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

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ABSTRACT

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,

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

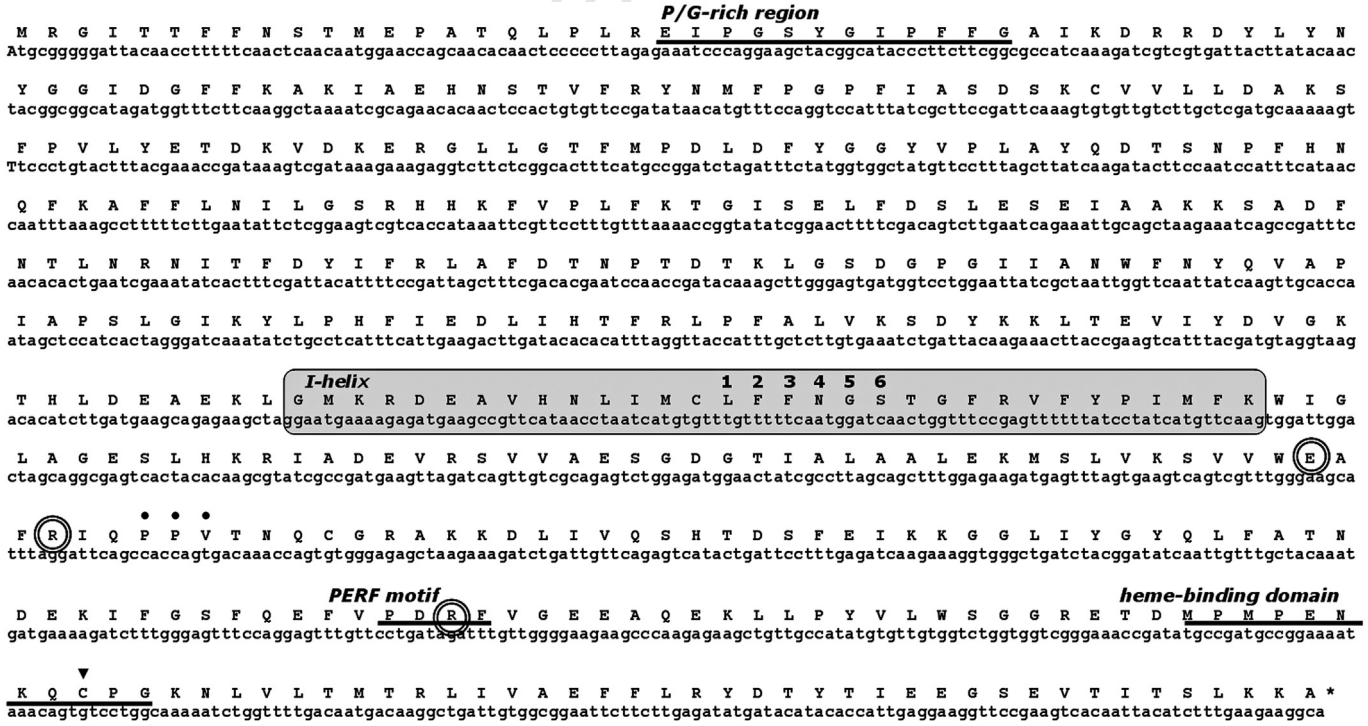

Fig. 1. Nucleotide and the deduced amino acid sequences of the target *Ranunculus acris CYP74Q1* cDNA. Conservative domains are marked as follows: the I-helix is outlined with gray rounded rectangle; the IHCD domain is numbered 1-6; ERR-triad is outlined with double circles; P/G-rich region, PERF-motif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

