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Note

Functional Identification of *Ginkgo biloba* 1-Deoxy-D-xylulose 5-Phosphate Synthase (DXS) Gene by Using *Escherichia coli* Disruptants Defective in DXS Gene

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DXS catalyzes the first step of MEP pathway. *Escherichia coli* disruptants defective in *dxs* were constructed by insertional mutation and characterized. Selected disruptant, DXM3, was auxotrophic for DX or ME. Putative class 1 *DXS* ORF from *Ginkgo biloba* was shown to rescue DXM3 grown without DX or ME supplementation. The putative ORF was thus confirmed as *DXS1*. The disruptant was demonstrated to be useful for *DSX* screening.

Key words: *1-Deoxy-D-xylulose 5-phosphate synthase,* Ginkgo biloba, dxs *disruptant*

Isoprenoids are assembled from the five-carbon building units, IPP and its isomer DMAPP. Biosynthesis of isoprenoids is essential for the survival of all organisms. IPP and DMAPP were initially thought to be biosynthesized only through the mevalonate pathway discovered in 1950s. However, recent discovery of the non-mevalonate pathway led to the study of terpenoid biosynthesis in new perspective.[1)] The new MEP pathway, named based on its key intermediate MEP, was completely elucidated in *E. coli*.[2-6)] Mevalonate pathway was found to operate in animal cells, whereas most eubacteria and cyanobacteria employ MEP pathway. On the other hand, plants can use both pathways for the synthesis of isoprenoids.[9)] MEP pathway has since become a new target for herbicides, antibiotics, and antimalarial drugs.[10,11)]

Seven biochemical steps are necessary for the conversions of pyruvate and GAP into IPP.[2-8)] Briefly, DXP is produced from condensations of pyruvate and GAP by DXS, and DXP is reductively isomerized to produce MEP. In the next step, cytidyl group is transferred to MEP, and MEP moiety is then phosphorylated and cyclized to yield MECDP. Finally, IPP is synthesized from 1-hydroxy-2-methyl-2-(*E*)-butenyl 4-diphosphate, a reductive product of MECDP (Fig. 1).

As stated above, DXS is responsible for the first reaction of MEP pathway. *DXS*'s from several plants were cloned and classified into classes 1 and 2, *DXS1* and *DXS2*.[12)] *DXS1*, mainly expressed in the plant leaves, was postulated to function as a household enzyme, whereas *DXS2* is expressed in the roots for carotenoid biosynthesis.[12)] The plant *DXS* having plastid targeting sequence at the N-terminal region posed a difficulty in over-expression of the catalytically competent enzyme in the *E. coli* system.[13,14)] Though the complementation of *E. coli dxs* disruptant with a putative *DXS* ORF is a facile test to confirm the function, this strategy does not enjoy wide use compared to *dxr* disruptant.[15,16)] Recently, a putative ORF of *DXS1* from ginkgo (*GbDXS1*) was reported (Genbank accession number, AY505128). The present study was aimed to construct an *E. coli dxs* disruptant and identify the function of this ORF.

Materials and Methods

Construction of the disruptant. To construct an *E.coli dxs* disruptant, insertional knock-out strategy was employed by inserting a kanamycin-resistant gene *aphII* inside the *E.coli dxs* gene (Fig. 2). A 3-kb long fragment named g*dxs*, which had additional nucleotides (5'-direction, 568 nts; 3'-direction, 569 nts) at both sides of *E.coli dxs*, was amplified from *E.coli* W3110 chromosomal DNA through PCR with gDXS-F/gDXS-B primer pair (gDXS-F: 5'-ATCCCAGCGATGAATT TGCAG-3', gDXS-B: 5'-GCTGGCGTTCTCGATTTTAAG-3'). It was cloned into pT7Blue vector (Novagene, USA) to construct g*dxs*-pT7, which was then digested with *Nru*I and dephosphorylated by alkaline phosphatase (Takara, Japan). A 1.3-kb *aphII* fragment, cloned to pUC19 from *Tn5*, was isolated by *Hind*III and *Sma*I treatments, and blunted with T4 DNA polymerase (Qiagen, Japan).[17)] The fragment was

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Abbreviations: DMAPP, dimethylallyl pyrophosphate; DX, 1-deoxy-D-xylulose; DXP, 1-deoxy-D-xylulose 5-phosphate; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GAP, D-glyceraldehyde 3-phosphate; IPP, isoprenyl pyrophosphate; LB, Luria-Bertani; ME, 2-*C*-methyl-D-erythritol; MECDP, 2-*C*-methyl-D-erythritol 2,4-cyclodiphosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; nts, nucleotides; ORF, open reading frame.

