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Contribution of hydroxymethylbutenyl diphosphate synthase to carotenoid biosynthesis in bacteria and plants

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ABSTRACT

The methylerythritol 4-phosphate (MEP) pathway synthesizes the precursors of carotenoids and other isoprenoids in bacteria and plant plastids. Despite recent progress in the identification of rate-determining steps, the relative contribution of most pathway enzymes to flux control remains to be established. In this work we investigated whether upregulated levels of hydroxymethylbutenyl diphosphate synthase (HDS) could increase the metabolic flux through this pathway, as judged by endpoint (carotenoid) measurements. Unlike other MEP pathway enzymes, however, increasing the levels of an active HDS protein in carotenoid-producing *Escherichia coli* cells and transgenic *Arabidopsis thaliana* plants did not result in an enhanced accumulation of MEP-derived isoprenoids. Our data suggest that enhanced flux through the MEP pathway for peak demand periods in bacteria and plastids does not require increased HDS activity.

Isoprenoids (also called terpenoids) are essential in all organisms but are particularly abundant and diverse in plants. Some act as primary metabolites in respiration, photosynthesis, and in the regulation of growth and development, but the bulk of plant isoprenoids are secondary metabolites that modulate the interaction of plants with their environment [1–3]. Despite their functional and structural diversity, all isoprenoids derive from the same five-carbon building blocks, isopentenyl diphosphate (IPP) and its double-bound isomer dimethylallyl diphosphate (DMAPP). Consecutive condensation of one or several IPP units with DMAPP results in the production of prenyl diphosphates of increasing size which constitute branch points for the biosynthesis of isoprenoid endproducts such as sterols, carotenoids, hormones, and the prenyl moiety of chlorophylls, tocopherols, and quinones [2].

Plants synthesize IPP and DMAPP by two independent pathways: the cytosolic mevalonic acid (MVA) pathway and the plastidial methylerytritol 4-phosphate (MEP) pathway [1]. By contrast, the MEP pathway is the only one present in most eubacteria, including *Escherichia coli*. Upregulating the biosynthesis of MEP-derived prenyl diphosphate precursors has been shown to be a viable biotechnological strategy to increase the production of economically-important isoprenoid end-products in bacteria and plant plastids [4,5]. This has been achieved by overexpression of the enzymes catalyzing the first two and the last step of the pathway (Fig. 1), deoxyxylulose 5-phosphate (DXP) synthase (DXS), DXP reductoisomerase (DXR), and hydroxymethylbutenyl diphosphate (HMBPP) reductase (HDR). The enhanced accumulation of carotenoids and other MEP-derived isoprenoid products in transgenic bacteria and plants overexpressing DXS, DXR, or HDR indicates that their production is limited by the supply of precursors and that several enzymes share control over the metabolic flux of the MEP pathway, with different enzymes exhibiting different degrees of control [6]. The contribution of other enzymes of the pathway to flux control remains to be established. However, a regulatory role has been suggested for HMBPP synthase (HDS), the enzyme catalyzing the transformation of methylerythritol 2,4-cyclodiphosphate (ME-cPP) into HMBPP in the penultimate step of the MEP pathway (Fig. 1). The proposal that HDS might be a flux-controlling enzyme is based on the distinctive sequence features of the plant enzyme [7,8], its post-transcriptional regulation in plastids [9,10], its participation in defense mechanisms [11], and the photosynthesis-dependent control of its enzymatic activity in plant cells [12]. In this work we have evaluated this hypothesis by upregulating HDS levels in transgenic bacteria (*E. coli*) and

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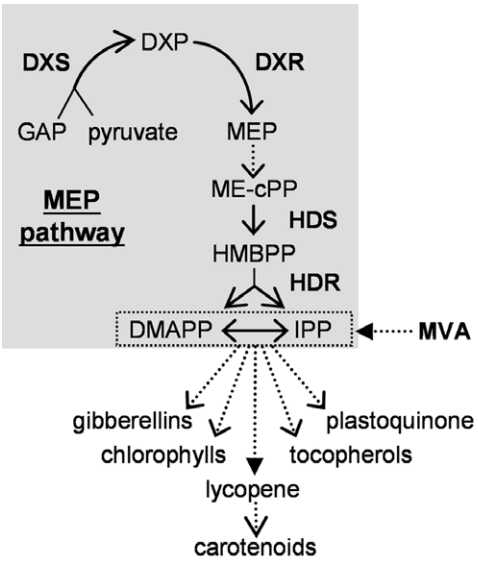