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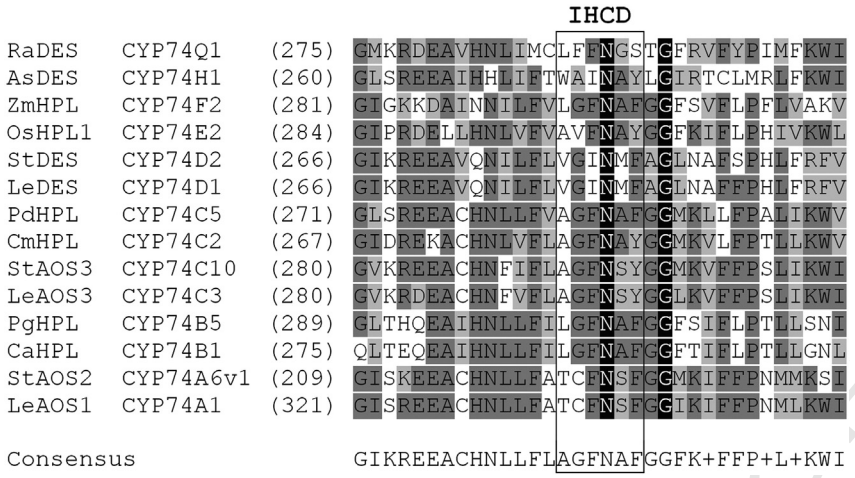

Fig. 2. The multiple alignments of I-helix domains of CYP74Q1 and selected enzymes of different CYP74 subfamilies. The IHCD domain is outlined with rectangle and signed on the top. The alignments were built with Clustal Omega tool.

ligand binding using the SigmaPlot 11 software (Systat Software Inc., USA). Five independent experiments were performed for each specified variant. The mean values and standard deviations are given.

2.6. Methods of spectral analyses

The UV spectra of products were scanned during the incubations of the recombinant RaDES with fatty acid hydroperoxides with Varian Cary 50 spectrophotometer. The UV spectra of purified enzyme products were recorded with the same instrument. Alternatively, the UV spectra of products were recorded on-line during the HPLC separations using an SPD-M20A diode array detector (Shimadzu). Products were analyzed as methyl esters or methyl esters/TMS derivatives by GC-MS as described before [9]. The GC-MS analyses were performed using a Shimadzu QP5050A mass spectrometer connected to Shimadzu GC-17A gas chromatograph equipped with a Supelco MDN-5S (5% phenyl 95% methylpolysiloxane) fused capillary column (length, 30 m; ID 0.25 mm; film thickness, 0.25 μm). Helium at a flow rate of 30 cm/s was used as the carrier gas. Injections were made in the split mode using an initial column temperature of 120 °C, injector temperature 230 °C. The column temperature was raised at 10 °C/min until 240 °C. The electron impact ionization (70 eV) has been used. The equivalent chain lengths (ECL) values were estimated as described before [28]. The ^{1}H NMR and 2D-COSY spectra were recorded with a Bruker Avance III 600 instrument (600 MHz, [$^{2}H_{6}$]benzene, 296 K).

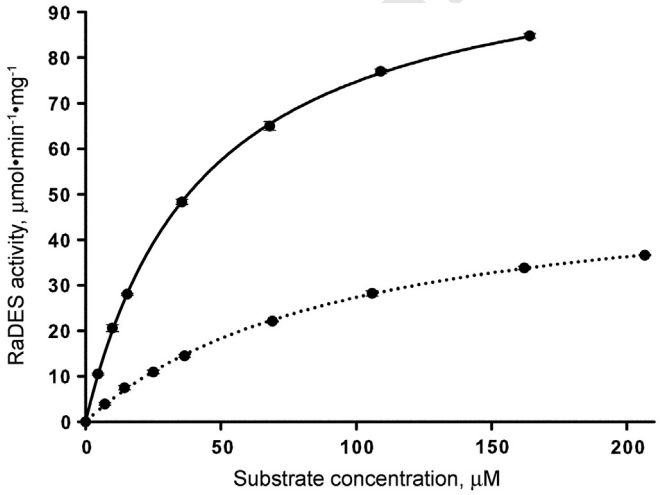

Fig. 3. Substrate saturation curves for the recombinant RaDES. Kinetic data were recorded with double beam spectrophotometer as described in Materials and methods. The quantitative kinetic parameters for the data representing conversions of 13-HPOT (solid line) and 13-HPOD (dotted line) were fitted to a one-site saturation model for simple ligand binding using the SigmaPlot 11 software as described in Materials and methods.

