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PII: S0168-9452(21)00098-4

DOI: https://doi.org/10.1016/j.plantsci.2021.110905

Reference: PSL 110905

To appear in: *Plant Science*

Received Date: 19 January 2021

Revised Date: 17 March 2021

Accepted Date: 2 April 2021

Please cite this article as: Pineau E, Sauveplane V, Grienenberger E, Bassard J-Etienne, Beisson F, Pinot F, CYP77B1 a fatty acid epoxygenase specific to flowering plants, *Plant Science* (2021), doi: https://doi.org/10.1016/j.plantsci.2021.110905

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CYP77B1 a fatty acid epoxygenase specific to flowering plants

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HIGHLIGHTS:

- Data mining pointed toward CYP77B1 as a potential fatty acid metabolizing enzyme
- *In vitro* biochemical studies confirmed that CYP77B1 is a fatty acid epoxygenase
- RT-qPCR and promoter-driven GUS studies showed highest expression in flower
- Flower cutin of loss-of-function mutants *cyp77b1* was unchanged compared to wild type
- Phylogeny shows gene conservation in flowering plant, suggesting a specific function

ABSTRACT:

Contrary to animals, little is known in plants about enzymes able to produce fatty acid epoxides. In our attempt to find and characterize a new fatty acid epoxygenase in *Arabidopsis thaliana*, data mining brought our attention on CYP77B1. Modification of the N-terminus was necessary to get enzymatic activity after heterologous expression in yeast. The common plant fatty acid C18:2 was converted into the diol 12,13-dihydroxy-octadec-*cis*-9-enoic acid when incubated with microsomes of yeast expressing modified CYP77B1 and AtEH1, a soluble epoxide hydrolase. This diol originated from the hydrolysis by AtEH1 of the epoxide 12,13-epoxy-octadec-*cis*-9-enoic acid produced by CYP77B1. A spatio-temporal study of *CYP77B1* expression performed with RT-qPCR revealed the highest level of transcripts in flower bud while, in open flower, the enzyme was mainly present in pistil. *CYP77B1* promoter-driven GUS expression confirmed reporter activities in pistil and also in stamens and petals. *In silico* co-regulation data led us to hypothesize that CYP77B1 could be involved in cutin synthesis but when flower cutin of loss-of-function mutants *cyp77b1* was analyzed, no difference was found compared to cutin of wild type plants. Phylogenetic analysis showed that *CYP77B1* is strictly conserved in flowering plants, suggesting a specific function in this lineage.

Key words: fatty acid, epoxide, diol, cytochrome P450, cutin, flower,

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1. INTRODUCTION:

In all organisms, enzymes able to oxidize fatty acids (FAs) have been the subject of an increasing number of studies because they produce compounds exhibiting fundamental as well as various biological activities [1-3]. Members of the cytochrome P450 (CYP450) superfamily which represents a highly diversified set of heme-containing protein found in all living kingdoms [4] play a major role in these oxidative processes.

In animals, hydroxylases of the CYP4A gene superfamily are involved in the formation of ω and ω-1 hydroxyl derivatives of arachidonic acid (eicosatetraenoic acid C20:4). Upregulation of these enzymes at the transcriptional level by peroxisome proliferator-activated receptors suggests a role in FA catabolism [5]. However, investigations showed biological activities of these hydroxylated derivatives demonstrating that hydroxylation can not only be considered as a catabolic step. Arachidonic acid can also be epoxidized and converted into epoxyeicosatrienoic acids (EETs) by CYP450 epoxygenases mainly belonging to CYP2C and CYP2J subfamilies [6, 7]. These epoxygenases have been deeply investigated because of evidence for their involvement in various aspects of metabolic diseases [6]. These diseases *i.e.* obesity, hypolipidemia, hypertension and diabetes [8] are linked to an increased risk of cardiovascular pathology and EETs exhibit cardiovascular effects and take part in signaling mechanism *via* interaction with G protein-coupled receptors [9].

