

Short sequence-paper

Cloning and expression of cDNAs encoding two enzymes of the MEP pathway in *Catharanthus roseus*¹

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Received 14 June 2000; received in revised form 29 August 2000; accepted 21 September 2000

Abstract

Two periwinkle cDNAs (*crdxr* and *crmeccs*) encoding enzymes of the non-mevalonate terpenoid pathway were characterized using reverse transcription-PCR strategy based on the design of degenerated oligonucleotides. The deduced amino acid sequence of *crdxr* is homologue to 1-deoxy-D-xylulose 5-phosphate reductoisomerases. *Crmeccs* represents the first plant cDNA encoding a protein similar to the 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase from *Escherichia coli*. Expression of *crdxr* and *crmeccs* genes was up-regulated in periwinkle cells producing monoterpenoid indole alkaloids. Involvement of the 2C-methyl-D-erythritol 4-phosphate pathway in alkaloid biosynthesis is discussed. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: 1-Deoxy-D-xylulose 5-phosphate reductoisomerase; 2C-Methyl-D-erythritol 2,4-cyclodiphosphate synthase; Monoterpenoid indole alkaloid; 2C-Methyl-D-erythritol 4-phosphate pathway; *Catharanthus roseus*

Until recently, the biosynthesis of isopentenyl diphosphate (IPP), the common precursor of all isoprenoids, was assumed to proceed exclusively via the cytosolic mevalonate pathway. It is now clear that in eubacteria, algae and higher plants, an alternative non-mevalonate pathway leads to IPP [1,2]. In plants, evidence has recently emerged that shikonin, sterols and many sesquiterpenes originate from the mevalonate pathway [3–5] whereas plastidic isoprenoids such as monoterpenes, carotenoids, plastoquinones and the phytol side-chain of chlorophylls derive from the alternative pathway [4,6]. For some isoprenoids, a mixed origin was reported. Thus, chamomile sesquiterpenes are formed via both pathways [7].

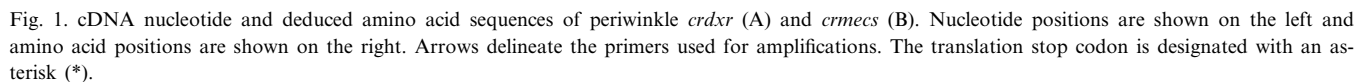
The first step of the non-mevalonate pathway (also designated 2C-methyl-D-erythritol 4-phosphate (MEP) pathway, see below) is the condensation of glyceraldehyde 3-phosphate and pyruvate into 1-deoxy-D-xylulose 5-phosphate (DXP) by the DXP synthase. The corresponding gene (*dxs*) has been isolated in bacteria [8,9] and several plant species [10–12]. In the second step, DXP is converted

into MEP. The reaction is catalyzed by the DXP reductoisomerase specified by the *dxr* gene, which has been characterized for bacteria [13], *Arabidopsis thaliana* [14] and *Mentha piperita* [15]. The following two steps have been elucidated in *Escherichia coli* and more recently in higher plants [16]. The sequential action of the two enzymes, 4-diphosphocytidyl-2C-methyl-D-erythritol synthase [17,18] and 4-diphosphocytidyl-2C-methyl-D-erythritol kinase [19,20], converts MEP into 4-diphosphocytidyl-2C-methyl-D-erythritol 2-phosphate. Thereafter, the latter compound is transformed into 2C-methyl-D-erythritol 2,4-cyclodiphosphate (MEC) by the MEC synthase specified in *E. coli* by the unannotated *ygbB* gene [21]. Other so far unknown steps lead to IPP.

We are currently studying the regulation of biosynthesis of the therapeutically valuable monoterpenoid indole alkaloids (MIAs) in periwinkle (*Catharanthus roseus* [L.] G. Don). Recent ¹³C nuclear magnetic resonance studies have given evidence that the MEP pathway is involved in the biosynthesis of the terpenoid moiety of the MIA precursors loganin [22] and secologanin [23]. We previously characterized the *dxs* cDNA in periwinkle [12]. We report here on the isolation of two other cDNAs of the MEP pathway, *crdxr* and *crmeccs*, encoding *C. roseus* DXP reductoisomerase and MEC synthase, respectively. This is the first characterization of a cDNA encoding MEC synthase in

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¹ The nucleotide sequence data reported in this paper have been submitted to the GenBank database under accession no. AF250235 for *crdxr* and AF250236 for *crmeccs*.



