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Overexpression of endogenous 1-deoxy-D-xylulose 5-phosphate synthase (DXS) in cyanobacterium *Synechocystis* sp. PCC6803 accelerates protein aggregation

Kai Kudoh,[1] Shingo Hotta,[2] Midori Sekine,[2] Rintaro Fujii,[2] Arisu Uchida,[2] Genma Kubota,[2] Yusuke Kawano,[3] and Masaki Ihara[2,4,5,*]

Department of Bioscience and Food Production Science, Interdisciplinary Graduate School of Science and Technology, Shinshu University, 8304 Minamiminowa, Nagano 399-4511, Japan,[1] Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Nagano 399-4511, Japan,[2] Innovation Medical Research Institute, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan,[3] JST, PRESTO, 4-1-8 Honcho, Kawaguchi, Saitama 332-0012, Japan,[4] and Department of Interdisciplinary Genome Sciences and Cell Metabolism, Institute for Biomedical Sciences, Interdisciplinary Cluster for Cutting Edge Research, Shinshu University, 8304 Minamiminowa, Kamiina, Nagano 399-4598, Japan[5]

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1-Deoxy-D-xylulose 5-phosphate synthase (DXS) is a rate-limiting enzyme in the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, which is responsible for the production of precursors of all isoprenoids. In a previous study, we had examined the overexpression of an endogenous DXS in a *Synechocystis* sp. PCC6803 mutant (DXS_ox), and found that the *dxs* mRNA level was 4-fold higher than that in the wild-type (WT) strain. However, the DXS protein level was only 1.5-fold higher, leading to the assumption that the level might be regulated by post-transcriptional events. In this study, we have additionally introduced an exogenous isoprene synthase (IspS; which can release MEP pathway products from the cell as gaseous isoprene) into the WT and DXS_ox strains (WT-isP and DXSox-isP strains, respectively), and their detailed DXS expression profiles were investigated from the induction phase through to the late-logarithmic phase. In the induction phase, the isoprene productivity of the DXSox-isP strain was slightly but significantly (1.4- to 1.8-fold) higher than that of the WT-isP strain, whereas the levels were comparable in the other phases. Interestingly, the ratios of soluble:insoluble DXS protein were remarkably low in the DXSox-isP strain during the induction phase to the early-logarithmic phase, resulting in a moderate level of soluble DXS. All our results suggested that the high translation rate of DXS disturbs the refolding process of DXS. To enhance the concentration of the active DXS in cyanobacteria, the enhancement of the DXS maturation system or the introduction of exogenous and robust DXS proteins might be necessary.

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[**Key words:** 1-Deoxyxylulose 5-phosphate synthase; MEP pathway; Protein solubility; Isoprene; Metabolic engineering]

Isoprene, the simplest member of the isoprenoids (1–3), is an important feedstock in the synthetic chemistry industry and is widely used in the production of synthetic rubber, medicines, pesticides, and synthetic oil additives, and as a vulcanizing agent for rubber. Isoprene also can be converted into biofuel, e.g., aviation fuel (4,5). Currently, isoprene used in industry is mainly produced from C5 petroleum distillate, as a byproduct of the oil refining process, *via* the fractionation of petroleum. However, in light of high oil prices and environmental concerns, isoprene from renewable materials is a potential substitute for a petroleum-based product (6).

In biology, isoprene is a member of the isoprenoid family and is synthesized *via* the mevalonic acid (MVA) pathway or the methyl-erythritol-4-phosphate (MEP) pathway (reviewed by Rodriguez-Concepcion and Boronat (7)) (Fig. 1, left). Through these biosynthetic pathways, two common isoprenoid precursors, dimethylallyl diphosphate (DMAPP) and isopentenyl diphosphate (IPP), are produced and converted into various isoprenoids. Isoprene is synthesized from DMAPP directly by isoprene synthase (IspS). The MVA pathway operates in eukaryotes (including all mammals), archaea, a few eubacteria, and in the cytosol and mitochondria of plants and fungi (8,9), whereas the MEP pathway is present in eubacteria including cyanobacteria, plant chloroplasts, algae, and apicomplexan parasites (10–12). Some eubacteria possess both of these pathways for the synthesis of isoprenoid feedstocks (13,14). The two pathways are quite different regarding stoichiometry, energy consumption, and the oxidation/reduction balance during the conversion of glucose to IPP (15). The MEP pathway has a higher theoretical yield, while the MVA pathway has a lower energy consumption.

