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Cloning and heterologous expression of a cDNA encoding 1-deoxy-D-xylulose-5-phosphate reductoisomerase of *Arabidopsis thaliana*[1]

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Abstract Various plant isoprenoids are synthesized via the non-mevalonate pathway of isopentenyl diphosphate formation. In this pathway, 1-deoxy-D-xylulose 5-phosphate (DOXP), the first intermediate, is transformed to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by an enzyme which was recently cloned from *Escherichia coli*. In order to find a plant homologue of this 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) we cloned a cDNA fragment from *Arabidopsis thaliana* which has high homology to the *E. coli* DXR. By expression of this fragment in *E. coli* we could demonstrate that it encodes a protein which transforms DOXP to MEP. The antibiotic fosmidomycin specifically inhibits this DXR enzyme activity.

Key words: 1-Deoxy-D-xylulose;
1-Deoxy-D-xylulose 5-phosphate reductoisomerase;
1-Deoxy-D-xylulose 5-phosphate pathway;
Isoprenoid biosynthesis; 2-*C*-Methyl-D-erythritol

1. Introduction

In plants there exist two different pathways for the formation of isopentenyl diphosphate (IPP), the common C_5 precursor of isoprenoids. Whereas the biochemical steps of the mevalonate pathway have been known for many years, the elucidation of the steps leading to IPP via the plastidic 1-deoxy-D-xylulose 5-phosphate (DOXP) pathway has just begun. The DOXP pathway is widespread in the plant kingdom [1] and in eubacteria [2]. In higher plants, chloroplast-bound isoprenoids (carotenoids, phytyl side chain of chlorophylls, nonaprenyl side chain of plastoquinone-9) are formed via the DOXP pathway [3–5]. The sterols of higher plants, in turn, are formed and accumulated in the cytosolic cell compartment via the acetate/mevalonate pathway [1,3–5].

The DOXP pathway of IPP formation starts with the formation of DOXP from D-glyceraldehyde 3-phosphate and pyruvate (Fig. 1). DOXP is subsequently transformed to 2-*C*-methyl-D-erythritol 4-phosphate (MEP) by a C-C skeletal rearrangement and a reduction step (Fig. 1) [1]. The operation of this pathway in plants was shown by the incorporation of ^{2}H-, ^{13}C- or ^{14}C-labeled 1-deoxy-D-xylulose into isoprene and phytol [4], into β-carotene [5] and into 2-*C*-methyl-D-erythritol of plants [6] as well as of ^{14}C-labeled DOXP and MEP into β-carotene of plastid preparations of different plants [7]. The 1-deoxy-D-xylulose 5-phosphate synthase, the first enzyme of the DOXP pathway, has been cloned and characterized for several plants [8–10]. A DOXP reductoisomerase (DXR), which transforms DOXP to MEP, was recently cloned in *Escherichia coli* [11,12], but not yet from a plant. In order to clone a plant gene encoding DOXP reductoisomerase, we searched in databases for plant nucleotide sequences with homology to the *E. coli* DXR.

2. Materials and methods

2.1. PCR conditions

Polymerase chain reaction (PCR) was carried out in a total volume of 50 μl with the following reagents: 20 mM Tris-HCl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphates, 2.5% dimethylsulfoxide, 50 pmol of each primer and 2.5 units *Pfu* polymerase (Peqlab, Erlangen, Germany). PCR cycling was as follows: 95°C (1 min); 33 cycles of 95°C (20 s), 57°C (15 s), 72°C (1 min); 72°C (7 min).

2.2. Vectors and bacterial strains used for cloning and expression

Restriction digestion of PCR products and vectors as well as ligation of DNA were performed according to standard protocols [13]. For cloning of PCR products into pBluescript II SK (+) (Stratagene, La Jolla, CA, USA) and into the expression vector pET5b (Promega, Mannheim, Germany) the PCR product and vector DNA were cut with *Eco*RI and *Bam*HI. Vector DNA was cut in the presence of shrimp alkaline phosphatase (Amersham Buchler, Braunschweig, Germany). For cloning and for propagation of plasmids, *E. coli* XL1 Blue MRF′ (Stratagene) was applied. For expression of the cloned DXS and DXR, *E. coli* JM109(DE3) (Promega) was used. LB-agar plates [13] were supplemented with ampicillin (100 mg/l).

