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

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3335**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* 

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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 20 (*Ranunculus acris* L.) and few other Ranunculaceae species. The nature of these DESs and their genes remained 21 unrevealed. The PCR with degenerate primers enabled to detect the transcript of unknown P450 gene assigned 22 as *CYP74Q1*. Besides, two more *CYP74Q1* isoforms with minimal sequence variations have been found. The full 23 length recombinant *CYP74Q1* protein was expressed in *Escherichia coli*. The preferred substrates of this enzyme 24 are the 13-hydroperoxides of  $\alpha$ -linolenic and linoleic acids, which are converted to the divinyl ether oxylipins 25 ( $\omega$ 5*Z*)-etherolenic acid, (9*Z*,11*E*)-12-[(1'*Z*,3'*Z*)-hexadienyloxy]-9,11-dodecadienoic acid, and ( $\omega$ 5*Z*)-etheroleic 26 acid, (9*Z*,11*E*)-12-[(1'*Z*)-hexenyloxy]-9,11-dodecadienoic acid, respectively, as revealed by the data of mass 27 spectrometry, NMR and UV spectroscopy. Thus, CYP74Q1 protein was identified as the *R. acris* DES (RaDES), a 28 novel DES type and the opening member of new CYP74Q subfamily. 29

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sinclairii [16] and red alga *Polyneura latissima* [17], although no CYP74 51 genes have been detected in algae yet [7]. DES genes of Solanaceae species 52 (CYP74D) [18–20], garlic (CYP74H1) [21], and flax (CYP74B16) [22] have 53 been cloned, and the properties of these recombinant DESs have been 54 characterized. All other described DES activities have been detected in 55 plant tissues, but the corresponding genes and proteins have not yet 56 been detected, sequenced and cloned. Divinyl ethers play the defensive 57 and antimicrobial role in plants [23–27]. 58

Majority of DESs are present in non-green plant tissues, e.g. potato 59 tubers, tomato roots, and garlic bulbs [7]. There are only two known 60 DESs present in plant leaves. These are flax [13,22] and Ranunculaceae 61 [10–12] DESs. Flax DES has been recently cloned and identified as 62 CYP74B16 [22], an unprecedented 13-DES member of the CYP74B sub- 63 family, while all other CYP74B members are 13-HPLs [7]. The nature of 64 Ranunculaceae DESs remains uncovered. This prompted us to look for 65 the CYP74 transcripts in *Ranunculus acris* leaves. Using the degenerate 66 primers, we succeeded to detect an unknown CYP74 transcript. The pres- 67 ent paper reports the cloning of corresponding full length cDNA and iden- 68 tification of the recombinant protein as *R. acris* DES (RaDES), CYP74Q1. 69

#### 2. Materials and methods

#### 2.1. Materials

The aerial parts of wild *R. acris* plants were collected near the lake 72 Sredny Kaban (Kazan) in Summer seasons 2012 and 2013. Linoleic 73 and  $\alpha$ -linolenic acids, as well as the soybean lipoxygenase type V, 74

Abbreviations: DES, divinyl ether synthase; RaDES, *Ranunculus acris* divinyl ether synthase; HPL, hydroperoxide lyase; AOS, allene oxide synthase; 13(S)-HPOT, (9Z,11E,13S,15Z)-13-hydroperoxyoctadecatrienoic acid; 13(S)-HPOD, (9Z,11E,13S)-13-hydroperoxyoctadecadienoic acid; 9(S)-HPOD, (9S,10E,12Z)-9-hydroperoxyoctadecadienoic acid; 1MAC, 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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#### t1.1 Table 1

t1.2 Degenerate oligonucleotide primers used in PCR for the detection of the CYP74 genes trant1.3 scripts in *Ranunculus acris* leaves transcriptome.