Fig. 1. Isoprenoid biosynthesis in plant cells. Dashed arrows represent multiple enzymatic steps. Pathways introduced in the *E. coli* strains used in this work are indicated with solid head arrows. GAP, glyceraldehyde 3-phosphate; DXP, 1-deoxyxylulose 5-phosphate; MEP, 2-methylerythritol 4-phosphate; ME-cPP, 2-methylerythritol 2,4-cyclodiphosphate; HMBPP, 1-hydroxy-2-methyl-2-butenyl 4-diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; MVA, mevalonic acid. MEP pathway enzymes are indicated in bold: DXS, DXP synthase; DXR, DXP reductoisomerase; HDS, HMBPP synthase; HDR, HMBPP reductase.

plants (*Arabidopsis thaliana*) and analyzed the effects on the levels of derived isoprenoids (carotenoids).

Materials and methods

Bacterial strains and growth conditions. The EcAB4-4 (*gcpE::CAT*) strain was constructed as described [13]. Briefly, after incorporating the MVA$^+$ synthetic operon into the genome of the K-12 MG1655 wild type strain, most of the coding region of the *gcpE* gene was replaced with the *CAT* gene encoding chloramphenicol acetyltransferase. Competent EcAB4-4 cells were transformed with the pQE32-GcpE plasmid [14] or an empty pQE32 vector (Qiagen) as a control and used for complementation experiments as described [8]. The same plasmids were also used to transform BL21(DE3) cells (Stratagene) together with plasmid pACCRT-EIB, which harbors the *Erwinia uredovora crtE, crtB, and crtI* genes for lycopene synthesis [15]. Double transformants were selected on plates with solid Luria broth (LB) medium supplemented with antibiotics to final concentrations of 17 μg/ml chloramphenicol (to select for the pACCRT-EIB plasmid) and 100 μg/ml ampicillin (to select for the pQE32 or pQE32-GcpE plasmids). Overnight cultures of several independent pink (lycopene-producing) colonies in antibiotic-supplemented medium were then used to inoculate fresh LB medium (1:100 dilution) containing the appropriate supplements with or without 0.5 mM IPTG. Aliquots of cultures grown at 37 °C for 15 h were used for protein and lycopene extraction as described below.

Plant material and growth conditions. Plasmid pCAMBIA-*35S-CSB3* (with the *A. thaliana* cDNA sequence encoding HDS under the transcriptional control of the constitutive *35S* promoter) was used for transformation of *Arabidopsis* plants of the Columbia-0 background harboring the *P69C*-GUS reporter or mutant *clb4-3* plants as described [11]. Plants were grown on plates and soil as described [16].

Analysis of transcript levels. Total RNA was isolated from 10-day-old seedlings grown on plates, copied to cDNA, and used for real-time quantitative RT-PCR as described [16]. A predesigned FAM-labeled TaqMan MGB probe and unlabeled primers (Applied Biosystems) were used for HDS (At02186785_g1). The threshold cycle for each probe assay was normalized using EF1α (elongation factor – 1α, At02337969_g1) and relative transcript levels were estimated as described [16].

Immunoblot analysis. After growing *E. coli* BL21(DE3) cells containing the pACCRT-EIB and pQE32-GcpE or pQE32 plasmids as described above, 1 ml aliquots were harvested and cells were pelleted by centrigugation. Protein crude extracts were made from 7.5 mg of cells and used for immunoblot analysis of RGS-His-GcpE levels as described [9,10] using a 1:2000 dilution of a commercial antibody against the RGS-His epitope (Qiagen). Immunoblot analysis of HDS levels in protein extracts of *Arabidopsis* 10-day-old seedlings was performed as described [9].