2.3. Preparation of RNA from Arabidopsis thaliana

Total RNA was isolated from 4 week old light grown *Arabidopsis thaliana* (var. Columbia) using silica gel based columns as described by the manufacturer (Qiagen, Hilden, Germany). The reverse transcriptase reaction was carried out with 25 μg of RNA, the oligonucleotide $(dT)_{16}$ (25 pmol) and 1000 units of Superscript II reverse transcriptase (Life Technologies, Rockville, MD, USA) for 2 h at 42°C as described by the manufacturer. cDNA synthesis product equivalent to 1 μg of RNA was used for PCR.

2.4. PCR based cloning of pET5b-DXR

In order to amplify the major part of a cDNA encoding *A. thaliana* DXR, we designed the following primers, based on the genomic DNA sequence AB009053 (GenBank) from *A. thaliana*: ATRV, reverse complement of bp 31 505–31 520 (5′-TCAGGATCCGGCGCCTCGTCAATCT-3′, sense oligonucleotide); ATRR, bp 28 974–28 993 (5′-GACGAATTCTTCTTCCAACAACCAATTCT-3′, antisense oligonucleotide). At the 5′-end of the oligonucleotides ATRV and ATRR, a *Bam*HI and a *Eco*RI restriction site were included, respec-

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[1] The sequence reported in this paper has been deposited in the EMBL database under accession number AJ242588.

Abbreviations: DOX, 1-deoxy-D-xylulose; DOXP, 1-deoxy-D-xylulose 5-phosphate; DXR, 1-deoxy-D-xylulose 5-phosphate reductoisomerase; DXS, 1-deoxy-D-xylulose 5-phosphate synthase; GAP, glyceraldehyde 3-phosphate; IPP, isopentenyl diphosphate; ME, 2-*C*-methyl-D-erythritol; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; Tris, tris-hydroxymethyl-aminomethane

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tively (the underlined sequences denote the engineered restriction sites). With these primers a fragment of about 1.2 kbp was amplified from cDNA of *Arabidopsis*. The fragment was gel purified, digested with *Eco*RI and *Bam*HI and ligated into pBluescript II SK (+). After transformation into *E. coli* XL1Blue MRF', the clone designated pBlue-DXR was sequenced by Seqlab GmbH (Göttingen, Germany) using M13 and M13-reverse primers. pBlue-DXR was cut with *Bam* and *Eco*RI and the 1242 bp insert was cloned into pET5b. The resulting clone was named pET5b-DXR and was sequenced using two sequencing primers which have been derived from the sequence of pBlue-DXR: DXRV (5′-AAGTAGCGGATGCGTTGAAG-3′) DXRR (5′-CGCTTCAATGACCTCAAGAC-3′).

2.5. Cloning of 1-deoxyxylulose-5-phosphate synthase into the expression vector pET5b

For the synthesis of unlabeled as well as ^{14}C-labeled DOXP, we constructed an expression clone for the *Chlamydomonas reinhardtii* 1-deoxyxylulose-5-phosphate synthase (DXS). The coding region of a cDNA clone of *Chlamydomonas reinhardtii* DXS (pBlue-DXS, accession number AJ007559) was amplified using the following primers: DXSEV1, bp 332–346 of AJ007559 (5′-TCAGGATCCGGAGGAGATTGATGA-3′, sense oligonucleotide, *Bam*HI restriction site added) and DXSER, reverse complement of bp 2373–2387 (5′-CTCGAATTCTGTGATTGCCGTCGT-3′, antisense oligonucleotide, *Eco*RI restriction site added). PCR with the plasmid pBlue-DXS (encoding AJ007559) and the primers DXSEV1 and DXSER resulted in a PCR product of approximately 2000 bp which was gel purified, cut with *Bam*HI and *Eco*RI and cloned into pET5b. The resulting plasmid was named pET5b-DXS.

