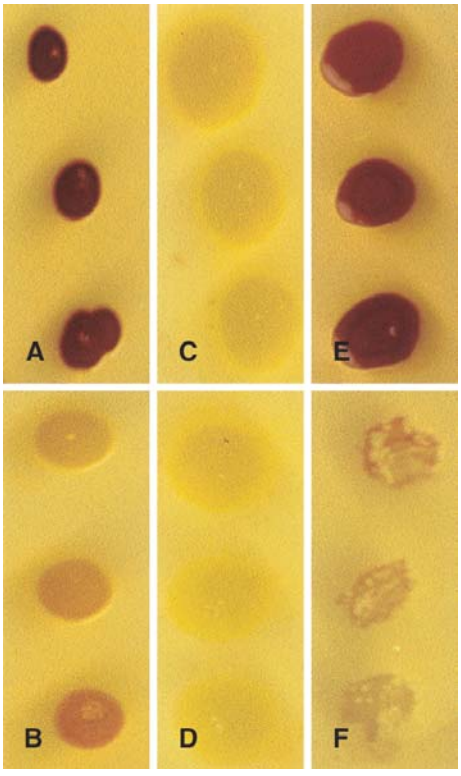

Fig. 3 Functional demonstration of ADPG activity, using iodine-staining of *E. coli glgC*$^-$ cells transformed with: *A* pBt2 and pSh2, *B* pΔBt2 and pSh2, *C* pAGP and pBt2, *D* pΔBt2 and pBluescript, *E* pAGP and pSh2, or *F* pBluescript and pΔSh2

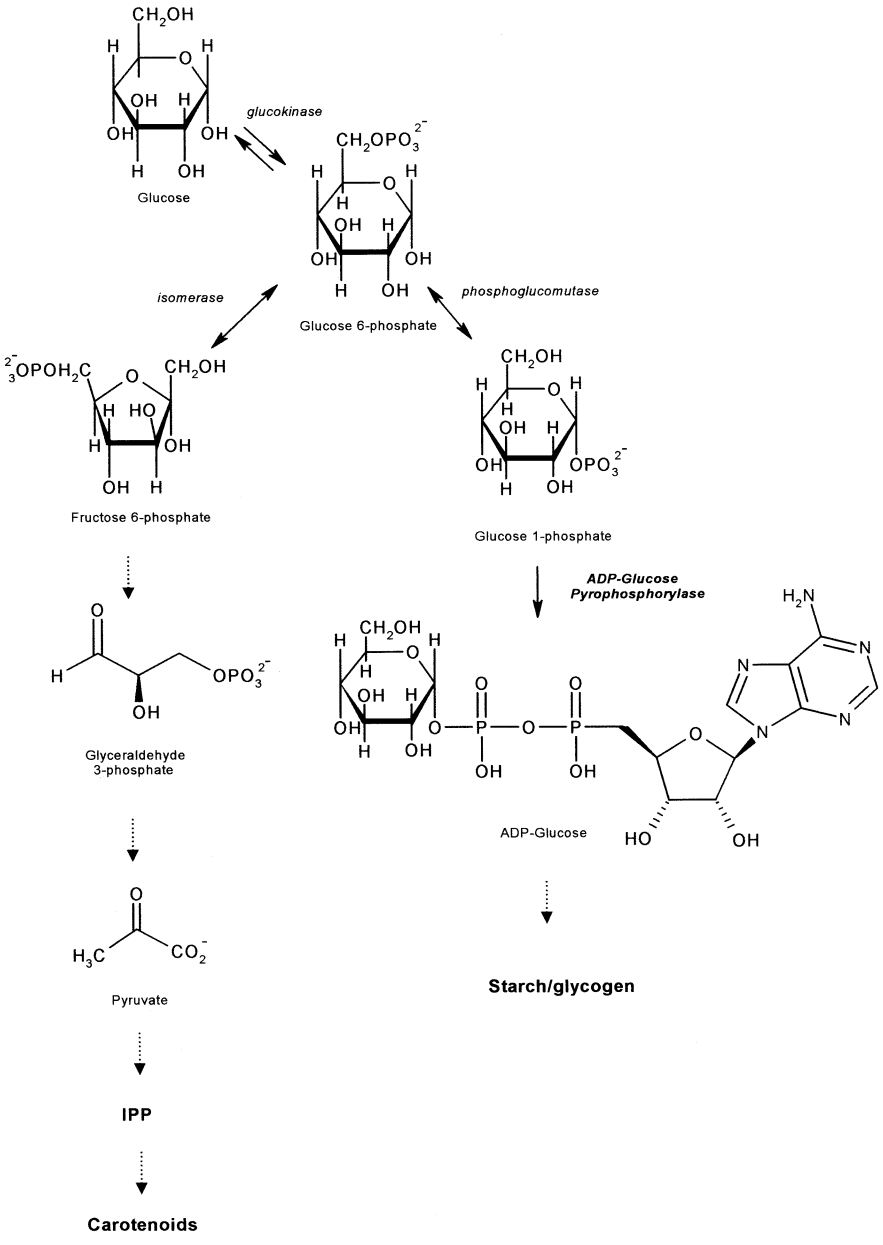

Fig. 4 Biosynthesis of carotenoids and starch/glycogen from common precursors

However, when AGP was expressed in combination with Sh2, staining was detected (Fig. 3E), a result similar to that obtained with the control, Bt2 plus Sh2 (Fig. 3A). These results indicate that AGP behaves as Bt2, in combination with Sh2, to form a functional ADPG enzyme in *E. coli*.