Quantification of pigment levels. For quantification of lycopene in *E. coli* strains harboring the pACCRT-EIB plasmid, the cell pellet of a 1.3 ml aliquot of cultures grown as described above was resuspended in 700 μl of acetone. Samples were then incubated at 55 °C for 15 min in the dark and centrifuged at 13,000*g* for 10 min to recover the supernatant with the pigment, which was placed in a clean tube. At least two samples were taken from each culture for lycopene extraction. Lycopene was quantified by measuring absorbance at 505 nm of diluted supernatants in acetone and comparing with a standard curve made with known concentrations of a lycopene standard (Sigma). Chlorophylls and carotenoids were extracted from 10-day-old seedlings, separated by HPLC, and quantified as described [16]. Statistical analysis of the data was performed using the Simple Interactive Statistical Analysis (SISA) *T*-test available online (http://home.clara.net/sisa/t-test.htm).

Results and discussion

Upregulated HDS levels in E. coli cells do not result in an enhanced production of isoprenoid precursors

A recent work showed that replacing the native promoter of the endogenous *E. coli gcpE* gene encoding HDS (also named GcpE or IspG in bacteria) with the strong bacteriophage T5 promoter in carotenoid-producing strains did not affect the accumulation of these MEP-derived isoprenoids [17]. Although the results suggested that the production of carotenoid precursors might not be limited by HDS activity in *E. coli* cells, no experiments were reported to confirm that increased levels of active enzyme were actually present in the engineered strains. As an alternative strategy to successfully upregulate HDS activity levels in bacteria, we used the pQE32-GcpE plasmid [14]. In this multi-copy vector, the T5 promoter (which can be induced by IPTG) drives the expression of a recombinant *E. coli* HDS protein with an N-terminal RGS-His epitope [14]. We first confirmed that the addition of the RGS-His tag did not impair enzyme activity by complementation of the lethal phenotype of HDS-defective cells. Disruption of the *gcpE* gene in the EcAB4-4 (*gcpE::CAT*) strain, which also harbors a synthetic MVA+ operon encoding heterologous MVA pathway enzymes that can transform exogenously supplied MVA into IPP and DMAPP, results in MVA auxotrophy [13]. As shown in Fig. 2A, transformation of EcAB4-4 cells with the pQE32-GcpE construct eliminated the requirement of MVA for survival, whereas cells from the same strain transformed with the empty pQE32 plasmid remained auxotrophic for MVA. These results confirmed that the recombinant RGS-His-GcpE protein retained HDS activity. We next co-transformed *E. coli* BL21(DE3) cells with the same constructs and a plasmid (pACCRT-EIB) encoding heterologous enzymes to synthesize the carotenoid lycopene from IPP and DMAPP [15] in order to eval-

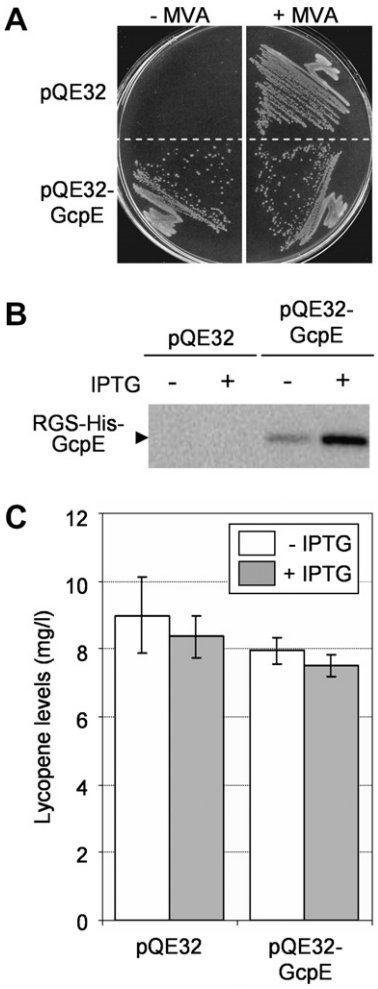

Fig. 2. Upregulation of HDS in bacteria. (A) Complementation of HDS-deficient *E. coli* cells. EcAB4-4 cells were transformed with a construct to express the recombinant RGS-His-GcpE protein (pQE32-GcpE) or an empty pQE32 vector and plated on LB medium supplemented with 1 mM MVA and antibiotics to recover transformants. Positive colonies were then streaked on new plates with (+) or without (−) MVA. (B) Immunoblot analysis of RGS-His-GcpE levels. BL21(DE3) cells were transformed with the indicated vectors together with plasmid pACCRT-EIB for lycopene synthesis. The resulting co-transformants (identified by their pink color) were grown in liquid medium supplemented (+) or not (−) with 0.5 mM IPTG for 15 h at 37 °C. Aliquots of the cultures were then used for protein extraction and immunoblot analysis with antibodies against the RGS-His epitope. Arrowhead indicates the position of the RGS-His-GcpE protein. (C) Lycopene content of the cultures described in (B). Mean values and standard errors of two replicates of at least four independent cultures are shown.