2.6. Expression of pET5b-DXS and pET5b-DXR

E. coli JM 109(DE3) cells harboring pET5b-DXS or pET5b-DXR were grown at 37°C in 100 ml of 2 YT medium (16 g/l tryptone, 10 g/l yeast extract, 10 g/l NaCl) with ampicillin (100 μg/ml) to an OD_{600} of 0.8. Then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a concentration of 0.4 mM and the cells were maintained for 4 h at 22°C. Bacteria were harvested by centrifugation (2000×*g*, 20 min) and washed with 10 ml of 1% NaCl. The cells were resuspended in 4 ml of extraction buffer (100 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 2 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride). After cell disruption with a French press at 0–4°C, the resulting homogenate was centrifuged at 0–4°C to pellet cell debris (10 000×*g*, 30 min). Protein concentration of cell-free extracts was determined and always between 2 and 5 μg/μl.

2.7. Synthesis of DOXP

Cell-free extract (5 μl) of DXS expressing cells (*E. coli* JM109(DE3)/pET5b-DXS) was added to the reaction mixture (20 μl total volume, containing 100 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM NaF, 2 mM dithiothreitol, 100 μM phenylmethylsulfonyl fluoride, 0.5 mM thiamine diphosphate, 5 mM Na-pyruvate, 10 mM DL-glyceraldehyde 3-phosphate). For the synthesis of ^{14}C-labeled DOXP, [2-^{14}C]pyruvate was added to the reaction (37 kBq). The assay mixture was incubated at 30°C for 30 min and then the reaction was stopped by heating to 95°C for 30 s. The formation of the radioactive product was checked by separation on silica gel with *n*-propanol/ethylacetate/H_2O 6:1:3 (v/v/v) and detection of radioactivity using a Packard Instant Imager (Canberra Packard). One radioactive product band was detected at $R_f = 0.36$ while pyruvate was found at $R_f = 0.61$. After treatment with alkaline phosphatase, the radioactive product was found at $R_f = 0.76$.

2.8. DXR enzyme assay

The supernatant of DXR expressing cells (*E. coli* JM109(DE3)/pET5b-DXR) (5 μl) was transferred to the reaction mixture (20 μl total volume, containing 100 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 5 mM NaF, 2 mM dithiothreitol, 0.5 mM NADPH and 2 μl of DXS synthesis mixture which is approximately 0.5 mM ^{14}C-labeled phosphate DOXP). The assay mixture was incubated for 30 min at 30°C and the reaction stopped by heating to 95°C for 30 s. $CaCl_2$ was added to a concentration of 50 mM and the assay mixture was incubated for 2 h at 30°C with 2 units of alkaline phosphatase (Sigma, Deisenhofen, Germany). The dephosphorylated reaction mixture was separated by TLC on silica gel with acetone/ethylacetate/H_2O 50:50:1 (v/v/v). Radioactivity was detected using a Packard Instant Imager (Canberra Packard). Non-radioactive products or reference substances (DOX, ME) were stained with methanol/acetic acid/sulfuric acid/*p*-anisaldehyde 85:10:5:0.5 (v/v/v). Authentic DOX ($R_f = 0.34$) was synthesized as described in [16] and methylerythritol ($R_f = 0.1$) was isolated from *Liriodendron tulipifera* [14].

