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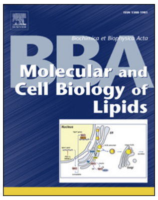

Detection and molecular cloning of *CYP74Q1* gene: Identification of *Ranunculus acris* leaf divinyl ether synthase

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Enzymes of the CYP74 family, including the divinyl ether synthase (DES), play important roles in plant cell signalling and defence. The potent DES activities have been detected before in the leaves of the meadow buttercup (*Ranunculus acris* L.) and few other Ranunculaceae species. The nature of these DESs and their genes remained unrevealed. The PCR with degenerate primers enabled to detect the transcript of unknown P450 gene assigned as *CYP74Q1*. Besides, two more *CYP74Q1* isoforms with minimal sequence variations have been found. The full length recombinant CYP74Q1 protein was expressed in *Escherichia coli*. The preferred substrates of this enzyme are the 13-hydroperoxides of α-linolenic and linoleic acids, which are converted to the divinyl ether oxylipins (ω5*Z*)-etherolenic acid, (9*Z*,11*E*)-12-[(1′*Z*,3′*Z*)-hexadienyloxy]-9,11-dodecadienoic acid, and (ω5*Z*)-etheroleic acid, (9*Z*,11*E*)-12-[(1′*Z*)-hexenyloxy]-9,11-dodecadienoic acid, respectively, as revealed by the data of mass spectrometry, NMR and UV spectroscopy. Thus, CYP74Q1 protein was identified as the *R. acris* DES (RaDES), a novel DES type and the opening member of new CYP74Q subfamily.

1. Introduction

The oxidative metabolism of polyenoic fatty acids through the lipoxygenase pathway is a source of numerous oxylipins, which play important roles in regulation of plant growth and development, cell signalling and defence [1,2]. Metabolism of fatty acid hydroperoxides and thus the diversity of oxylipins largely depend on the enzymes of CYP74 family [1,2]. These are allene oxide synthase (AOS), hydroperoxide lyase (HPL) and divinyl ether synthase (DES) [1–4]. Hitherto the CYP74s were known only as the constituents of plant species. New CYP74 genes and enzymes have been detected recently in some proteobacteria and metazoan species [5]. Thus, the CYP74 family has been extended to the CYP74 clan [6], which includes new bacterial and metazoan members. A novel CYP74 enzyme, the epoxy alcohol synthase, has been detected recently in the lancelet *Branchiostoma floridae* [5].

DESs have been detected in several flowering plant species [7–15]. Moreover, the divinyl ethers have been detected in brown alga *Laminaria sinclairii* [16] and red alga *Polyneura latissima* [17], although no CYP74 genes have been detected in algae yet [7]. DES genes of Solanaceae species (CYP74D) [18–20], garlic (CYP74H1) [21], and flax (CYP74B16) [22] have been cloned, and the properties of these recombinant DESs have been characterized. All other described DES activities have been detected in plant tissues, but the corresponding genes and proteins have not yet been detected, sequenced and cloned. Divinyl ethers play the defensive and antimicrobial role in plants [23–27].

Majority of DESs are present in non-green plant tissues, e.g. potato tubers, tomato roots, and garlic bulbs [7]. There are only two known DESs present in plant leaves. These are flax [13,22] and Ranunculaceae [10–12] DESs. Flax DES has been recently cloned and identified as CYP74B16 [22], an unprecedented 13-DES member of the CYP74B subfamily, while all other CYP74B members are 13-HPLs [7]. The nature of Ranunculaceae DESs remains uncovered. This prompted us to look for the CYP74 transcripts in *Ranunculus acris* leaves. Using the degenerate primers, we succeeded to detect an unknown CYP74 transcript. The present paper reports the cloning of corresponding full length cDNA and identification of the recombinant protein as *R. acris* DES (RaDES), CYP74Q1.