uate whether upregulated levels of HDS activity resulted in an enhanced production of isoprenoids using carotenoid (lycopene) accumulation as a reporter. Colonies of double transformants grown on plates supplemented with the appropriate antibiotics were visually identified based on the red color imparted by lycopene accumulation and used to inoculate liquid media. Grown cultures were then used to analyze the production of recombinant protein and to quantify the levels of lycopene. Immunoblot analysis of RGS-His-GcpE levels with an antibody specific for the RGS-His epitope showed that this protein was synthesized in BL21(DE3) cells harboring the pQE32-GcpE plasmid even in the absence of the IPTG inducer (Fig. 2B). However, lycopene accumulation was not increased compared to control cells transformed with the pQE32 vector (Fig. 2C). Addition of 0.5 mM IPTG to the growth medium resulted in a substantially increased production of RGS-His-GcpE protein (Fig. 2B), but no significant ($P < 0.05$) changes were observed in lycopene accumulation (Fig. 2C). Our data confirm that overproduction of an active HDS enzyme in *E. coli* cells does not result in increased carotenoid levels, consistent with a non-limiting role of this activity for isoprenoid biosynthesis in bacteria.

HDS levels are not limiting for plastid isoprenoid biosynthesis in wild type Arabidopsis plants

The control exerted by HDS over the metabolic flux to plastidial isoprenoids in plant cells was examined by upregulating its levels in *Arabidopsis* plants with a wild type *HDS* gene (At5g60600, named *GCPE*, *ISPG*, *CLB4*, or *CSB3* in the literature) and in mutants with reduced HDS activity. Complete loss of HDS function in the *chloroplast biogenesis 4* (*clb*4) null alleles *clb4-1* and *clb4-2* results in a seedling lethal albino phenotype, as expected for a knockout mutant of the MEP pathway [18]. A point mutation at this locus has been reported to result in a partial loss of function in the *constitutive subtilisin 3* (*csb3*, renamed *clb4-3*) mutant, causing a developmental delay and, unexpectedly, a strikingly enhanced resistance to biotrophic pathogens [11]. We also observed that *clb4-3* plants had a slightly pale phenotype (Fig. 3A) caused by a decreased accumulation of MEP-derived photosynthetic pigments, chlorophylls and carotenoids (Fig. 4). Transformation of *clb4-3* plants with a construct to constitutively express the *Arabidopsis* HDS enzyme under the control of the *35S* promoter restored a wild type phenotype in terms of growth and pigmentation (Fig. 3A), confirming that the mutant phenotypes were caused by reduced HDS levels and that the recombinant protein produced in these lines was active. Quantitative real-time PCR analysis of three of these *clb4-3 35S::HDS* lines (1.1, 5.6 and 4.2) confirmed that *HDS* transcripts were increased several fold in the rescued lines (Fig. 3B). Immunoblot analysis with HDS-specific antibodies demonstrated a corresponding accumulation of the encoded protein (Fig. 3C). But despite the observed upregulation of an active HDS enzyme, the levels of chlorophylls and carotenoids were unchanged ($P < 0.05$) compared to those observed in plants with wild type HDS activity levels (Fig. 4). The same construct was used for transformation of *Arabidopsis* plants with wild type HDS levels (line P69C-GUS) [11]. The generated lines (G5.A, G6.A, and G6.B) were visually undistinguishable from untransformed plants or *clb4-3 35S::HDS* lines (Fig. 3B). Also similar to *clb4-3 35S::HDS* plants, G5.A, G6.A, and G6.B lines displayed enhanced *HDS* transcript (Fig. 3B) and protein levels (Fig. 3C) but no significant ($P < 0.05$) changes in the accumulation of chlorophylls or carotenoids compared to untransformed controls (Fig. 4). Together, our data suggest that the production of carotenoids and other MEP-derived isoprenoids is not limited by the levels of HDS activity present in wild type *Arabidopsis* chloroplasts under normal growth conditions. Consistent with this observation, no major changes in the levels of HDS-encoding transcripts are detected when carotenoid biosynthesis is boosted during the transition from etioplasts to chloroplasts in deetiolating *Arabidopsis* seedlings and from chloroplasts to chromoplasts in ripening tomato fruit [6,7].