2.9. Identification of DOXP and MEP

Non-radioactive DOXP was synthesized with the recombinant *Chlamydomonas* DXS and used for a DOXP reductoisomerase reaction as described above in a total volume of 1 ml. After stopping the DXR reaction by heating, the assay mixture was separated by TLC on silica gel with *n*-propanol/ethylacetate/H_2O 6:1:3 (v/v/v). A band ($R_f = 0.36$) was recovered, dephosphorylated using alkaline phosphatase and acetylated for 12 h at room temperature using pyridine/acetic acid anhydride 1:1 (v/v). The fraction was analyzed by GC/MS (Hewlett Packard 5890 Series II, crosslinked methylsilicone 20 m×0.32 mm, coupled with a Hewlett Packard 5971A Mass Selective Detector). Temperature programming was set to 80°C for 3 min and heating to 280°C (20°C/min).

3. Results and discussion

3.1. Related protein sequences to the E. coli DXR

The DOXP reductoisomerase (DXR) gene of *E. coli* (accession number P45568) was compared to other known protein sequences via a data base search using the TBlastN program of the European Molecular Biology Laboratory Husar Package (European Molecular Biology Laboratory, Heidelberg, Germany). We found a group of highly related proteins from the following bacteria: *Synechocystis* PCC6803

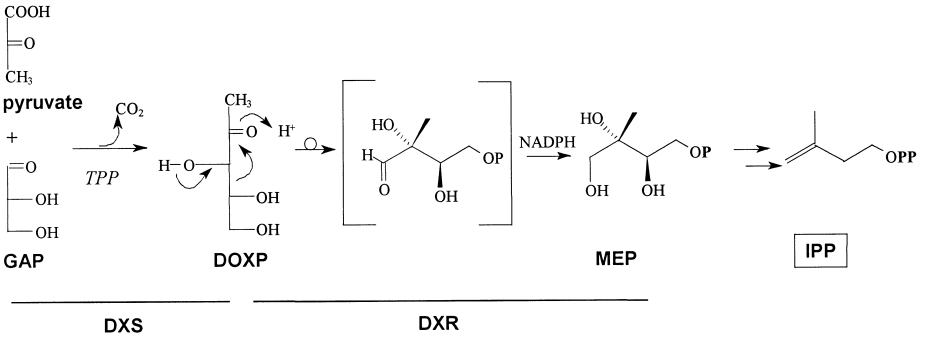

Fig. 1. Biosynthesis of IPP via the non-mevalonate DOXP pathway. DOXP is formed by the addition of a pyruvate derived C_2-unit to D-glyceraldehyde 3-phosphate (GAP) in a transketolase-like manner (TPP, thiamine diphosphate). DOXP is transformed by a C-C rearrangement and a subsequent reduction step to MEP. This enzymic step is catalyzed by DXR. MEP is transformed to IPP by a series of dehydration and reduction steps, including one kinase, that are not yet known.