3. Results

3.1. Design of degenerate primers for CYP74 transcript detection

The *R. acris* genome has not been sequenced yet. Only a very limited number of *R. acris* gene sequences is published, among them there are no P450 genes. No CYP74 ESTs of Ranunculaceae are currently present in the genomic databases. Thus, there was no genomic background that could facilitate the identification of *RaDES* gene. That is why an approach with degenerate primers was used in the present work. Recently we succeeded to detect and clone the before unknown flax CYP74B16 gene using degenerate primers although flax genome was not sequenced at that time [22]. In the present work, we used the same approach to detect the CYP74 mRNAs in *R. acris* leaf transcriptome. To design the degenerate primers, we analyzed the alignments of CYP74 amino acid sequences to find the most conserved domains.

The first step in the design of degenerate primers was a search for conservative motifs in sequences of the different plant CYP74s. Primer regions were chosen so that the resulting amplicons would contain the I-helix central domain (IHCD [29], which is referred to "the oxygen-binding domain" in the case of monooxygenases). The sequences of all constructed degenerate primers used for the PCR search of CYP74 transcripts are listed in Table 1.

Table 2
Kinetic parameters and substrate specificity of the recombinant RaDES.

Substrate	k_{cat} (s^{-1})	K_M (μM)	k_{cat}/K_M (μM^{-1} s^{-1})	Substrate specificity, % 13(*S*)-HPOT
13-HPOT	134.0 ± 1.4	43.1 ± 1.1	3.11	100
13-HPOD	67.7 ± 1.1	97.6 ± 3.5	0.69	22
9-HPOT	–	–	–	0[a]
9-HPOD	–	–	–	0[a]

[a] No divinyl ethers have been detected upon the incubations with 9-HPOT and 9-HPOD.

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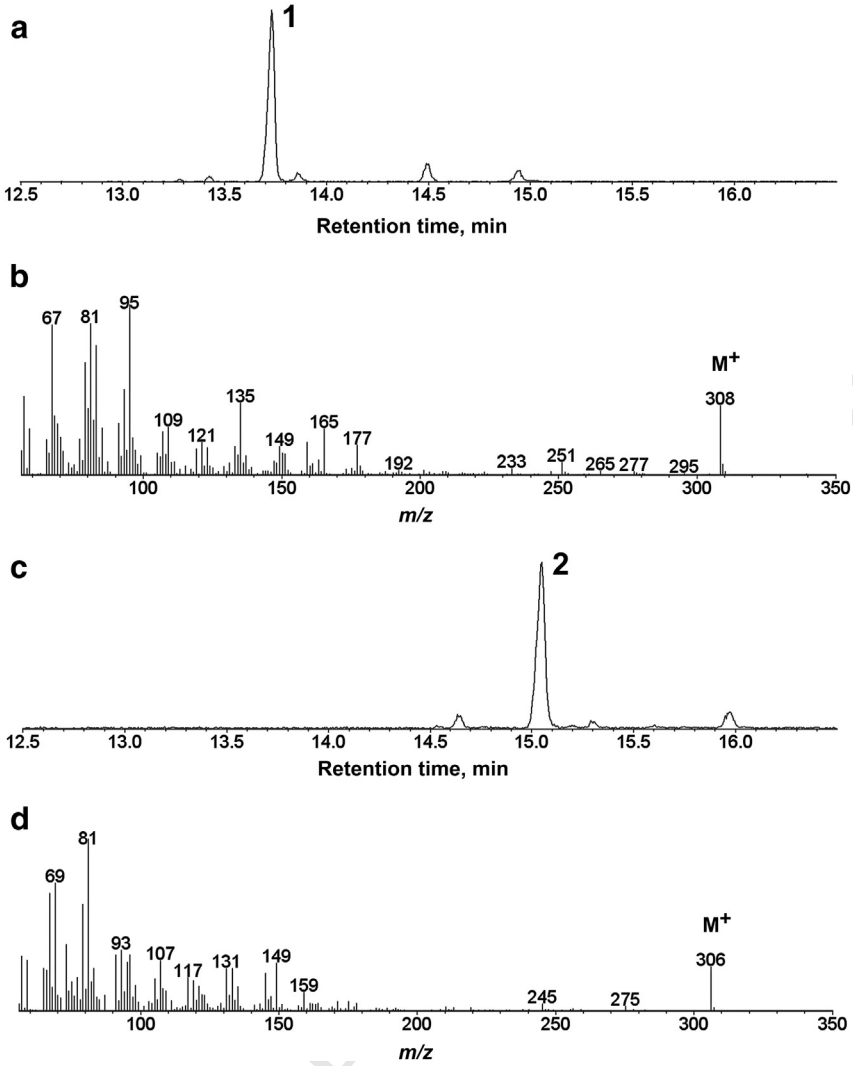