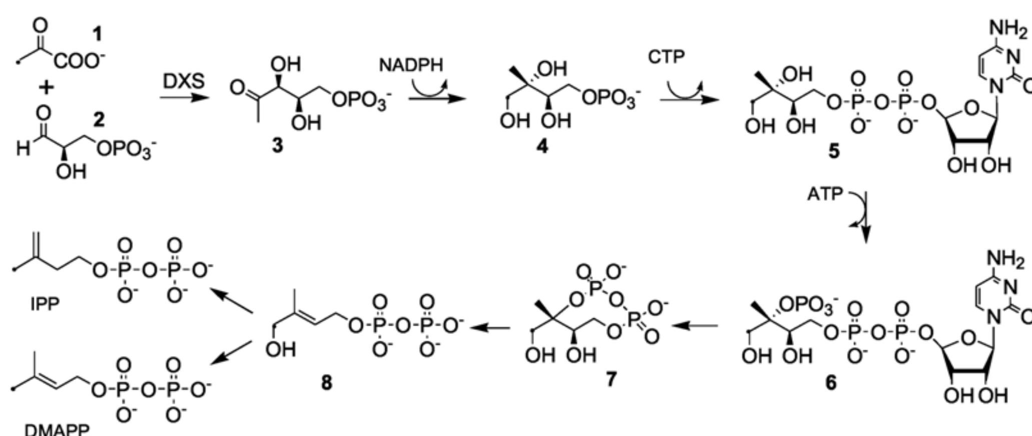

Fig. 1. MEP pathway. **1**, Pyruvate; **2**, glyceraldehyde 3-phosphate; **3**, DXP; **4**, MEP; **5**, 4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol; **6**, 2-phospho-4-(cytidine 5'-diphospho)-2-*C*-methyl-D-erythritol; **7**, MECDP; **8**, 1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate, HMBPP.

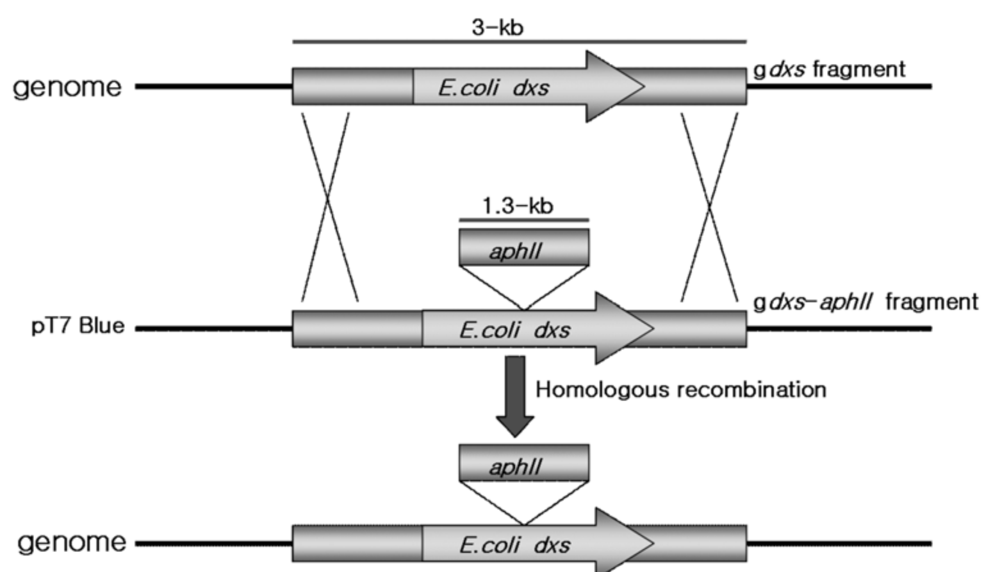

Fig. 2. A construction strategy of the *E. coli dxs* disruptant. Homologous recombination occurred between the g*dxs* region and g*dxs-aph*II fragment.