CYP450 dependent FA hydroxylases and epoxygenases have also been described in plants [10]. Contrary to animals, majority of investigations focused on hydroxylases, particularly ω-hydroxylases, which play a key role in the synthesis of plants protective polymers [10]. Indeed, FA ω-hydroxylases of the CYP86 family are involved in cutin and suberin synthesis covering aerial parts and root surfaces respectively [11, 12] and members of the CYP703 and CYP704 families participate to sporopollenin synthesis, protecting the pollen [13, 14]. The major components of these polymers are FA, essentially C16 and C18, linked together in a three dimensional network *via* ester bonds involving carboxyl and mainly ω-hydroxyl groups [15, 16]: by introducing hydroxyl groups on the last methyl of FAs (ω-position), CYP450 dependent FA ω-hydroxylases will allow their condensation to occur leading to polymer extension *via* primary ester bonds formation.

Even though the first description of a plant CYP450 dependent FA epoxygenase was performed more than four decades ago in a spinach leave extract [17] only few information is available concerning these enzymes in plants today. In the 90's biochemical studies performed with synthetic unsaturated analogs of lauric acid (C12:0) confirmed existence of CYP450 dependent FA epoxygenases in microsomes of *Vicia sativa* [18], Jerusalem artichoke [19] and wheat [20]. More recently we characterized CYP77A4 from *Arabidopsis thaliana* and showed that when heterologously expressed in yeast, it can epoxidize *in vitro*, C18:1 (oleic acid), C18:2 (linoleic acid) and C18:3 (linolenic acid) the 3 major C18 FAs in plants [21]. We also described CYP77A19 from *Solanum tuberosum* an enzyme

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also able to produce *in vitro* epoxides of C18:2 [22]. Roles and presence of FA epoxides has been scarcely studied in plants. They have been reported in seed oil. For example, the two monoepoxides from common C18:2, vernolic acid (12,13-epoxy-octadec-*cis*-9-enoic acid) and coronaric acid (9,10-epoxy-octadec-cis-12-enoic acid) account for 80% and 15 % of the total FA in seed oil of *Vernonia anthelmintica* and *Chrysanthemum coronarium* respectively and are found in seed oil of many other plants [23]. In *Euphorbia lagascae,* vernolic acid which represents up to 90% of FA in some Euphorbiaceae is produced by CYP726A1 [24]. Epoxides of unusual plant FA have also been reported, e.g. seed oil of *Alchornea cordifolia* contains more than 50% of 14,15-epoxyeicosa-11-enoic acid [23]. Signification of FA epoxides accumulation in seed oil still needs to be elucidated. They might represent a reservoir of molecules used in plant pathogen interaction, indeed vernolic and its derivatives have been shown to have antifungal properties [25, 26].

Epoxides of C18 FA and their corresponding diols derivatives, together with ω-hydroxyl mentioned above, are also constituent of plant protective polymers [15, 16]. This presence confers to CYP450 dependent FA epoxygenases a potential role to play in protective envelope synthesis which still needs to be assessed. Recent observations are in favor of such a role. Indeed, regarding CYP77A4 described above, Kawade et al., (2018) [27] observed a developmental defect in *cyp77a4 Arabidopsis thaliana* embryos. Based on toluidine blue permeability test, authors demonstrated importance of CYP77A4 in cuticle production and properties, in embryos. CYP77A19, a FA epoxygenase characterized in *Solanum tuberosum* [22] partially restored the wild type phenotype in an *Arabidopsis thaliana* cutin mutant, also suggesting a possible role in cutin synthesis. FA epoxides are also precursors of diols which are produced by epoxide hydrolase like AtEH1 recently described in *Arabidopsis thaliana* [28]. These diols are key components in term of cutin structure: they are secondary hydroxyls and are engaged into secondary ester bond bringing reticulation to cutin [29]. Importance of secondary hydroxyls in cutin organization is illustrated by the example of CYP77A6. This enzyme produces a secondary hydroxyl by hydroxylation of the carbon 10 of 16-hydroxypalmitic acid leading to the formation of 10,16-dihydroxypalmitic acid (diOH) [30]. Scanning electron microscopy observation of *cyp77a6* flowers revealed that absence of diOH led to disappearance of nanoridges in the cutin on the surface of petals compared to petals of WT plants [30].