Fig. 4. The GC-MS analyses of RaDES products. (a) The GC-MS chromatogram of products (Me/TMS) of 13-HPOD incubation with CYP74Q1. (b) The mass spectrum for product **1**. (c) The GC-MS chromatogram of products (Me/TMS) of 13-HPOT incubation with CYP74Q1. (d) The mass spectrum for product **2**.

3.2. Detection and cloning of CYP74Q1 gene

The total RNA was isolated from the leaves of *R. acris* plants. The cDNA obtained after the reverse transcription was used as the template for PCR with the constructed primers (Table 1). The resulting ca. 1000 bp products obtained in PCR with primers RaF1-RaR1 were initially cloned into pGEM-T vector (Promega, USA) and subjected to DNA sequencing to verify the presence of any CYP74 fragments. The detected partial CYP74 sequences were used to construct the gene-specific primers (supplementary Table S1, supplementary Fig. S1) for the detection of 3′ and 5′ cDNA ends by RACE method. 3′ and 5′ cDNA ends were obtained in reaction with "Mint RACE cDNA amplification set" (Evrogen, Russia) in three serial PCRs using gene-specific primers and universal Step-out primer mix1, Step-out primer mix2, Step-out primer mix3 (supplementary Fig. S1) according to the manufacturer's protocol. Thus, the novel full length CYP74 gene was sequenced and cloned. Its nucleotide and deduced amino acid sequences are presented in Fig. 1. The screening of cDNA library revealed two additional isoforms possessing some minor sequence variations as shown in supplementary Fig. S2.

The ORF (Fig. 1) of isoform 1 consisted of 1449 nucleotides and encoded 483 amino acid polypeptide (Fig. 1). The sequence alignments of isoform 1 demonstrated that the novel *R. acris* CYP74 protein does not belong to any known CYP74 subfamily. Thus, this is the first representative of novel subfamily CYP74Q. The name CYP74Q1 has been assigned to this new sequence [Professor David R. Nelson, personal communication]. The multiple alignment of CYP74Q1 (I-helix domain) and other CYP74s is illustrated in Fig. 2. The deduced CYP74Q1 amino acid sequence does not belong to any previously known CYP74 subfamily. It has 52% identity to CmHPL (CYP74C2), 51% to putative AOS (CYP74C9) of *Petunia integrifolia* subsp. *inflata*, 49% to LeDES (CYP74D1) and 49% to HbAOS (CYP74A9).

3.3. Substrate specificity and kinetics of the recombinant CYP74Q1

Recombinant CYP74Q1 (RaDES) efficiently utilized 13-HPOT and 13-HPOD (Fig. 3). At the same time, the enzyme was quite inactive towards 9-HPOD and 9-HPOT in full agreement with the literature data [11]. RaDES exhibited the hyperbolic kinetic profiles for both 13-HPOT and 13-HPOD conversions (Fig. 3). The K_M values (Table 2) demonstrated that RaDES affinity for 13-HPOT was over twice higher than for 13-HPOD. The estimated turnover rate (k_{cat}) of 13-HPOT was about twice higher than that of 13-HPOD (Table 2). As judged by specificity constant (k_{cat}/K_M) values (Table 2), 13-HPOT was the preferential substrate for RaDES.