| Name | Primer sequence 5' to 3'                 | T <sub>m,</sub> °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  |
|      | Name<br>RaF1<br>RaR1<br>RaF2<br>RaR2     | Name     Primer sequence 5' to 3'       RaF1     gA(A/g)AAg(C/g)ACAAgAgCAC(g/C)gT(g/T)TTC       RaR1     CA(T/A)Ag(A/C)A(g/A)CTC(C/g/A)CCTTTCTTg       RaF2     CT(T/C)gT(T/C)gg(T/C)g)gA(T/C)TTCATgCC       RaR2     ggCATgAA(g/A)TC(C/g/A)CC(g/A)AC(g/A)Ag |

were purchased from Sigma. Adams's catalyst and silvlating reagents 75 76 were purchased from Fluka (Buchs, Switzerland). (95,10E,12Z)-9-Hydroperoxy-10,12-octadecadienoic acid (9-HPOD) was prepared by in-77 cubation of linoleic acid with tomato fruit lipoxygenase at 0 °C, pH 6.0, 78 79under continuous oxygen bubbling. (9Z,11E,13S,15Z)-13-Hydroperoxy-80 9,11,15-octadecatrienoic (13-HPOT) and (9Z,11E,13S)-13-hydroperoxy-81 9,11-octadecadienoic (13-HPOD) acids were obtained by incubation of  $\alpha$ -linolenic and linoleic acids, respectively, with the soybean lipoxygenase 82 83 type V. All hydroperoxides were purified by normal phase HPLC.

#### 84 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.

#### 89 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- $\beta$ -D-1-thiogalactopyranoside to the medium. Histagged recombinant protein was purified by immobilized metal affinity 96 chromatography (IMAC) using Bio-Scale Mini Profinity IMAC cartridge 97 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 100 measured with Lambda 25 spectrophotometer (Perkin-Elmer, USA) by 101 the decrease of fatty acid hydroperoxides absorbance at 234 nm [22]. 102

#### 2.4. Incubations of recombinant enzyme with fatty acid hydroperoxides 103

The recombinant enzyme (10 µg) was incubated with 100 µg of fatty 104 acid hydroperoxide in Na-phosphate buffer (2 ml), pH 7.0, 4 °C, for 105 15 min. The products were extracted, purified with solid phase cartridges, 106 methylated with diazomethane and trimethylsilylated as described be-107 fore [22], followed by GC-MS analysis. When specified, the products 108 were reduced with NaBH<sub>4</sub> and hydrogenated over PtO<sub>2</sub>, then methylated 109 and trimethylsilylated. Products (without or with the preliminary hydro-110 genation and reduction) were analyzed as Me esters/TMS derivatives 111 (Me/TMS) by GC-MS as described before [22].

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#### 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  $\mu$ M. 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 ( $\omega$ 5*Z*)the molar extinction coefficients 41,200 M<sup>-1</sup> cm<sup>-1</sup> and 25,700 M<sup>-1</sup> cm<sup>-1</sup>, respectively. The latter value is the difference between the molar extinction coefficients 32,100 M<sup>-1</sup> cm<sup>-1</sup> and 6400 M<sup>-1</sup> cm<sup>-1</sup> for ( $\omega$ 5*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