With the aim of identifying a FA epoxygenase potentially involved in cutin synthesis, and based on phylogenetic and *in silico* studies, we focused our attention on *CYP77B1* (*At1g11600*) from *Arabidopsis thaliana*. *CYP77B1* is a single gene in its family and this absence of duplication suggests a strong selection pressure due to important function in the plant physiology. We report here the heterologous expression and functional characterization of CYP77B1. Catalytic properties of the protein were explored using recombinant CYP77B1, expressed in an engineered yeast strain. Modification of the N-terminus was necessary to get enzymatic activity. Our study confirms that this enzyme is a fatty acid metabolizing enzyme and is able to produce 12,13-epoxy-octadec-*cis*-9-enoic acid by epoxidation of linoleic acid. RT-qPCR spatio-temporal and promoter-driven GUS expression studies revealed the

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higher level of transcripts in flower at early stage while, in open flower, the transcript is mainly present in pistil and stamens. However, when cutin of loss-of-function mutants *cyp77b1* was analyzed, no difference was found compared to cutin of wild type plants. We discuss the possible biological roles of CYP77B1.

2 EXPERIMENTAL PROCEDURES:

2.1 Chemicals

Radiolabeled [1-^{14}C]lauric acid (45 Ci/mol) was from CEA (Gif sur Yvette, France). The silylating reagent N,O-bistrimethylsilyltrifluoroacetamide containing 1% of trimethylchlorosilane was from Sigma (Steinheim, Germany). NADPH and linoleic acid (C18:2) were from Sigma (Steinheim, Germany). Thin layer plates (Silica Gel G60 F254; 0.25mm) were from Merck (Darmstadt, Germany).

2.2 Cloning of CYP*77B1* and modification of N-terminal sequence

The coding sequence of *CYP77B1* (*At1g11600*) was cloned by PCR from a cDNA library of *Arabidopsis thaliana* ecotype Columbia-0 using primers 5'-CCCCAGATCTATGGATCTCACCGACGTAATC-3' and 5'-CCCCGGTACCTTAGGTCCTTGACCTGATTTG-3', adding respectively BglII and KpnI sites. *CYP77B1* cDNA was amplified with the Phusion High-Fidelity PCR Master Mix (Thermo Fisher Scientific, Illkirch France) using the following conditions: initial denaturation of 30 s at 98 °C, 30 thermal cycles (15 s at 98 °C, 15 s at 61 °C, 1 min 45 s at 72 °C), final extension 5 min at 72 °C. The PCR product was cloned into the pYeDP60 vector [31] using BamHI and KpnI restriction sites. The program TOPCONS (https://topcons.net/) was used to compare the prediction of protein membrane topology of CYP77A4 (*At5g04660*), CYP77A6 (*At3g10570*) and CYP77B1 (*At1g11600*). CYP77B1 N-terminal transmembrane domain was swapped by the one of CYP73A92 from *Brachypodium distachyon* (*Bradi2g53470*) besides optimization of the first 100 codons for improved yeast expression (http://www.encorbio.com/protocols/Codon.htm). gBlocks (IDT) sequence coding for the transmembrane domain and the 100 first codons was ordered (5'-GCCCTATAGTGAGTCGTATTACCCCGGGATGGATGTATTATTACTGGAAAAGGCGCTGTT
AGGTTTATTCGCTGCTGCTGTCTTAGCTATTGCAGTAGCAAAACTGACAGGTAAAAGATT
TAGACTTAAAGGTTGGCCATTGGTTGGTAATTTGTTGCAAGTTATTTTCCAAAGAAGACA
TTTCGTTTTCTTGATGAGAGATTTGAGAAAAAAATATGGTCCAATTTTCACTATGCAAATG
GGTCAAAGAACTATGATTATTATTACTGATGAAAAATTGATTCATGAAGCTTTGGTTCAA