Cyanobacteria are not only widely used model phototrophs for basic biological studies, but are also attractive candidates for use in bio-industrial applications because of their high photosynthetic capability. Cyanobacteria can convert captured solar energy into biomass at efficiencies exceeding those of terrestrial plants (cyanobacteria, 3–9%; terrestrial plants, 0.25–3%) (16). As cyanobacteria possess the MEP pathway but lack the *isps* gene, Lindberg et al. (5) introduced an *isps* gene from *Pueraria montana* (*skisps*) into cyanobacteria, and successfully detected isoprene in the headspace of a closed-cultivation system. However, the isoprene yield was low, constituting only a few percent of the total cyanobacterial

* Corresponding author at: Faculty of Agriculture, Shinshu University, 8304 Minamiminowa, Nagano 399-4511, Japan. Tel./fax: +81 265 77 1518.
E-mail address: m_ihara@shinshu-u.ac.jp (M. Ihara).

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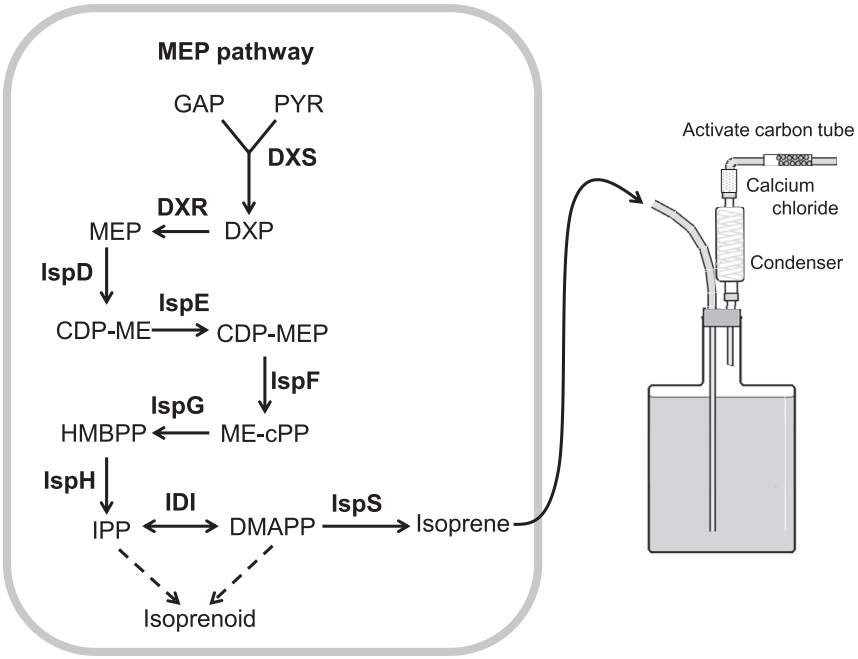

FIG. 1. The methyl-erythritol-4-phosphate (MEP) pathway (left) and a diagram of the apparatus for capturing volatile isoprene (right). GAP, glyceraldehyde-3-phosphate; PYR, pyruvate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2C-methyl-D-erythritol 4 phosphate; CDP-ME, 4-diphosphocytidyl-2C-methyl D-erythritol; CDP-MEP, 4-diphosphocytidyl-2C-methyl D-erythritol 2-phosphate; ME-cPP, 2Cmethyl-D-erythritol 2,4-cyclodiphosphate; HMBPP, (E)-4-hydroxy-3-methylbut-2-enyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; DXR, 1-deoxy-D-xylulose 5-phosphate reductase; IspD, 4-diphosphocytidyl-2C-methyl-derythritol synthase; IspE, 4-diphosphocytidyl-2-C-methylerythritol kinase; IspF, 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase; IspG, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase; IspH, 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase; IDI, isopentenyl diphosphate isomerase; IspS, isoprene synthase.