|  | P/G-rich region |      |       |           |     |        |           |              |          |          |          |          |           |           |          |          |           |          |          |           |          |          |          |           |          |          |          |            |            |           |              |      |          |           |      |   |           |           |           |           |     |              |    |
|--|-----------------|------|-------|-----------|-----|--------|-----------|--------------|----------|----------|----------|----------|-----------|-----------|----------|----------|-----------|----------|----------|-----------|----------|----------|----------|-----------|----------|----------|----------|------------|------------|-----------|--------------|------|----------|-----------|------|---|-----------|-----------|-----------|-----------|-----|--------------|----|
| М  | R               | G    | I     | т         | т   | F      | F         | N            | S        | т        | М        | Е        | P         | A         | т        | Q        | L         | P        | L        | R         | E        | I        | P        | G         | ร        | Y        | G        | I          | P          | F         | F            | G    | A        | I         | к    | D   | R         | R         | D         | Y         | г   | Y N          |    |
| Atgcgggggattacaacetttttcaaceaatggaaccagcaacacaactcececttagagaaateecaggaagetaeggeataecettetteggegeeateaaagategtegtgattaettataeaae           |                 |      |       |           |     |        |           |              |          |          |          |          |           | 3         |          |          |           |          |          |           |          |          |          |           |          |          |          |            |            |           |              |      |          |           |      |   |           |           |           |           |     |              |    |
| v  | G               | G    | т     | ъ         | G   | F      | F         | v            | 7        | v        | т        | 7        | F         | u         | N        | c        | m         | v        | F        | ъ         | v        | N        | м        | F         | ъ        | G        | ъ        | F          | т          | 7         | c            | n    | e        | v         | c    | v   | v         | т         | т         | ъ         |     | v c          |    |
| tad  | aad             | aad  | ata   | at        | aat | ttet   | tte       | aad          | act      |          | atc      | aca      | aaa       | cac       | aac      | tcc      | act       | ata      | ttc      | caa       | tat      | aac      | ata      | ttt       | cca      | aat      | cca      | ttt        | atc        | act       | tcc          | ratt | caa      | aat       | tate | atte  | atci      | ttad      | ctco      | dato      | caa | aaadt        |    |
|  |                 |      |       |           | 99- |        |           |              | 9        |          |          | <b>y</b> | <b>J</b>  |           |          |          |           | 9-9      |          | - 9       |          |          |          |           |          | 55-      |          |            |            | <b>J</b>  |              | ,    |          |           |      | J   | ,         |           |           |           |     |              |    |
| F  | P               | v    | г     | Y         | Е   | т      | D         | к            | v        | D        | к        | Е        | R         | G         | г        | г        | G         | т        | F        | М         | Р        | D        | L        | D         | F        | Y        | G        | G          | Y          | v         | Р            | г    | A        | Y         | Q    | D   | т         | s         | N         | P         | F J | H N          |    |
| ${\tt Ttccctgtactttacgaaaccgataaagtcgataaagaagaggtcttctcggcactttcatgccggatctagatttctatggtggctatgttcctttagcttatcaagatacttccaatccatttcataac$ |                 |      |       |           |     |        |           |              |          |          |          |          |           | J         |          |          |           |          |          |           |          |          |          |           |          |          |          |            |            |           |              |      |          |           |      |   |           |           |           |           |     |              |    |
| •  |                 |      |       |           |     | -      | w         | -            | -        | ~        | ~        |          |           |           | v        |          | .,        | -        |          |           |          | -        | ~        | -         | ~        |          | ÷        |            |            | ~         |              |      | ~        |           | -    |   |           |           |           | ~         |     |              |    |
| Ca   | 2<br>+++        |      | A     | •+++      | ++c | ц<br>т | N<br>aat: | ⊥<br>attr    | сt с     | d da     | ant      | cat.     | п<br>сас  | n<br>rat  | л<br>ааа | г<br>+†с | v<br>at t | r<br>cct | ц<br>++а | +++       | л<br>ааа | T<br>acc | aat      | ⊥<br>ata  | 5<br>tca | na a     | с±+      | ++c        | nac        | ant       | -1-1-        | naat | ъ<br>сас | E<br>taa: | ++/  | A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A<br>A | A<br>act: | n<br>aan: | n<br>aaat | ъ<br>tcan |     | o r<br>attto |    |