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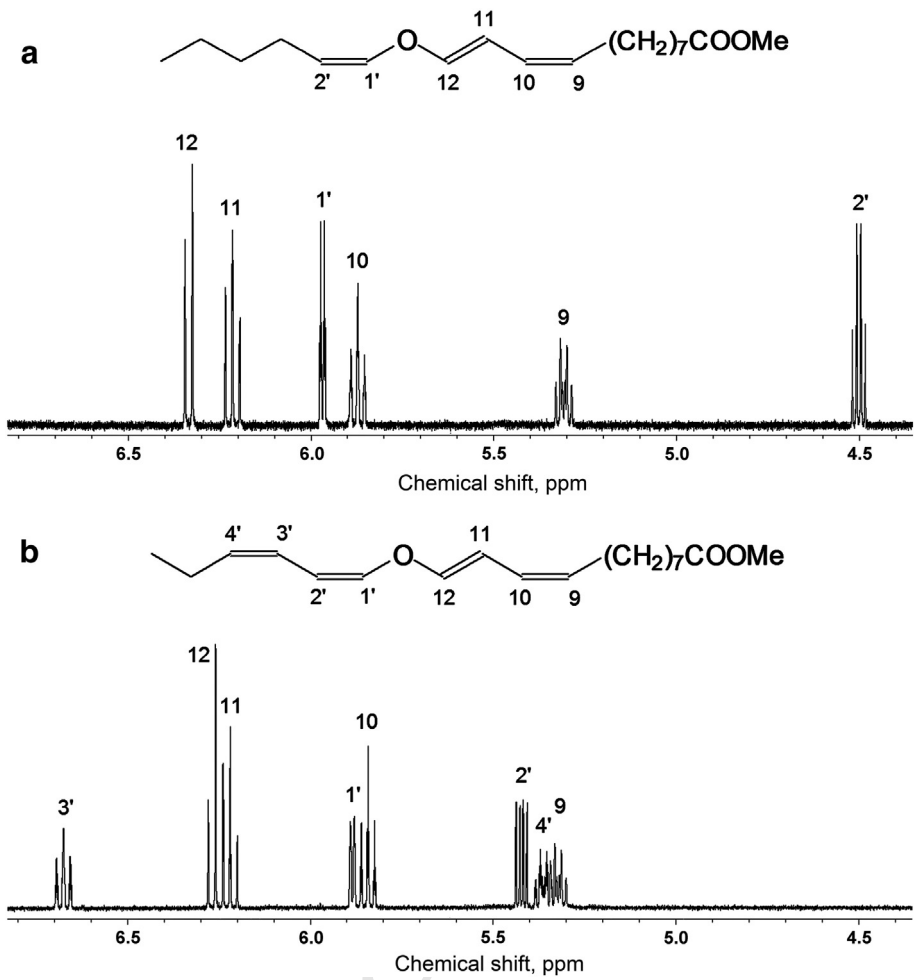

Fig. 5. The olefinic regions of ^{1}H-NMR spectra (600 MHz, [$^2H_6$]benzene, 296 K) of products **1** (a) and **2** (b). Methine position number is specified at the top of each multiplet. Insets, the structural formulae of products **1** and **2** with numbered positions of olefinic protons.