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AGAGGTCCAACTTTCGCTTCTAGACCACCAGATTCTCCAATTAGATTGATGTTCTCTGTTG GTAAATGTGCTATTAATTCTGCTGAATATGGTTCTTTGTGGAGAACTTTGAGAAGAAATTT CGTTACTGAATTGGTTACTGCTCCAAGAGTTAAACAATGTTCTTGGATTAGATCT-3'), primers 5'-TAAACAATGTTCTTGGATTAGATCTGGCTATGCAGAATCATATG-3'- and 5'-CAAGCTATTTAGGTGACACTATAGAGGTACCTTAGGTCCTTGACCTGATTTG-3' were used to amplify *CYP77B1* C-terminal coding sequence. Then Gibson cloning (ThermoFisher) was performed to assemble the two DNA fragments in pGEMT vector (Promega) following manufacturer's instructions. After SmaI/KpnI digestion the mutated CYP77B1 coding sequence was inserted in pYeDP60 vector [31].

2.3 Heterologous expression of CYP77B1 in ye*ast*

For expression of the full-length CYP77B1 protein, we used a yeast expression system originally developed for the expression of P450 enzymes and consisting of plasmid pYeDP60 and *Saccharomyces cerevisiae WAT11* strain [31]. Yeast cultures were grown and CYP77B1 expression was induced from one isolated transformed colony as described in [31]. After growth, cells were harvested by centrifugation and manually broken with glass beads (0.45 mm diameter) in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA and 600 mM sorbitol. The volume of buffer was proportional to the weight of yeast pellet: 6g were homogenized in *ca*. 30mL of buffer. The homogenate was centrifuged for 20 min at 10,000 *g*. The resulting supernatant was centrifuged for 1 h at 100,000 *g*. The pellet consisting of microsomal membranes was resuspended in 50 mM Tris-HCl (pH 7.4), 1 mM EDTA and 30% (v/v) glycerol with a Potter-Elvehjem homogenizer and stored at -30°C. The volume of resuspension buffer was proportional to the weight of yeast pellet: microsomes extracted from 6 g of yeast were resuspended in 3 mL of buffer. All procedures for microsomal preparation were carried out between 0-4°C.

Recombinant epoxide hydrolase AtEH1 from *Arabidopsis thaliana* was produced as described in [28].

2.4 Enzyme activity

Incubations were performed in 5 mL glass tubes (VSM, Andeville, France). Radiolabeled substrate dissolved in ethanol (10 µL of 1mM solution) was added in the tube and then ethanol was evaporated. Resolubilization of the substrate was confirmed by measuring the radioactivity of the incubation media. Enzymatic activity of yeast-expressed CYP77B1 proteins were determined by following the formation rate of metabolites. The standard assay (0.1 mL) contained 20 mM sodium phosphate (pH 7.4), 1mM NADPH and radiolabeled substrate (100 µM). For lauric acid metabolism

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measurements, the reaction was initiated by addition of the microsomal fraction from yeast expressing CYP77B1 (0.4 mg of protein) and was stopped after 10 min by the addition of 20 µl acetonitrile (containing 0.2% acetic acid). The reaction products were resolved by thin layer chromatography (TLC) (Silica Gel G60 F254, 0.25mm, Merck Darmstadt, Germany) as described below. For linoleic acid metabolism measurement, the same procedure was followed but 10 min after initiation of the reaction with addition of microsomes, cytosolic fraction from yeast expressing AtEH1 (0.4 mg of protein) was added to the incubation. After another 10 min, metabolites were extracted from incubation media with 1mL of diethyl ether/hexane (50:50, v/v) which was then evaporated, metabolites were then methylated with diazomethane, trimethylsilylated with BSTFA containing 1% (v/v) TMS (1:1, v/v) and subjected to GC/MS analysis.

2.5 Thin layer chromatography (TLC) methods

Incubation media was directly spotted on Thin Layer Chromatography (TLC) plates. For separation of metabolites from residual substrate, TLC plates were developed with a mixture of diethyl ether/light petroleum (boiling point, 40-60°C)/formic acid (50:50:1, v/v/v). The plates were scanned with a radioactivity detector (Raytest Rita Star, Straubenhardt, Germany). The silica corresponding to the radiolabeled metabolites were scraped and metabolites were eluted from the silica with 10 mL of diethyl ether, which was removed by evaporation, derivatized with diazomethane and BSTFA as described above and subjected to GC/MS analysis.