We first isolated a *C. roseus* cDNA fragment by a strategy based on identifying sequence similarities among pre-

viously cloned genes encoding DXP reductoisomerases from *A. thaliana* [14], *M. piperita* [15], and *Synechocystis* sp. PPC6803 (accession number Q55663). Regions of high homology were used to design the two degenerated

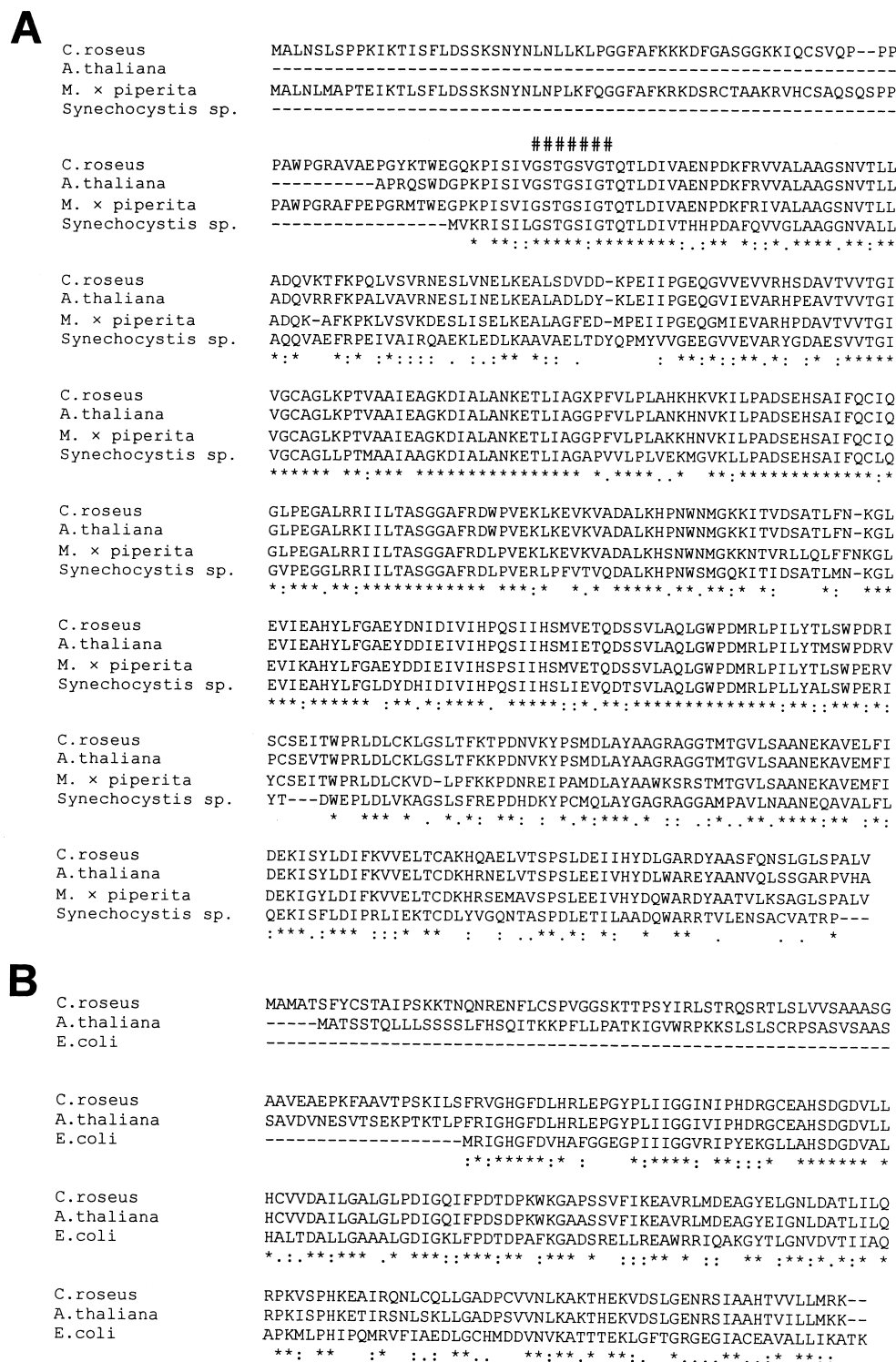


Fig. 2. Alignments of protein sequences. A: Comparison of the deduced amino acid sequence CRDXR from *C. roseus* with DXP reductoisomerase from *A. thaliana* (accession number AJ242588), *M. piperita* (accession number AF116825) and *Synechocystis* sp. PCC6803 (accession number Q55663). The putative NADPH binding motif is indicated by the # signs. B: Comparison of the deduced amino acid sequence CRMECS from *C. roseus* with the *E. coli* MEC synthase (accession number AF230738) and the homologue predicted protein of *A. thaliana* (accession number AF07360).