3.4. Identification of the recombinant CYP74Q1 as divinyl ether synthase

The GC-MS analyses of the products (Me/TMS) revealed a specific conversion of 13-HPOD to product **1** (Fig. 4a). Its mass spectrum (Fig. 4b) possessed M^+ at *m/z* 308 (21%), $[M - MeO]^+$ at *m/z* 277 (2%), $[M - Me(CH_2)_3]^+$ at *m/z* 251 (4%), *m/z* 219 (3%), *m/z* 177 (15%), and $[M - (CH_2)_6COOMe]^+$ at *m/z* 165 (19%). The spectral patterns were identical to those of (ω5*Z*)-etheroleic acid [10]. The equivalent chain length (ECL) value (19.05) of product **1** peak corresponded to (ω5*Z*)-etheroleic acid. It is much smaller compared to other geometric isomers of etheroleic acid, having the ECL values above 19.5. To substantiate the structural identification including the double bond geometry, we purified product **1** (Me ester) by HPLC and recorded its ^{1}H-NMR and 2D-COSY spectra (Fig. 5a and supplementary Table S2). The data fully confirmed the identification of product **1** as (ω5*Z*)-etheroleic acid, (9*Z*,11*E*)-12-[(1′*Z*)-hexenyloxy]-9,11-dodecadienoic acid.

RaDES was most efficient towards 13-HPOT. 13-HPOT was quite specifically converted to a single predominant product **2** (detected as Me ester), as illustrated in Fig. 4c. Mass spectrum of compound **2** (Fig. 4d) exhibited M^+ at *m/z* 306 (27%), $[M - Et]^+$ at *m/z* 277 (2%), $[M - MeO]^+$ at *m/z* 275 (2%), $[277 - MeOH]^+$ at *m/z* 245 (4%), and $[M - (CH_2)_7COOMe]^+$ at *m/z* 149 (25%). The spectrum matched that of (ω5*Z*)-etherolenic acid. The ECL value 20.03 of this peak also corresponded to that of (ω5*Z*)-etherolenic acid. It should be noted that (ω5*Z*)-etherolenic and etherolenic acids coelute during the GC separation on the nonpolar stationary phases. Thus, to reveal the exact double bond geometry, the product **2** (Me) was isolated and purified by RP-HPLC and NP-HPLC. Then the ^{1}H-NMR and 2D-COSY data (Fig. 5b and supplementary Table S3) were recorded. These data unambiguously revealed the structure of compound **2** as (ω5*Z*)-etherolenic acid, (9*Z*,11*E*)-12-[(1′*Z*,3′*Z*)-hexadienyloxy]-9,11-dodecadienoic acid.

4. Discussion

Ranunculaceae species (Ranunculales, basal Eudicots) are phylogenetically distant from other plants possessing DESs. Although DESs were detected in tissues of few Ranunculaceae species [10–12], neither DES proteins nor their genes have been sequenced and cloned. RaDES (CYP74Q1) presents a new extension to the known diversity of DESs. DESs of Solanaceae species belong to CYP74D subfamily, while garlic (*Allium sativum* L.) DES is the only member of CYP74H subfamily. The recently described flax enzyme LuDES (CYP74B16) is an unusual DES, belonging to CYP74B subfamily [22]. All other known members of this subfamily are 13-HPLs. DESs of CYP74D subfamily, as well as DES of garlic, are expressed in non-green plant organs [8]. In contrast, CYP74Q1 and CYP74B16 [22] are expressed in the leaves of *R. acris* and flax, respectively. Both enzymes preferentially utilize 13-hydroperoxides of linolenic and linoleic acids, while 9-hydroperoxides are poor substrates. Despite these similarities, CYP74Q1 and CYP74B16 are phylogenetically distant from each other. They possess only 36% identity.

Various DESs differ in respect of substrate and product specificities. The "leaf DESs" RaDES (CYP74Q1), as well as LuDES (CYP74B16), are