2.6 *cyp77b1* characterization and GC/MS analysis

For polyester analysis, ten flowers were used for each biological replicate. Polyesters of whole flowers were analyzed by GC-MS as described in [32]. A T-DNA insertion line was obtained from Salk seed stock. Correct T-DNA insertion in the *cyp77b1* gene was confirmed for the SALK_106298 line by PCR on genomic DNA from leave using a T-DNA specific primer: 5'-GCGTGGACCGCTTGCTGCAACT-3' and CYP77B1 specific primer: 5'-TGATTACGTCGGTGAGATCC-3'. The lack of *CYP77B1* transcripts in homozygous individuals (Supplemental figure 1) was confirmed by RT-qPCR using primers: 5'-CGGAGTACGGATCTTTGTGG-3' and 5'-CGCGGTCACTAGCTCTGTC-3'.

GC-MS analysis of metabolites obtained from incubations were carried out on a gas chromatograph (Agilent 6890 Series) equipped with a 30-m capillary column with an internal diameter of 0.25 mm and a film thickness of 0.25 µm (HP-5MS). The gas chromatograph was coupled with a

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quadrupole mass selective detector (Agilent 5973N). Mass spectra were recorded at 70 eV and analyzed as in [33].

2.6.1 Metabolites of lauric acid: For analysis of products generated by recombinant modified CYP77B1 in incubation with lauric acid, metabolites of peaks 1 and 2 (Fig. 2B) were eluted from silica, derivatized and subjected to GC/MS analysis which revealed the presence of 3 metabolites.

Mass spectrum of first metabolite derivatized showed ions at *m/z* (relative intensity %): 73 (54%) ($(CH_3)_3Si^+$), 75 (23%) ($(CH_3)_2Si^+=O$), 117 (100%), 255 (8%) (M-47) (loss of methanol from the (M-15) fragment), 271 (2%) (M-31) (loss of OCH_3 from the methyl ester), 287 (3%) (M-15) (loss of a methyl from the TMSi group). This fragmentation pattern is characteristic of the derivative of 11-hydroxylauric acid (M = 302g/mol) (Fig 2C, compound (a)).

Mass spectrum of second metabolite derivatized showed ions at *m/z* (relative intensity %): 73 (100%) ($(CH_3)_3Si^+$), 75 (25%) ($(CH_3)_2Si^+=O$), 131 (54%), 255 (3%) (M-47) (loss of methanol from the (M-15) fragment), 271 (1%) (M-31) (loss of OCH_3 from the methyl ester), 273 (13%) (M-29). This fragmentation pattern is characteristic of the derivative of 10-hydroxylauric acid (M = 302g/mol) (Fig 2C, compound (b)).

Mass spectrum of third metabolite derivatized showed ions at *m/z* (relative intensity %): 73 (100%) ($(CH_3)_3Si^+$), 75 (31%) ($(CH_3)_2Si^+=O$), 145 (95%), 255 (4%) (M-47) (loss of methanol from the (M-15) fragment), 259 (35%), 271 (2%) (M-31) (loss of OCH_3 from the methyl ester), 287 (2%) (M-15) (loss of a methyl from the TMSi group). This fragmentation pattern is characteristic of the derivative of 9-hydroxylauric acid (M = 302g/mol) (Fig 2C, compound (c)).

2.6.2 Metabolite of linoleic acid: Mass spectrum of the derivatized metabolite formed in incubation of C18:2 with microsomes of yeast expressing CYP77B1 in presence of NADPH followed by addition of epoxide hydrolase AtEH1 showed ions at *m/z* (relative intensity %): 73 (100%) ($(CH_3)_3Si^+$), 75 (14%) ($(CH_3)_2Si^+=O$), 441 (4%) (M-31) (loss of OCH_3 from the methyl ester), 457 (1%) (M-15) (loss of a methyl from the TMSi group). It also showed ions at *m/z* (relative intensity %): 173 (85%), 275 (67%) and 299 (60%) resulting from cleavage in the vicinity of hydroxyls carrying the trimethylsilyl (Fig 4). This fragmentation is characteristic of the derivative of 12,13-dihydroxy-octadec-*cis*-9-enoic acid (Fig 5 compound (c)) generated by hydrolysis of 12,13-epoxy-octadec-*cis*-9-enoic acid (Fig 5 compound (b)) formed by CYP77B1 (M = 472g/mol).