biomass. Following this pioneering work, Bentley et al. (17) successfully improved the isoprene productivity of the SKIspS-expressing cells by heterologous introduction of the MVA pathway genes. Pade et al. (18) also tried to enhance the carbon flux into SKIspS by overexpression of the endogenous *dxs* gene, which encodes 1-deoxy-D-xylulose 5-phosphate synthase (DXS), the initial enzyme in the MEP pathway. However, the isoprene productivity was, unexpectedly, decreased. Interestingly, they also reported in the same article that the highest isoprene production rate (336 μg isoprene/g dry cell weight per day) was observed in the strain expressing only SKIspS when cultured in the open-cultivation system, in which the medium is continuously aerated and isoprene is detected at the outlet. They had assumed that in the open-cultivation system, the isoprene concentration would be lower than in the closed system, which would lower the inhibitory effects of the isoprene molecule. Very recently, seminal studies were published by Gao et al. (19) and Chaves et al. (20). They clearly demonstrated that the ratio of IPP to DMAPP is the most critical factor affecting isoprene production. Gao et al. (19) showed that IPP works as an inhibitor of IspS. They then dramatically enhanced isoprene production in *Synechococcus elongatus* by inducing heterologous expression of isopentenyl diphosphate isomerase (IDI) from *Eucalyptus globulus*. They finally succeeded in redirecting up to 65% of the carbon flow towards isoprene production by genetically modifying the latter half of the MEP pathway (downstream of 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (IspG)).

The key enzyme in the first half of the MEP pathway is DXS. However, many attempts to enhance isoprenoid productivities in cyanobacteria by overexpressing DXS have encountered difficulties (18,21). Kiyota et al. (21) successfully observed limonene production from the limonene-synthase expressing *Synechocystis* sp. PCC6803 (*S. 6803*) strain in an open-cultivation system, but the co-expression of DXS had only a small effect. We also previously attempted the overexpression of endogenous DXS in *S. 6803*, and found that the mRNA level increased 4-fold compared with the wild-type strain, but the increase in the soluble protein levels of DXS was only 1.5-fold (22). This result suggests that the amount of soluble DXS was regulated by the events downstream of transcription, for example, mRNA degradation, protein degradation, protein aggregation, and feedback inhibition of the enzymatic activities.

A study of post-transcriptional regulation of DXS in the chloroplasts of *Arabidopsis thaliana* identified a role for the heat shock proteins (Hsps) J-protein J20 and Hsp70 in the activation of DXS (23). However, we have no information about the post-transcriptional regulation of DXS in cyanobacteria. To resolve this situation, we quantified mRNA levels, soluble and insoluble protein levels, and isoprene production of the DXS-SKIspS co-expressing strains from induction phase to late-logarithmic phase. To minimize the potential inhibitory feedback effect of isoprene, we used the newly developed open-cultivation system equipped with an activated carbon tube at the outlet for stoichiometric isoprene harvest (Fig. 1, right).

MATERIALS AND METHODS

Strains and growth conditions *S. 6803* strains were cultivated at 25°C in BG-11 medium (24) buffered with 5 mM *N*-Tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH (pH 8.0), at a photon flux density of 100 μmol photons·m^{-2}·s^{-1}, and aerated using air bubbles at a rate of 200 mL·min^{-1} under these conditions. Antibiotics were added into each culture according to the resistance of the strains at the following concentrations: kanamycin (20 μg·L^{-1}) (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan), spectinomycin (5 μg·L^{-1}) (LKT Laboratories, St. Paul, MN, USA), streptomycin (10 μg·L^{-1}) (Nacalai Tesque, Kyoto, Japan). The optical density at 730 nm (OD_{730}) was measured using a UV–VIS spectrophotometer (model V630; JASCO, Tokyo, Japan).

Plasmid construction and transformation All primer sequences are shown in Table S1. The sequences of the *trc* promoter and the *ispS* gene were amplified from the laboratory plasmids pT31CTH_TePixJ (25) and pUC19-trc-SkIspS, which harbors *skisps* from pBA2SkIKmA2 (addgene: Cambridge, MA, USA) (5), respectively, using

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