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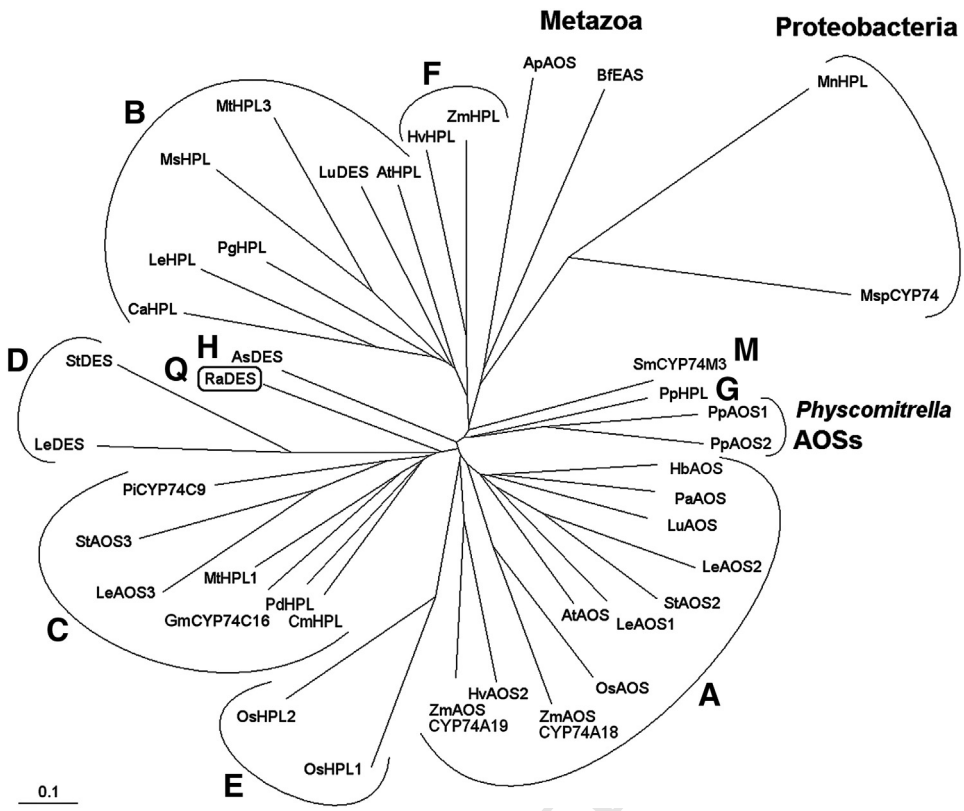

Fig. 6. The unrooted phylogenetic tree of CYP74 clan. Classified CYP74 subfamilies are marked with their letter designations (**A**, **B**, **C** etc.). Subfamilies consisting of more than one member are outlined with unclosed curves (semi-ellipses). RaDES (CYP74Q1) is outlined with rounded rectangle. CYP74s of flowering plants: As, *Allium sativum*; AsDES, GI:83414021; At, *Arabidopsis thaliana*; AtAOS, CYP74A1, GI:15239032; AtHPL, CYP74B2, GI:3822403; Ca, *Capsicum annuum*; CaHPL, CYP74B1, GI:1272340; Cm, *Cucumis melo*; CmHPL, CYP74C2, GI:14134199; Gm, *Glycine max*; GmCYP74C16, GI:100037482; Hb, *Hevea brasiliensis*; HbAOS, CYP74A9, GI:84028363; Hv, *Hordeum vulgare*, HvAOS2, GI:7452981; HvHPL, CYP74F3, GI: 22265998; Le, *Lycopersicon esculentum*; LeAOS1, GI:7581989; LeAOS2, CYP74A2, GI:7677376; LeAOS3, CYP74C3, GI:25991603; LeHPL, GI:7677378; LeDES, GI:11991245; Lu, *Linum usitatissimum*; LuAOS, CYP74A1, GI:1352186; LuDES, CYP74B16, GI:310687282; Ms, *Medicago sativa*; MsHPL, GI:5830465; Mt, *Medicago truncatula*; MtHPL1, CYP74C12, GI:33504430; MtHPL3, CYP74B4, GI:63081244; Os, *Oryza sativa*; OsAOS, CYP74A4, GI:115455571; OsHPL1, CYP74E2, GI:115445057; OsHPL2, CYP74E1, GI:125538638; Pa, *Parthenium argentatum*; PaAOS, CYP74A1, GI:218511958; Pd, *Prunus dulcis*; PdHPL, CYP74C5, GI:33300600; Pg, *Psidium guajava*; PgHPL, CYP74B5, GI:13183137; Pi, *Petunia inflata*; PiCYP74C9, GI:85720841; Sm, *Selaginella moellendorfii*; SmCYP74M3, GI: 9654395; St, *Solanum tuberosum*; StAOS2, GI:86769479; StAOS3, GI:56605358; StDES, CYP74D2, GI:12667099; Zm, *Zea mays*; ZmAOS, CYP74A18, GI:223947589; ZmAOS, CYP74A19, GI: 223947589; ZmHPL, CYP74F2, GI:162462890. CYP74s of mosses: Pp, *Physcomitrella patens*; PpAOS1, CYP74A1, GI:22217985; PpAOS2, CYP74A8, GI:168014176; PpHPL, CYP74G1, GI:76057841. CYP74 clan members of proteobacteria: Mn, *Methylobacterium nodulans*; MnHPL, GI:220926268; Msp, *Methylobacterium* sp. 4-46; MspHPL (putative HPL), GI:170743950. CYP74 clan members of Metazoa: Ap, *Acropora palmata* (coral); ApAOS, GI:187948710; Bf, *B. floridae* (lancelet); BfEAS, GI:189312561. The tree was built with the Clustal Omega and the TreeView software using the alignments of full amino acid sequences of the above listed CYP74 clan members. The preliminary BLAST analyses of CYP74s were performed using the protein NCBI BLAST tool.