ligated to *Nru*I-digested g*dxs*-pT7 plasmid, resulting in g*dxs-aphII*-pT7. The 4.3-kb g*dxs-aphII* fragment was isolated by *Bam*HI and *Nde*I digestions, and transformed into *E. coli* FS1576, an *recD* mutant.[18] A transformant, which grew on LB plates containing kanamycin and 0.01% DX or ME,[19,20] but not on LB medium supplemented only with kanamycin, was selected and designated as DXM3.

Cloning of *Ginkgo biloba DXS1* and complementation. The ORF was amplified from the embryonic root cDNA and cloned to pMW118 (Nippon Gene, Japan) to obtain *GbDXS1*-pMW plasmid. DXM3 was transformed with Gb*dxs*1-pMW, and the resulting transformants were incubated at 37°C on LB plate containing kanamycin.

Results and Discussion

The transformant DXM3 grew on LB plates containing kanamycin and DX or ME but not on kanamycin LB medium. Utilization of DX or ME by *E. coli* for the production of MEP[20] thus confirmed the auxotrophic requirement of DXM3 for MEP. The wild-type strain FS1576 could grow on the plate without DX or ME as expected (Fig. 3). The correct homologous recombination in the expected site was confirmed by sequencing. To this end, a 4.3-kb DNA fragment was amplified from DXM3 genomic DNA using gDXS-F/gDXS-B primer pair, cloned to pGEM-T easy (Promega, USA), and sequenced. Upon the performance of PCR using the primer pair of T7 and M4 (T7, 5'-AATACGACTCACTATAG-3'; M4, 5'-GTTTTCCCAGTCACGACGT-3') to exclude the chance presence of g*dxs-aphII*-pT7 in DXM3, amplification of products were not found (data not shown), which is an indication that homologous recombination indeed occurred on the DXM3 chromosome.

With the *dxs* disruptant DXM3 at hand, we set to

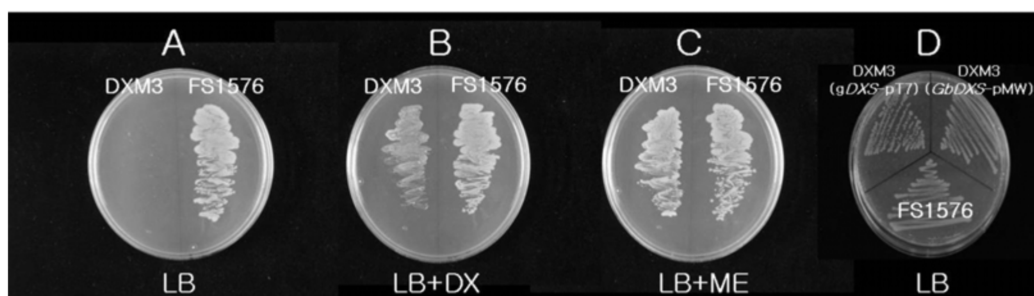

Fig. 3. Complementation assay. (A) The wild-type strain FS1576 grew on the LB plate without DX or ME. (B) The *E.coli dxs* distuptant, DXM3, grew only with DX or (C) ME supplementation. (D) The transformant g*dxs*-pT7 could grow without DX or ME and complementation of the disruptant with Gb*DXS*-pMW could rescue the DXM3 mutant.

functionally identify ORF of the putative *GbDXS1*. The putative *GbDXS1* could rescue the disruptant in the absence of DX or ME supplementation (Fig. 3), which confirmed the identity of ORF as *DXS1*. The disruptant would be useful in screening of putative DXS ORFs, particularly when the overexpression of *DXS* or enzymic assay is difficult.

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