2.7 Analysis of gene expression

2.7.1 Real Time-quantitative PCR analysis of *CYP77B1* expression

Tissues from 6-week-old *Arabidopsis thaliana* plants ecotype Col-0 were collected (approx. 50 mg) and snap frozen in liquid nitrogen. Total RNA was extracted using the Trizol kit, following the

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manufacturer's instructions. cDNA was synthesized from 1 µg total RNA of each sample, using ImPromII Reverse Transcription System (Promega), following the manufacturer's instructions. RT-qPCR assays were performed using 1 µL of cDNA (diluted five times) together with appropriate primer pairs (2.5 µM each), SYBR Green PCR master mix (Applied Biosystems) in a final volume of 10 µL. Primers were designed with the software Universal Probe Library (Roche). Assays were run in triplicates on a Light Cycler 480 (Roche). The amplification program consisted of a denaturation step at 98°C for 5 min followed by 45 cycles (95°C for 10 s, 60°C for 15 s, 72°C for 15 s). Relative quantification of gene expression was performed using the $2^{-\Delta Ct}$ method in which the amount of target is normalized to endogenous references. The *Arabidopsis thali*ana genes *EXP* (*At4g26410*) and *GAPDH* (*At1g13440*) were used as endogenous references using primers pair 5'-GAGCTGAAGTGGCTTCAATGAC-3', 5'-GGTCCGACATACCCATGATCC-3'and 5'-TTGGTGACAACAGGTCAAGCA-3', 5'-AAACTTGTCGCTCAATGCAATC-3', respectively. The amplification reaction on *CYP77B1* was carried out using the primers 5'-CGGAGTACGGATCTTTGTGG-3' and 5'-CGCGGTCACTAGCTCTGTC-3'.

2.7.2 *ProCYP77B1::*GUS experiment

The 2000 bp region upstream of the CYP77B1 translation initiation codon was amplified by PCR using primers 5'-GGGGACAACTTTGTATAGAAAAGTTGGTATATACTTTACCTTCTTCCAGTAAAA-3' and 5'-GGGGACTGCTTTTTTGTACAAACTTGTGTTTATTATTATTGAAGAGAGAAAGA-3'.
Amplicons were cloned into pDONRP4-P1R entry vector by BP recombination (Invitrogen). Generated entry vector was sequence verified. To generate the *ProCYP77B1::GUS* fusion construct, entry vector containing *proCYP77B1* and pEN-L1-S-L2 vector containing *GUS* ORF were recombined with pHbm42GW7 by LR clonase (Invitrogen). *Agrobacterium tumefaciens* strain C58C1 was transformed with the generated expression construct and *Arabidopsis thaliana* ecotype Col-0 plants transformed by floral dip. Transgenic plants were selected on 1/2 Murashige and Skoog plates supplemented by 2% Saccharose and 50 mg/mL kanamycin. After two weeks, plants were transferred in pots and maintained on soil under 16h/8h day/night cycle in a growth chamber with 21°C/18°C day/night temperature cycle.

The histochemical GUS essays were carried out by incubating flowering stems in GUS assay buffer (0.5 mM Ferrocyanide, 0.5 mM Ferricyanide, 0.1% Triton X-100, 0.5 mg/mL X-Gluc in 50 mM Phosphate Buffer pH7) at 37°C overnight and decolorizing with 75% ethanol. Prior the observation, tissues were rinsed 3 times with water. Imaging was performed using a LEICA binocular microscope with following setup: 0.5X objective, automatic exposure, maximum saturation, gain 2.5X.

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2.8 Phylogeny analysis

CYP77 putative homologs were searched in Phytozome 12 databases (http://www.phytozome.org) by selecting all *viridiplantae*. Sequences were aligned using MUSCLE 3.7 program [34]. The resulting multi-alignment was cured using Gblocks 0.91b with the following settings: minimum number of sequences for a conserved position of 4, minimum number of sequences for a flanking position of 5, minimum number of contiguous nonconserved position of 8, minimum length of block of 5 and gap positions allowed with half use similarity matrices. A Maximum likelihood phylogenic tree was constructed using PhyML 3.0 [35] using the WGA amino acid substitution model. Statistical branch support was obtained by bootstrapping 500 replicates.