2. Materials and methods

2.1. Materials

The aerial parts of wild *R. acris* plants were collected near the lake Sredny Kaban (Kazan) in Summer seasons 2012 and 2013. Linoleic and α-linolenic acids, as well as the soybean lipoxygenase type V,

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Abbreviations: DES, divinyl ether synthase; RaDES, *Ranunculus acris* divinyl ether synthase; HPL, hydroperoxide lyase; AOS, allene oxide synthase; 13(*S*)-HPOT, (9*Z*,11*E*,13*S*,15*Z*)-13-hydroperoxyoctadecatrienoic acid; 13(*S*)-HPOD, (9*Z*,11*E*,13*S*)-13-hydroperoxyoctadecadienoic acid; 9(*S*)-HPOD, (9*S*,10*E*,12*Z*)-9-hydroperoxyoctadecadienoic acid; IMAC, immobilized metal affinity chromatography (IMAC); TMS, trimethylsilyl; GC-MS, gas chromatography-mass spectrometry; ECL, equivalent chain lengths; ORF, open reading frame; IHCD, I-helix central domain, the catalytically important six amino acid domain in the centre of P450 I-helix

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Table 1
Degenerate oligonucleotide primers used in PCR for the detection of the CYP74 genes transcripts in *Ranunculus acris* leaves transcriptome.

Name	Primer sequence 5′ to 3′	$T_{m,}$ °C
RaF1	gA(A/g)AAg(C/g)ACAAgAgCAC(g/C)gT(g/T)TTC	58.2/60.2
RaR1	CA(T/A)Ag(A/C)A(g/A)CTC(C/g/A)CCTTTCTTg	43.6/57.9
RaF2	CT(T/C)gT(T/C)gg(T/C/g)gA(T/C)TTCATgCC	50.3/59.0
RaR2	ggCATgAA(g/A)TC(C/g/A)CC(g/A)AC(g/A)Ag	50.3/60.2

were purchased from Sigma. Adams's catalyst and silylating reagents were purchased from Fluka (Buchs, Switzerland). (9*S*,10*E*,12*Z*)-9-Hydroperoxy-10,12-octadecadienoic acid (9-HPOD) was prepared by incubation of linoleic acid with tomato fruit lipoxygenase at 0 °C, pH 6.0, under continuous oxygen bubbling. (9*Z*,11*E*,13*S*,15*Z*)-13-Hydroperoxy-9,11,15-octadecatrienoic (13-HPOT) and (9*Z*,11*E*,13*S*)-13-hydroperoxy-9,11-octadecadienoic (13-HPOD) acids were obtained by incubation of α-linolenic and linoleic acids, respectively, with the soybean lipoxygenase type V. All hydroperoxides were purified by normal phase HPLC.

2.2. Bioinformatic methods for the CYP74 structure analysis

The primary structures of the CYP74s were aligned using NCBI and PlantGDB BLAST searches, as well as the Clustal Omega tool. The phylogenetic tree of selected CYP74 clan members was built with the Clustal Omega and the TreeView software.

2.3. Expression and purification of recombinant enzymes

The open reading frame (ORF) of gene *RaDES* has been cloned into the vector pET32-Ek/LIC (Novagen, USA) to yield the target recombinant protein with His-tags at N- and C-termini. The resulting construction was transformed into *Escherichia coli* host strain Rosetta-gami(DE3)pLysS B. The expression of recombinant gene in *E. coli* cells was induced by adding 0.5 mM isopropyl-β-*D*-1-thiogalactopyranoside to the medium. His-tagged recombinant protein was purified by immobilized metal affinity chromatography (IMAC) using Bio-Scale Mini Profinity IMAC cartridge and BioLogic LP chromatographic system (Bio-Rad, USA) [22]. The homogeneity of purified protein was confirmed by SDS-PAGE. Protein concentration was estimated as described before [22]. The enzyme activity was measured with Lambda 25 spectrophotometer (Perkin-Elmer, USA) by the decrease of fatty acid hydroperoxides absorbance at 234 nm [22].

2.4. Incubations of recombinant enzyme with fatty acid hydroperoxides

The recombinant enzyme (10 μg) was incubated with 100 μg of fatty acid hydroperoxide in Na-phosphate buffer (2 ml), pH 7.0, 4 °C, for 15 min. The products were extracted, purified with solid phase cartridges, methylated with diazomethane and trimethylsilylated as described before [22], followed by GC-MS analysis. When specified, the products were reduced with $NaBH_4$ and hydrogenated over PtO_2, then methylated and trimethylsilylated. Products (without or with the preliminary hydrogenation and reduction) were analyzed as Me esters/TMS derivatives (Me/TMS) by GC-MS as described before [22].