Several MEP pathway enzymes, but not HDS, control isoprenoid precursor supply during peak demand periods in bacteria and plastids

Accumulation of ME-cPP, the substrate of HDS (Fig. 1), has been observed in bacteria under oxidative stress [19], whereas in *Arabidopsis* plants it appears to be involved in resistance to some pathogens [11]. On the other hand, HMBPP, the product of HDS, acts as a potent antigen that triggers human immunological responses [20]. Furthermore, plant HDS has been shown to be an essential enzyme [18] whose activity is post-transcriptionally regulated in plastids [9,10] in part by a photosynthesis-dependent control [12]. These observations suggest that a certain level of HDS activity is maintained under normal growth conditions but it might be changed

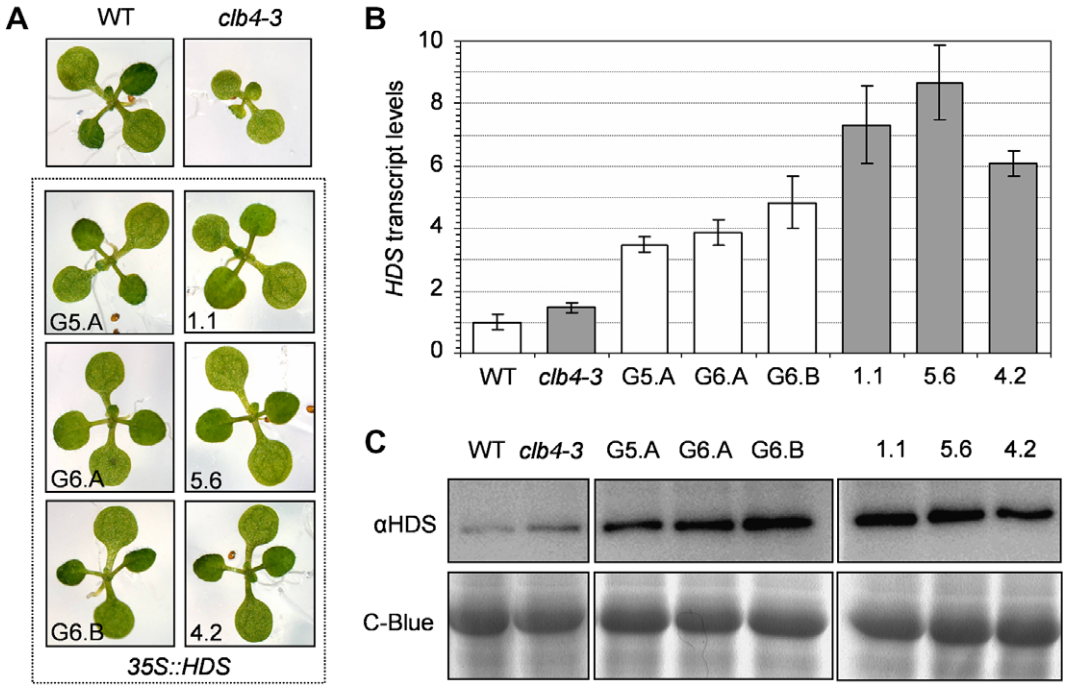

Fig. 3. Phenotype of *Arabidopsis* lines with altered levels of HDS activity. *Arabidopsis* plants with a wild type (WT) gene encoding HDS or a partial loss of function mutant allele (*clb4-3*) were transformed with a construct to constitutively overproduce the enzyme (*35S::HDS*). Several independent lines with the WT (G5.A, G6.A, G6.B) or mutant (1.1, 5.6, 4.2) genomic locus were selected. (A) Representative 10-day-old seedlings of the indicated lines. All panels are to the same scale. (B) Real-time quantitative RT-PCR analysis of *HDS* transcript levels in seedlings of lines shown in (A). Mean and standard error of duplicates of two independent experiments is shown. Levels are represented relative to those in untransformed WT seedlings. (C) Immunoblot analysis of HDS protein levels in 10-day-old seedlings of the indicated lines with a HDS-specific antibody (αHDS). Coomassie Blue (C-Blue) staining is shown as an estimate of equal protein loading.