| cu   |                 | uuu  | .gee  |           |     | cege   | 440       |              |          | ggu      | uge      | cgc      | cuc       | cut       | uuu      |          | 900       |          | ceg      |           | uuu      | 400      | 990      | ucu       | ceg      | guu      |          |            | gue        | uge       |              | Juui | -cu      | Juui      |      | Jou   | 9000      | augu      | auu       | ceug      | ceg |              | 2  |
| N  | т               | L    | N     | R         | N   | I      | т         | F            | D        | Y        | I        | F        | R         | L         | A        | F        | D         | т        | N        | Р         | т        | D        | т        | к         | L        | G        | s        | D          | G          | P         | G            | I    | I        | A         | N    | W   | F         | N         | Y         | Q         | v x | A P          |    |
| aa   | caca            | ct   | gaat  | cga       | aat | atca   | act       | ttc          | gat      | tac      | att      | ttc      | cga       | tta       | gct      | ttc      | gac       | acg      | aat      | cca       | acc      | gat      | aca      | aag       | ctt      | ggg      | agt      | gat        | ggt        | cct       | ggaa         | atta | tco      | gcta      | aat  | tggt  | ttca      | aatt      | tato      | caag      | ttg | cacca        | ł. |
| -  | -               | -    | ~     | -         | ~   | -      |           |              | -        | -        |          | _        | -         | _         | _        | -        | -         |          | _        | -         | _        | -        | _        | _         | -        | -        |          |            | ~          | _         |              |      |          | -         | _    | -   |           | -         |           | -         |     |              |    |
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| ale  | igei            | .004 | 1100  | ICLA      | 999 | alca   | aaa       | Lali         | cug      | CCL      | Cal      | LLC      | all       | yaa       | yac      | LLY      | aca       | Cac      | aca      |           | ayy      | LLA      | cca      |           | get      | CLL      | grg      | aaa        | LCL        | gaı       | LaCa         | aga  | iaac     |           | acce | Jaaq  | Juca      | 1000      | Lacy      | yary      | Lag | graag        | 1  |
|  |                 |      |       |           |     |        |           | 1            |          | -he      | lix      |          |           |           |          |          |           |          |          |           |          |          | 1        | 2         | 3        | 4        | 5        | 6          |            |           |              |      |          |           |      |   |           |           |           |           |     |              |    |
| т  | H               | L    | D     | Е         | A   | Е      | к         | L            | G        | М        | K        | R        | D         | Е         | A        | v        | н         | N        | L        | I         | м        | с        | L        | F         | F        | N        | G        | s          | т          | G         | F            | R    | v        | F         | Y    | Р   | I         | м         | F         | к         | w : | IG           |    |
| aca  | acat            | ctt  | tgat  | gaa       | gca | gaga   | aago      | cta          | gga      | atg      | aaa      | aga      | gat       | gaa       | gcc      | gtt      | cat       | aac      | cta      | atc       | atg      | tgt      | ttg      | ttt       | ttc      | aat      | gga      | tca        | act        | ggt       | ttco         | gag  | gttt     | tt        | tate | ccta  | atca      | atgi      | ttca      | aagt      | gga | ttgga        | ı  |
|  |                 |      |       |           |     |        |           |              | _        |          |          |          |           |           |          |          |           |          |          |           |          |          |          |           |          |          |          |            |            |           |              |      |          |           |      |   |           |           |           | _         | C   | 3            |    |
| L  | A               | G    | E     | s         | L   | H      | K         | R            | I        | A        | D        | E        | V         | R         | s        | V<br>~++ | V<br>ata  | A        | E        | S         | G        | D        | G        | T         | .I       | A        | L<br>++- | A          | A          | L<br>++~  | E            | ĸ    | M        | S         | L    | V   | K         | S         | v         | V<br>~+++ | WU  | E A          |    |
| CL   | igea            | igge | gag   | JLCa      | CLA | Caca   | aage      | gu           | alco     | gee      | gaı      | gaa      | guu       | aga       | LCa      | gıı      | gre       | gca      | gag      | LCL       | gga      | gaı      | gga      | acı       | alc      | gee      | ιιa      | gca        | get        | ιιg       | gaga         | aga  | ilga     | igu       | LLag | Juga  | agi       | LCag      | gree      | giii      | 999 | aagca        |    |