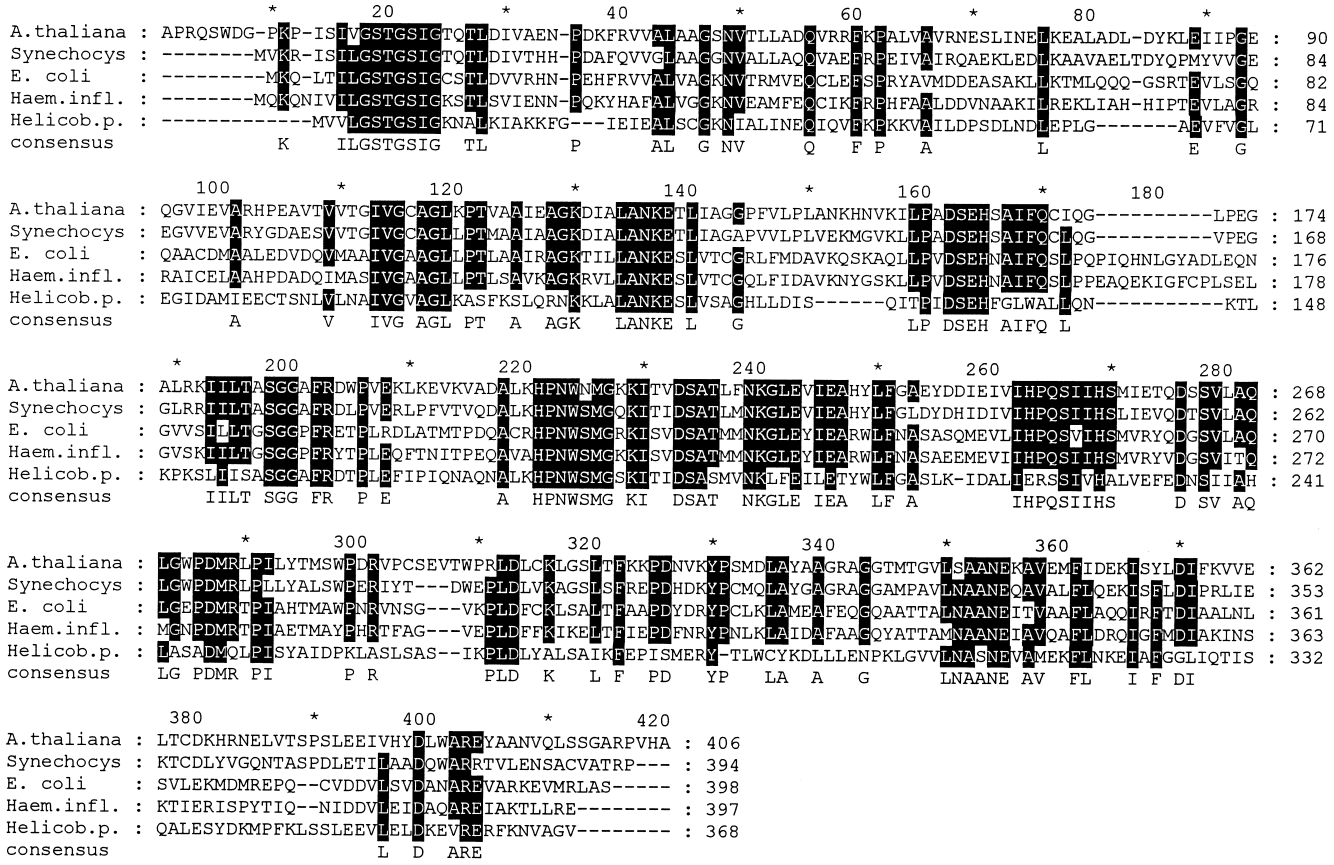

Fig. 2. Alignment of the protein sequences of the *A. thaliana* DXR with *E. coli* DXR (accession number P45568) and the predicted protein sequences of *Synechocystis* PCC6803 (Synechocys, Q55663), *Haemophilus influenzae* (Haem. infl., p44055) and *Helicobacter pylori* (Helicob. p., P56139). Hyphens denote the absence of amino acid residues. The shaded amino acid residues denote those which are highly conserved in at least four of the five sequences.

(Q55663), *Bacillus subtilis* (B69881), *Haemophilus influenzae* (p44055), *Mycobacterium tuberculosis* (q10798), *Chlamydia trachomatis* (A71562), *Aquifex aeolicus* (O66722), *Treponema pallidum* (ae001235) and *Helicobacter pylori* (P56139). All these protein sequences were about 400 amino acids in length and no function has yet been assigned to all these bacterial DXR homologues. Highly conserved amino acid residues were found spread over the entire protein sequence and the sequence '(I/V)(L/V)GSTGSIG' was found close to the N-terminus of the *E. coli* DXR and all bacterial homologues (Fig. 2).