primers DXR5 5'-AT(A,C,T)GG(A,T,G,C)AC(A,T,G,C)-CA(A,G)AC(A,T,G,C)(C,T)T(A,T,G,C)GA-3' (forward primer) and DXR4 5'-C(T,G)CAT(C,A)TC(A,T,G,C)-GGCCA(A,T,G,C)CC-3' (reverse primer). We amplified

an internal fragment by reverse transcription (RT)-PCR using 1 µg of total RNA as template. After cloning into pGEM-T vector (Promega) and sequence analysis, the amplicon was used to isolate the entire coding region of the

cDNA. The 3'-end of the cDNA was characterized through 3'-rapid amplification of cDNA ends (RACE)-PCR (Gibco BRL) with the sense primer DXR12, 5'-GCTTTGAAGCATCCCAACTGGAAT-3' and an anti-sense (dT)-adapter primer. To isolate the 5'-upstream terminal region of the cDNA, an asymmetric PCR was performed on a λ -ZAPII-oriented cDNA library with the antisense primer DXR10, 5'-TGACAGAACTAACTGAGGCTTGA-3' and the M13 reverse universal primer (sense orientation). The resulting PCR fragments were cloned and sequenced. The full-length cDNA (designated *crdxr*) was achieved by a final PCR using primers from the 5'- and 3'-untranslated regions and the oriented cDNA library as template. The cDNA sequence is 1740 nucleotides long and consists of an open reading frame encoding a peptide of 474 amino acids (Fig. 1A), with a calculated molecular mass of 51 kDa and an isoelectric point of 6.1. The predicted CRDXR amino acid sequence presents high homologies with *A. thaliana* and *M. piperita* DXP reductoisomerases (89% and 76% identity, respectively) (Fig. 2A). Moreover, CRDXR like other DXP reductoisomerases contains in its N-terminal region a conserved motif with some homology to the NADPH binding site of ketol acid reductoisomerase [24] (Fig. 2A).

For the characterization of the second cDNA (*crmees*), a BLAST search using the *E. coli* MEC synthase [21] allowed identification of EST clones from *Zea mays* (accession number AI712133), *Glycine max* (accession number AW202270) and an *A. thaliana* genomic sequence (accession number AAF07360). On the basis of their highly conserved amino acid sequences, two degenerated oligonucleotides were synthesized. The sense primer YB2, 5'-GA(T,C)(C,A)G(G,A,C,T)GG(G,A,C,T)TG(T,C)GA(A,G)GC-3' and the antisense primer YB4, 5'-TA(G,A,C,T)CC(G,A,C,T)GC(C,T)TC(G,A)T(G,C)CAT-3' were used to amplify an internal fragment by RT-PCR. This cDNA was cloned and sequenced. The deduced amino acid sequence revealed 53% identity with the MEC synthase from *E. coli* (AF230738). As previously described for *crdxr*, the 3'- and 5'-ends of the cDNA were obtained through 3'-RACE-PCR and 5'-asymmetric PCR using the specific primers YB8, 5'-GGGTTACCGACATAGGGCAAA-3' (sense orientation) and YB6, 5'-TGAAGAAGGTGCTCCTTTCCATT-3' (antisense orientation), respectively. Amplification with primers designed in the 5'- and 3'-untranslated regions allowed us to obtain the full-length sequence. The 994 bp long cDNA (referred to as *crmees*) contains an open reading frame encoding a peptide of 236 amino acids (Fig. 1B) with a calculated molecular mass of 25 kDa and an isoelectric point of 9.0. The deduced amino acid sequence shows 48% identity with the *E. coli* MEC synthase and 90% identity with an unknown protein specified by an *A. thaliana* ortholog (accession number AAF07360) (Fig. 2B).

The N-termini of periwinkle DXP reductoisomerase and MEC synthase revealed typical features of plastidial tar-