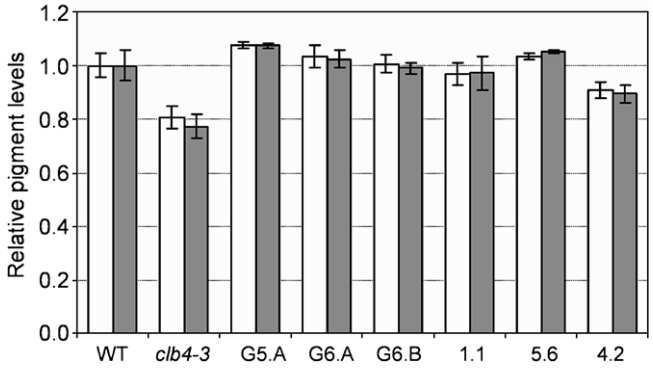

Fig. 4. Accumulation of plastidial isoprenoid pigments in transgenic *35S::HDS* lines. Total carotenoid (white columns) and chlorophyll (gray columns) levels of 10-day-old seedlings of the indicated lines are shown. Pigment levels are represented relative to those in untransformed WT plants. Mean values and standard errors correspond to two experiments.

in response to different stimuli in bacteria and plant cells. Consistently, our results with *clb4-3 35S::HDS* lines suggest that the production of wild type levels of plastidial isoprenoids such as chlorophylls and carotenoids requires a threshold level of HDS enzyme activity. However, two observations imply that the flux control coefficient of HDS could be quite low. First, the effect of the *clb4-3* mutation on carotenoid and chlorophyll production is relatively slight (Fig. 4), suggesting the flux control coefficient of this step does not rise to a significant level until HDS activity has decreased below its wild type level. By contrast, similar point mutations in the gene encoding DXS produce a far more dramatic phenotype [21,22]. Secondly, that overexpression has no effect on endproduct accumulation (Fig. 4) indicates that the flux control coefficient (under wild type expression levels) is too low to increase flux by upregulating this step.

Alternatively, it is possible that the availability of enzyme cofactors required for HDS activity might limit the production of plastidial prenyl diphosphates upon upregulation of enzyme levels. Both HDS and HDR, the last two enzymes of the MEP pathway (Fig. 1), are iron–sulfur reductases that require a reduced $[4Fe–4S]^{1+}$ cluster for enzymatic activity [23–26]. The oxidized $[4Fe–4S]^{2+}$ form of the two enzymes can be reduced *in vitro* by a flavodoxin/flavodoxin reductase/NADPH system, probably the *in vivo* reducing system in *E. coli* [24,25]. In plants, electron flow from photosynthesis can directly provide the electrons required for HDS activity in chloroplasts *via* ferredoxin in the absence of any reducing cofactor [12]. However, it is not likely that this reducing system is limiting for HDS activity or/and the production of MEP-derived isoprenoid precursors in chloroplasts because overexpression of HDR (another enzyme that most likely requires the same reducing system) successfully increased the accumulation of chlorophylls and carotenoids in *Arabidopsis* [27]. Moreover, upregulated HDR levels in *E. coli* also resulted in enhanced accumulation of carotenoids [28], indicating that the reducing power required for the activity of both HDS and HDR is also non-limiting in bacteria.

Although it is possible that another unknown factor required for the activity of HDS (but not HDR) might prevent an increased production of carotenoids in transgenic bacteria and plants with enhanced levels of active HDS protein, it is more likely that the activity of a different MEP pathway enzyme becomes limiting when HDS activity is upregulated. Here, we have shown for the first time that not all the MEP pathway enzymes cause a significant change in the pathway flux when their concentration is altered, i.e. not all of them might be considered rate-determining according to metabolic control analysis (MCA) terminology. Based on overexpression studies, the control of flux through the MEP pathway

should be shared by the rate-determining enzymes DXS, DXR, and HDR [6,16], whereas the contribution of HDS to overall flux appears to be comparatively much lower. A thorough examination of the relevant MCA parameters is now in progress in our lab to confirm this model, which has important implications for metabolic engineering of carotenoids and other isoprenoids of interest in bacterial and plant systems.

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