Discussion

The heterologous expression of biosynthetic pathways in *E. coli* continues to be a powerful approach for developing metabolic engineering applications in plants. The utility of the bacterial system lies in its inherent similarity to the biochemistry of the plant plastid. Though the plant and bacterial systems have obvious differences, studies using the bacterial system can provide the foundation needed to justify and strategize for the more laborious testing in plants. As demonstrated by functional screening and sequence analysis, we isolated the first functional maize IPPI cDNAs and further confirmed the role of this enzyme in promoting pathway flux. In the case of IPPI, there is a body of work supporting the importance of IPPI in providing precursors for isoprenoid metabolism: IPP and its isomer, dimethylallyl pyrophosphate. Other attempts using *E. coli* to screen for rate-controlling enzymes in carotenoid biosynthesis have also yielded IPPI cDNAs (Cunningham Jr. and Gantt 2000). Prior efforts to test pathway flux also pointed to IPPI as a key enzyme. For example, co-expression of IPPI from *Rhodobacter capsulatus* together with enzymes encoded by carotenoid gene clusters resulted in a 2-fold increase in

carotenoid accumulation in *E. coli* (Albrecht et al. 2000). When geranylgeranyl pyrophosphate synthase (GGPPS) and IPPI were overexpressed in *E. coli*, together with enzymes encoded by carotenoid gene clusters, the yield of the carotenoid astaxanthin increased 9-fold, compared with over-expression of GGPPS alone (Wang et al. 1999). It has been shown that maize IPPI activity in planta is critical in mediating the light-stimulated increase in carotenoid accumulation that occurs during the conversion of etioplasts to chloroplasts. Such activity appears more important in controlling carotenoid pathway flux than the prenyl transfer reactions mediated by GGPPS or the synthesis of phytoene that is catalyzed by the first carotenoid pathway enzyme, PSY (Albrecht and Sandmann 1994).

These and other studies imply that metabolic engineering of carotenoid accumulation may be more successful when implementing overexpression of upstream enzymes, such as IPPI, as compared with the pathway enzymes themselves. When testing other upstream enzymes, such as deoxyxylulose phosphate synthase, the bacterial system used here also demonstrated the importance of this upstream enzyme in controlling the flux of a downstream pathway (Matthews and Wurtzel 2000); and this observation was later supported by evidence in planta (Estevez et al. 2001). One limitation of the bacterial system is that identification of critical genes affecting pathway flux depends on mRNA prevalence, an important factor in the choice of plant materials for cDNA library construction.

The second class of cDNAs identified here caused decreased carotenoid accumulation. Through the use of sequence analysis and further functional testing, the cDNA isolated was unambiguously shown to encode the small subunit of ADPG, an enzyme that catalyzes a rate-controlling step in starch biosynthesis and therefore an important target for starch-engineering in plants (Stark et al. 1992; Giroux et al. 1996; Smidansky et al. 2002). At first thought, it was puzzling that an enzyme of the starch pathway might impact carotenoid accumulation. However, the recently discovered non-mevalonate IPP biosynthetic pathway (Rohmer et al. 1993; Lichtenthaler et al. 1997; Lichtenthaler 1999), found both in plastids and *E. coli*, provides a clue. As seen in Fig. 4, both IPP, the carotenoid precursor, and the starch produced in plants or the glycogen produced in bacteria, are derived from glycolytic intermediates. For IPP biosynthesis, glycolytic intermediates are used in the non-mevaloate, DOXP pathway; and the ADPG substrate, glucose-1-phosphate, is also derived from glucose. Since glycogen is present in bacteria, the effect is the same: AGP may be competing for a common substrate, thereby causing the diminution in carotenoid accumulation.

In maize, ADPG enzymes exist both as a major, extraplastidial form and as a minor, plastidial form (Denyer et al. 1996); and the externally produced ADPG product is transported into the plastid, where it contributes to starch accumulation (Shannon et al. 1998). That being the case, even if AGP is expressed extraplastidially, it may impact the carotenoid accumulation occurring in the plastid. Therefore, it is possible to predict that efforts to manipulate the starch pathway in plants may be at odds with manipulation of the carotenoid pathway. Recent efforts to produce carotenoid-rich golden rice (Ye et al. 2000) are now being commercialized, but the trait must next be introduced into local rice varieties. As this is only one of several important traits to be introduced, it is quite possible to predict potential challenges for pyramiding multiple traits, such as carotenoids and starch, not to mention the limited knowledge that we have regarding the expression of endogenous genes encoding the carotenoid biosynthetic pathway enzymes.

In summary, the bacterial system provides an expedient tool for obtaining results that can then be applied to testing in important crop species. The observations obtained with genes, such as those encoding IPPI, followed by demonstrated parallel effects in plants, justify future investigation and consideration of the potential competition for common substrates by the starch and carotenoid pathways. The results shown here provide clues to potential problems when engineering multiple pathways in plants and other organisms.

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