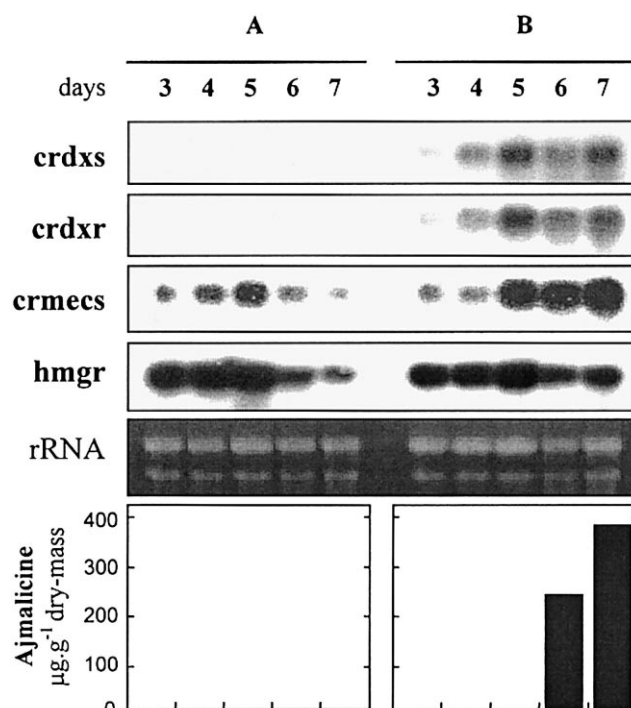


Fig. 3. Time course of *crdxs*, *crdxr*, *crmees* and *hmgr* mRNA levels (upper) and ajmalicine contents (lower) in *C. roseus* cells grown on MM (A) or a MIA production medium (B). Total RNA (30 µg) was fractionated by denaturing agarose gel electrophoresis, transferred onto nylon membranes and hybridized with ³²P-radiolabeled cDNA probes (*crdxs*: the full-length cDNA, *crdxr*: a 800 bp internal fragment, *crmees*: a 600 bp 3'-end fragment, *hmgr*: a 1500 bp 3'-end fragment) at 42°C for 12 h. Membranes were washed twice for 30 min at low stringent washing conditions (2×SSC and 0.1% sodium dodecyl sulfate (SDS) at 42°C) and 10 min at high stringency (0.1×SSC and 0.1% SDS at 65°C) and then autoradiographed (Biomax, Amersham-Pharmacia Biotech). Ethidium bromide-stained gel picture indicated uniform loading and integrity of RNA samples. Indole alkaloids were extracted with methanol from 25 mg freeze-dried cells. Concentration of ajmalicine, the chosen marker of alkaloid accumulation, was estimated spectrofluorometrically.

getting sequences [25] which were confirmed by the ChloroP neural network program [26]. This prediction is consistent with the proposed subcellular localization of the MEP pathway for IPP biosynthesis in plants [2].

The expression of both corresponding genes, and also that of the *crdxs* gene encoding *C. roseus* DXP synthase [12], was investigated by Northern blot experiments in periwinkle cell cultures. We previously reported that periwinkle cells grown in a maintenance medium (MM) containing 4.5 µM 2,4-dichlorophenoxyacetic acid (2,4-D) did not accumulate MIAs. However, transferring the cells in a 2,4-D-free medium (PM) induced MIA production [27] (Fig. 3). Cells grown in MM and PM were harvested from day 3 to day 7 for RNA extraction and alkaloid quantitation [28]. Expression to the MEP pathway genes was studied in both culture conditions. In the same way, we looked at the expression of the periwinkle *hmgr* gene [29], which encodes the key enzyme of the classical mevalonate pathway [30]. As shown in Fig. 3, neither *crdxs* nor

crdxr transcripts were found in cells grown in MM. In cells cultured in PM, *crdxs* and *crdxr* transcripts accumulated from day 4 onward. *CrmeCs* transcripts were present in cells grown in both media, but their abundance was greatly increased after 2,4-D suppression. No significant difference was observed in the *hmgr* expression profile of cells grown in MM or PM.

The presence of two pathways leading to IPP raises the question about the origin of MIAs in periwinkle. Our results show that the *hmgr* gene cannot be regarded as controlling a key step (at the transcriptional level) in the production of MIAs in periwinkle, although we cannot exclude a minor participation of the mevalonate pathway to the production of MIAs [12]. However, we clearly demonstrate a correlation between MEP pathway gene expression and MIA accumulation. Our results corroborate recent studies showing that the MIAs are mainly formed via the MEP pathway [23,24]. This view is reinforced by a preliminary result (Courtois, M., unpublished data) showing that treatment of MIA accumulating cells with fosmidomycin, a specific inhibitor of DXP reductoisomerase [31], led to a complete inhibition of MIA production without affecting the cell growth. The Northern blot profiles also suggest that the genes encoding CRDXS and CRDXR enzymes are good candidates for a regulatory control of MIA production.

This work was supported by a grant from a Ministère de l'Éducation Nationale, de la Recherche et de la Technologie (France) and by a doctoral fellowship from the Région Centre (to B.V.). We thank Dr. J. Memelink for the gift of the cDNA library and Dr. C.L. Nessler for the *hmgr* cDNA clone.

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