2.5. Kinetic studies

The enzymatic activity of the purified recombinant RaDES was determined by monitoring the increase of the signal at 250 nm or 267 nm (for 13-HPOD and 13-HPOT, respectively) in a Varian 50 Bio UV–VIS spectrophotometer with substrate concentrations ranging from 5 to 210 μM. The analyses were performed in 0.6 ml of 0.05 M Na phosphate buffer (pH 7.5) at 25 °C. The initial linear regions of the kinetic curves were used to calculate the rates. The amounts of products, namely (ω5*Z*)-etherolenic and (ω5*Z*)-etheroleic acids, formed were estimated using the molar extinction coefficients 41,200 M^{-1} cm^{-1} and 25,700 M^{-1} cm^{-1}, respectively. The latter value is the difference between the molar extinction coefficients 32,100 M^{-1} cm^{-1} and 6400 M^{-1} cm^{-1} for (ω5*Z*)-etheroleic acid and 13-HPOD at 250 nm, respectively. Kinetic parameters were calculated by fitting the datasets to a one-site saturation model for simple

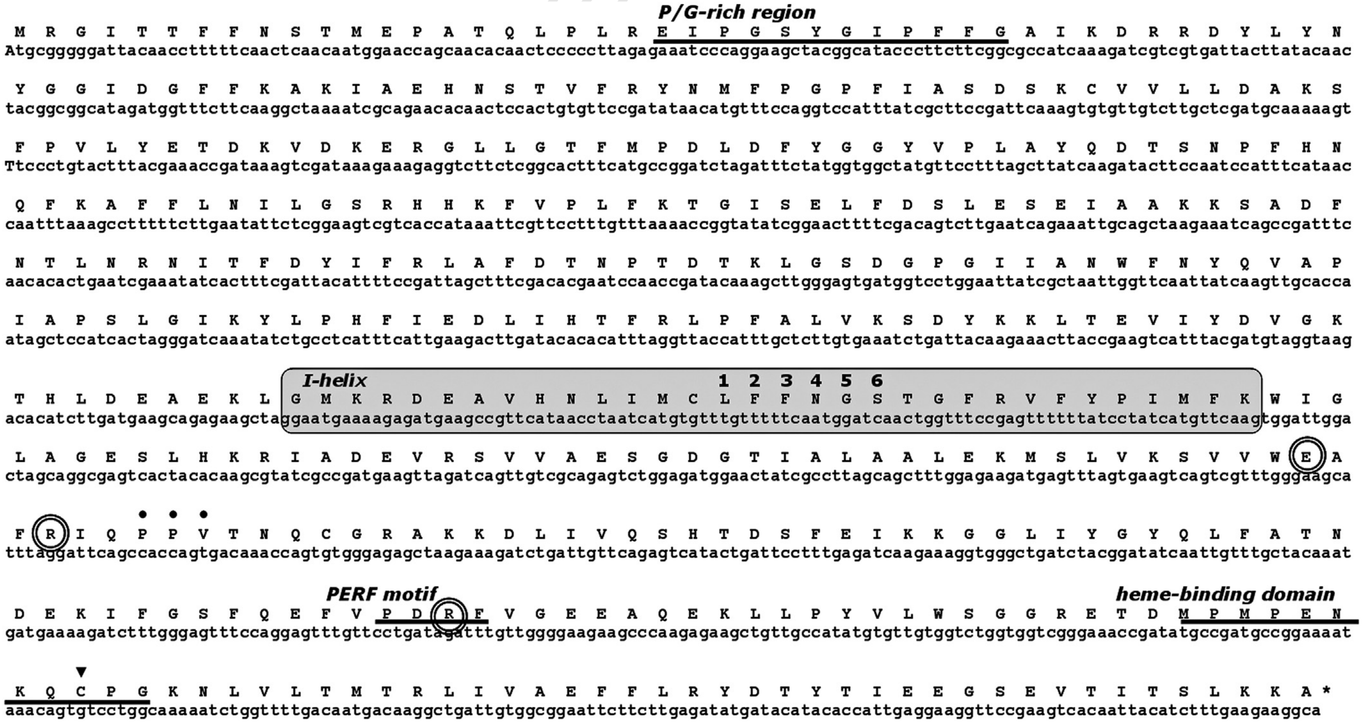

Fig. 1. Nucleotide and the deduced amino acid sequences of the target *Ranunculus acris CYP74Q1* cDNA. Conservative domains are marked as follows: the I-helix is outlined with gray rounded rectangle; the IHCD domain is numbered 1-6; ERR-triad is outlined with double circles; P/G-rich region, PERF-motif and haem-binding domain are underlined with bold lines; black triangle shows the position of the conserved cysteinyl haem ligand.

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