We also found in data bases two DNA sequences from *A. thaliana* which obviously encode a protein related to the bacterial DXR sequences: in a genomic DNA sequence from chromosome 5 of *A. thaliana* (accession number AB009053), the reverse complement of bp 28 974–31 520 seemed to encode a DXR homologous protein sequence, which was, however, disrupted by several introns. The amino acid residues 'IVGSTGSIG', which are highly conserved near the N-termini of the bacterial DXR sequences, are encoded by nucleotides 31 461–31 487 (reverse complement) of this sequence. This highly conserved amino acid sequence motif was supposed also to be close to the N-terminus of a mature DXR protein of *Arabidopsis* (after cleavage of a possible transit peptide sequence). In addition to the genomic DNA sequence, we also found DXR homology in a cDNA sequence of *A. thaliana* (EMBL database accession number AA586087) which has been cloned as an expressed sequence tag [17]. We obtained the corresponding cDNA-clone (named 56F11) from the Arabidopsis Biological Resource Center (DNA Stock Center, Columbus, OH, USA). After complete sequencing of this clone, we found that the 722 bp cDNA can be assigned to bp 28 799–29 947 (reverse complement) of the genomic sequence AB009053. It obviously represents a part of the transcript of the putative DXR gene, including an open reading frame of about 200 amino acids, followed by a 3′ untranslated sequence and the 3′ poly-A tail. With the N-terminal conserved amino acid residues and the 3′ end of a corresponding cDNA, enough information was provided to isolate a cDNA fragment from *Arabidopsis* encoding a DXR homologous protein. We cloned a cDNA fragment of the putative *Arabidopsis* DXR gene (designated pET5b-DXR) by a PCR based cloning method which subsequently allowed us to check its enzymic activity by heterologous expression in *E. coli*.

In order to produce the substrate 1-deoxy-D-xylulose-5-phosphate for the DXR reaction, we constructed the expression vector pET5b-DXS, with a 2055 bp insert derived from a cDNA encoding DXS of *C. reinhardtii* (this sequence has been deposited to the EMBL database under accession number AJ007559). We previously cloned this cDNA that encodes a protein highly homologous to the amino acid sequences of other plant DXS [8–10].

3.2. Sequence analysis of pET5b-DXR

The cDNA insert of pET5b-DXR (1242 bp) has an open reading frame encoding 406 amino acids with a predicted

molecular mass of 44 212 Da (Fig. 2). The predicted protein sequence shares 44% and 66% identical amino acid residues with the *E. coli* DXR (P45568) and the *Synechocystis* PCC 6803 DXR homologue (Q55663), respectively (cf. Fig. 2). Alignment of the cDNA nucleotide sequence with the genomic sequence AB009053 (reverse complement of bp 28 974–31 520) showed that this part of the gene consists of 11 exons and 10 introns. All exon/intron junctions follow the GT/AG rule [18].

3.3. Expression of A. thaliana DXR

Cultures of *E. coli* JM109(DE3)/pET5b-DXR were induced with IPTG, the bacterial cells were harvested and the cell-free extracts were separated by SDS-polyacrylamide gel electrophoresis. A prominent band of about 46 kDa was visible, which was not found in control cells (*E. coli* JM109(DE3) transformed with pET5b without insert, data not shown). The size of the 46 kDa protein band, found by SDS-polyacrylamide gel electrophoresis, is in accordance with the calculated size of 45 661 Da of the expressed protein. This fusion protein consists of a N-terminal T7-tag (14 amino acids encoded by pET5b) and of the cDNA encoded protein (406 amino acids, Fig. 2).

3.4. DXR enzyme assay

Cell extracts of induced *E. coli* JM109(DE3)/pET5b-DXR were assayed using ^{14}C-labeled DOXP and NADPH as substrates. After dephosphorylation, a radioactive product was found (Fig. 3, lane 1) which cochromatographed with authentic methylerythritol (ME) (R_f 0.1). Under the same assay conditions, no formation of ME was detected, if *E. coli* JM109(DE3) cells were transformed with pET5b without insert (not shown). When NADPH was omitted, no ME product could be detected (Fig. 3, lane 2). In addition, the formation of MEP could be fully inhibited by adding fosmidomycin (1 μM) to the assay mixture (Fig. 3, lane 3). Fosmidomycin is a specific inhibitor of the *E. coli* DXR [19] and of plant terpenoid biosynthesis [20]. Thus the formation of the radioactive DXR product is dependent on NADPH and can be inhibited by fosmidomycin, an antibiotic and herbicidal substance.