the specific 13-DESs. Their distinguishing feature is the production of (ω5*Z*)-etherolenic and (ω5*Z*)-etheroleic acids. In contrast, the "classic" DESs of Solanaceae (CYP74D) are 9-DESs and produce the colneleic and colnelenic acids. The garlic DES (CYP74H1) utilizes both 13- and 9-hydroperoxides of linoleic and linolenic acids and produces either etheroleic and etherolenic or colneleic and colnelenic acids, respectively.

CYP74Q1 has 52% identity to CmHPL (CYP74C2), 51% to putative AOS (CYP74C9) of *P. integrifolia* subsp. *inflata*, 49% to LeDES (CYP74D1) and 49% to HbAOS (CYP74A9). Thus, CYP74Q is nearly equally distant towards CYP74C, CYP74D and CYP74A subfamilies; see the phylogenetic tree of CYP74 clan, Fig. 6. Interestingly, RaDES (CYP74Q1), AsDES (CYP74H1) and CYP74D subfamily DESs are grouped into a cluster on the phylogenetic tree (left side of tree, Fig. 6).

It is worthwhile that plant evolution created a big diversity of distinct DES types. Known DESs belong to subfamilies CYP74B (LuDES, CYP74B16), CYP74D (Solanaceae DESs), CYP74H (AsDES), CYP74M3 and CYP74Q (RaDES). DESs are not as widespread as AOSs and HPLs. Some of the sequenced plant genomes like those of *Arabidopsis thaliana* and *Oryza sativa* do not possess any DES gene. At the same time, it is too early to make conclusions on the commonness of DES genes. The current state of genomic knowledge is too incomplete. One can expect that further progress will uncover many new DESs not only in Plant Kingdom, but possibly also in some metazoans. The tree of CYP74 clan continues to grow, and there are certainly still large gaps to be filled.

Regardless of the limited occurrence of DES genes, each separate DES type has evidently been evolved from gene duplication and independent evolution targeted to the creation of DES catalysis. For instance, the recently detected LuDES although belonging to CYP74B subfamily, possesses only ca. 70% identity to the putative flax HPL (Lus10030029, CYP74B). These two flax genes were evidently evolved through the duplication and divergence. Indeed, the qualitative changes of CYP74 catalysis can occur due to site-directed mutagenesis. For instance, the AOSs to HPLs [5,30] as well as DESs to AOSs [30] conversions have been demonstrated.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbalip.2014.05.005.

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