3 RESULTS:

3.1 Identification of CYP77B1 as a potential FA-metabolizing enzyme.

An approach based on an *in silico* analysis of publicly available transcriptome data has been developed to investigate potential involvement of cytochrome P450s in specific metabolic pathways [36]. This analysis identifies genes that are co-expressed with a given bait CYP450 in different conditions (*i.e.* development, stress, hormonal regulation….) and based on the functional annotation of co-expressed genes, a metabolic pathway in which the bait CYP450 could be involved, is predicted. This strategy allowed us to highlight *CYP77B1* (*At1g11600*) with a potential implication in fatty acid metabolism. Indeed, it is developmentally co-expressed with several characterized enzymes involved in lipid metabolism. Among the most similarly expressed genes are *At1g06350, At1g06100* and *At1g06120* coding for fatty acid desaturases which introduce unsaturation in FA required for an enzymatic epoxidation to occur. *AtEH1* (*At3g05600*) coding for a fatty acid epoxide hydrolase that we recently characterized [28] is also co-regulated with *CYP77B1*. For a complete list of co-expressed genes, see: http://www-ibmp.u-strasbg.fr/~CYPedia/CYP77B1/CoExpr_CYP77B1_Organs.html. These *in silico* observations support the hypothesis of a role for CYP77B1 to play in fatty acid metabolism more particularly in epoxidation.

3.2 Cloning and expression of *CYP77B1,* incubation with the model substrate lauric acid.

The coding sequence of *CYP77B1* was amplified by PCR from a cDNA collection from *Arabidopsis thaliana* and cloned into pYeDP60, a yeast expression vector [31]. Characterization of CYP77B1 was performed using microsomes from the yeast strain *WAT11* transformed with pYeDP60

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carrying *CYP77B1* coding sequence. *WAT11* is engineered for *CYP450* expression: it over-expresses a plant CYP450 reductase leading to a better electron transfer and to an increase of the expressed CYP450 stability. Furthermore, *WAT11* possesses only three *CYP450* not expressed or expressed at a negligible level in our growth condition, ensuring that the metabolism described here results from enzymatic reactions catalyzed by CYP77B1. To test the hypothesis of CYP77B1 being a FA-metabolizing enzyme, we first incubated the model substrate lauric acid (C12:0) [10] with microsomes of transformed yeast. However we could not detect any metabolite, neither in absence nor in presence of NADPH, the cofactor required for CYP450 activity (not shown).

3.3 Hydrophobicity analysis and modification of CYP77B1 N-terminal extremity.

To investigate if the lack of enzymatic activity could originate from a bad anchoring of the protein in the yeast ER membrane, we analyzed its hydrophobicity pattern and compared with the ones of two members of the same family, CYP77A4 and CYP77A6 both shown to be catalytically active with FAs after heterologous expression in *WAT11* yeast strain [21, 30]. As shown in figure 1, hydrophobicity of the amino acids 1-40 of CYP77B1 is about half of that of CYP77A4 or CYP77A6. Furthermore, hydrophobicity patterns of these latter enzymes suggest the existence of 2 and 3 transmembrane domains in CYP77A4 (Domains 1 and 2) and CYP77A6 (Domains 1, 2 and 3) respectively which are not present in CYP77B1. To further investigate on the importance of CYP77B1 anchoring on enzymatic activity, we replaced its native N-terminal extremity with the one of *Brachypodium distachyon* BdCYP73A92 which has been shown to insure both efficient anchoring and enzymatic activity during CYP450 heterologous expression [37]. As shown on figure 2B, two major peaks of radioactivity (Fig 2B, peak 1 and 2) appear when the mixture resulting from incubation of radiolabeled lauric acid with microsomes of yeast expressing modified CYP77B1 is loaded on TLC. These peaks are not formed in absence of NADPH (Fig 2A). Metabolites present in these peaks were purified and derivatized (see Experimental Procedure). GC/MS analysis revealed that they consist in a mixture of 9-, 10- and 11-hydroxylauric acids (Fig 2C).