3.5. Identification of DOXP and MEP

In addition to the radioactive DXR assay, non-radioactive DOXP was used as a substrate for the recombinant *A. thaliana* DXR and both DOXP and MEP were identified by GC/MS. After separation of the DXR reaction mixture by TLC (*n*-propanol/ethylacetate/H_2O 6:1:3, v/v/v), a band at $R_f = 0.36$ was recovered. Alkaline phosphatase treatment yielded a dephosphorylated fraction which was acetylated and analyzed by GC/MS. Both 1-deoxyxylulose triacetate and methylerythritol triacetate could be identified by comparison with the retention times and MS spectra of authentic DOX triacetate and ME triacetate: DOX triacetate (7.86 min), GC/MS: *m/z* = 217 (2%), 158 (23%), 145 (11%), 115 (100%), 103 (27%), 74 (9%), 73 (12%), 61 (11%). Methylerythritol triacetate (8.17 min), GC/MS: *m/z* = 187 (2%), 159 (31%), 145 (5%), 140 (10%), 129 (94%), 117 (100%), 103 (18%), 87 (26%), 86 (13%), 75 (20%). The MS data found for ME triacetate were also in accordance with those reported in [15].

Our results described here show that we have cloned a cDNA fragment from *A. thaliana* which encodes a protein very similar to the *E. coli* DXR and a family of homologous bacterial proteins (Fig. 2). By expression of this cDNA in *E. coli* we could demonstrate that it encodes the enzyme which catalyzes the NADPH dependent transformation of DOXP to MEP. According to this biochemical property, this enzyme is obviously the DOXP reductoisomerase involved in the plastidic IPP and isoprenoid biosynthesis of *Arabidopsis*. According to Southern blot analysis (data not shown), the *Arabidopsis* DXR seems to be a single copy gene as is the case for the *Arabidopsis* DXS [8]. For the first enzyme of the DOXP pathway, DXS, it has been shown in *Arabidopsis* that it is needed for plastid greening and development [8]. A similar dependence of the plastidic isoprenoid formation and chloroplast development on the DXR enzyme is to be expected and will be checked by antisense plants. The existence of a possible N-terminal plastid targeting sequence of the *Arabidopsis* DXR is under investigation. Fosmidomycin, an inhibitor of plant isoprenoid biosynthesis [20] and bacterial DXR [11], strongly inhibits the recombinant *Arabidopsis* DXR, proving that the plant DXR is the target of this herbicidal substance. The high sequence homology of the *Arabidopsis* DXR sequence with the sequences in the photosynthetic blue-green bacterium *Synechocystis* and the heterotrophic bacteria, shown in Fig. 2, strongly suggests that these genes with so far unknown function encode the bacterial DXR. It is of interest in this context that the existence of the DOXP pathway in isoprenoid biosynthesis in *E. coli* [21] and *Synechocystis* [22] has been shown before via labeling studies.

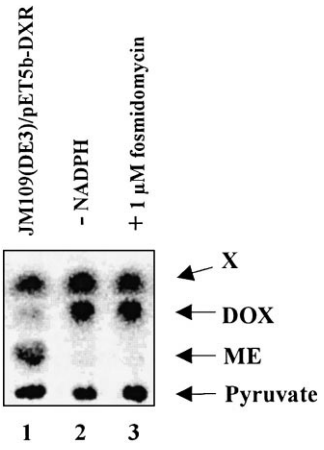

Fig. 3. After induction of *E. coli* JM109(DE3)/pET5b-DXR with IPTG, cell free extracts were incubated with ^{14}C-labeled DOXP and NADPH. After a successive treatment of the assay mixture with alkaline phosphatase and separation by TLC, the radioactive product ME was detected with an instant imager (lane 1). When NADPH was omitted (lane 2) or the inhibitor fosmidomycin was added to the assay mixture (lane 3), no ME could be detected. X = unknown product of ^{14}C-pyruvate resulting from the DXS reaction.

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