|  | 6               |      |       | •         | ٠   | •      |           |              |          |          |          |          |           |           |          |          |           |          |          |           |          |          |          |           |          |          |          |            |            |           |              |      |          |           |      |   |           |           |           |           |     |              |    |
| F  | (R              | ) і  | Q     | P         | Р   | v      | т         | N            | Q        | С        | G        | R        | A         | к         | к        | D        | L         | I        | v        | Q         | s        | н        | т        | D         | s        | F        | Е        | I          | к          | к         | G            | G    | L        | I         | Y    | G   | Y         | Q         | L         | F         | A   | T N          |    |
| tt   | age             | jatt | cag   | jcca      | cca | gtga   | acaa      | aac          | cag      | tgt      | ggg      | aga      | gcta      | aag       | aaa      | gat      | ctg       | att      | gtt      | cag       | agt      | cat      | act      | gat       | tcc      | ttt      | gag      | atc        | aag        | aaa       | ggt          | gggd | tga      | tct       | tace | ggat  | tato      | caat      | ttgi      | tttg      | cta | caaat        |    |
|  |                 |      |       |           |     |        |           |              |          |          |          |          |           | _         |          |          |           |          |          |           |          |          |          |           |          |          |          |            |            |           |              |      |          |           |      |   |           |           |           |           | -   |              |    |
| _  | _               |      | _     | _         | -   |        | _         |              | _        | P        | ERI      | Fm       | otif      | 0         | _        |          | _         | _        | _        |           |          | _        |          |           | _        | _        |          |            | _          |           | -            |      |          | _         | _    | he  | me        | -biı      | ndii      | ng d      | lom | ain          |    |
| D  | E               | K    | I<br> | F<br>.+++ | G   | S      | F         | Q            | E        | F<br>+++ | V<br>~++ | P        | D (       |           | <u>F</u> | v<br>~++ | G         | E        | E        | A         | Q        | E        | K        | ь<br>~+~  | L<br>++~ | P        | ¥<br>+-+ | V          | ь<br>++    | W         | S            | G    | G        | R         | E    | T   | D         | M         | P         | M         | P 1 | E N          | -  |
| ga   | gad             | aaq  | Jaco  |           | 999 | agu    |           | cage         | gag      |          | guu      | CCL      | gati      | aya       |          | gıı      | 999       | yaa      | yaa      | gee       | Caa      | yay      | aay      | cug       | LLG      | cca      | Lal      | gug        | ιιg        | Lgg       | LCL          | gu   | guu      | ggg       | Jaao | 1000  | Jaco      | augu      | ccga      | augo      | cgg | aaaau        | 1  |
|  |                 | ▼    |       |           |     |        |           |              |          |          |          |          |           |           |          |          |           |          |          |           |          |          |          |           |          |          |          |            |            |           |              |      |          |           |      |   |           |           |           |           |     |              |    |
| ĸ  | Q               | С    | P     | G         | к   | N      | L         | v            | L        | т        | М        | т        | R         | L         | I        | v        | A         | Е        | F        | F         | L        | R        | Y        | D         | т        | Y        | т        | I          | Е          | Е         | G            | s    | Е        | v         | т    | I   | т         | s         | L         | к         | ĸ   | A *          |    |
| aaa  | acaç            | ıtgt | cct   | ggc       | aaa | aato   | ctg       | gtt          | ttg      | aca      | atg      | aca      | agg       | ctg       | att      | gtg      | gcg       | gaa      | ttc      | ttc       | ttg      | aga      | tat      | gat       | aca      | tac      | acc      | att        | gag        | gaa       | ggti         | ccq  | gaag     | ,tca      | acaa | atta  | acat      | tctt      | ttga      | aaga      | agg | ca           |    |
|  |                 |      |       |           |     |        |           |              |          |          |          |          |           |           |          |          |           |          |          |           |          |          |          |           | _        |          |          |            |            |           |              |      |          |           |      |   |           |           |           |           |     |              |    |

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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