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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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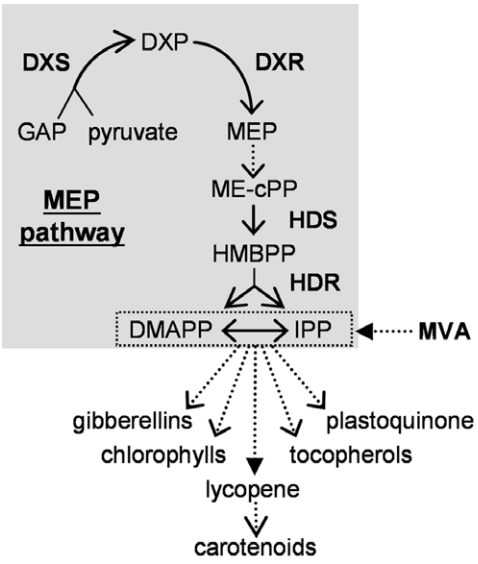

Fig. 1. Isoprenoid biosynthesis in plant cells. Dashed arrows represent multiple enzymatic steps. Pathways introduced in the *E. coli* strains used in this work are indicated with solid head arrows. GAP, glyceraldehyde 3-phosphate; DXP, 1-deoxyxylulose 5-phosphate; MEP, 2-methylerythritol 4-phosphate; 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-