3.4 Epoxidation of linoleic acid by CYP77B1.

Carbons 9, 10 and 11 hydroxylated by CYP77B1 in lauric acid, correspond, in the abundant C18:2 plant FA, to carbons sp2 (engaged in unsaturation) or to carbons located in the vicinity of unsaturation. Knowing that attack of a sp2 carbon by a CYP450 usually results in epoxide formation, we tested the capabilities of CY77B1 to catalyse formation of epoxides when incubated with C18:2. This potential substrate was incubated with microsomes of yeast expressing modified CYP77B1 in

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absence or presence of NADPH. Incubation media also contained heterologous AtEH1, an epoxide hydrolase recently characterized in *Arabidopsis thaliana* [28]. This later enzyme was introduced in incubation media in order to hydrolyze into diol, putative epoxides produced by CYP77B1. Indeed, diols of FA give a more characteristic fragmentation pattern than the parent epoxides [33]. After extraction and derivatization, metabolites were subjected to GC/MS analysis. As shown in figure 3B addition of NADPH in the incubation leads to the formation of a metabolite (Fig 3B, Peak 1, Rt:45.82 min). It is not produced in incubation with microsomes from yeast transformed with a void plasmid (Fig 3C). Its fragmentation pattern (Fig 4) is characteristic of the derivative of 12,13-dihydroxy-octadec-*cis*-9-enoic (Fig 5 compound (c)) coming from hydrolysis by AtEH1 of 12,13-epoxy-octadec-*cis*-9-enoic (Fig 5 Compound (b)) generated by CYP77B1. Interestingly, a compound present in control incubation (Fig 3A Peak X Rt: 45.90 min) was absent in presence of NADPH (Fig 3B). According to its fragmentation pattern (Supplemental figure 2) it is not related to FA and according to NIST98 library, it might be linked to grandirubrine. Its absence in figure 3B might reflect its metabolization by microsomal enzyme.

In preliminary experiments we also tested C18:1 as a potential substrate. This FA was metabolized by CYP77B1, however we did not pursue investigation because relative mobility of major metabolites on TLC (Supplemental figure 3B peaks 1-5) was lower than what was previously described for the corresponding epoxide [21] and suggests that these metabolites are hydroxyl derivatives of C18:2.

3.5 Localization of CYP77B1 expression and flower cutin analysis.

RT-qPCR and GUS reporter gene constructs were performed to determine the expression pattern of *CYP77B1* at the organ and tissue level. As shown in figure 6, RT-qPCR revealed the highest expression of *CYP77B1* in flowers buds. *CYP77B1* is also expressed in pistil, petals and to a lesser extent in root, leave and silique. To study the expression of *CYP77B1* in flower with higher resolution, the 2kb putative promoter region upstream of the predicted *CYP77B1* start codon was fused in frame to the *β-glucuronidase* (GUS) gene and introduced in *Arabidopsis thaliana* genome by transformation. Microscopic examination of GUS staining in the flowers (Figure 7) showed coloration in the pistil, stamens filaments and petals. In order to test the hypothesis of an involvement in cutin biosynthesis, we analyzed and compared cutin of Col0 and loss of function mutants *cyp77b1* in flowers, which showed the highest expression of *CYP77B1*. We could not detect any fatty epoxides or corresponding diols in constituting monomers and we did not see any significant difference between both genotypes in the typical C16 and C18 monomers found in flowers (Supplemental figure 4).

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3.6 Phylogenetic analysis.

CYP77B1 belongs to the cytochrome P450 family 77. In *Arabidopsis thaliana* genome, five *CYP77A* are found (*CYP77A4, CYP77A5, CYP77A6, CYP77A7, CYP77A9*) while only one member of the subfamily *CYP77B* is found (*CYP77B1*). Both *CYP77A6* [30] and *CYP77A4* [27] have been shown to be involved in cutin formation.

Cutin analysis performed in the present work seems to rule out the participation of CYP77B1 in its synthesis. However, the present work shows that similarly to CYP77A6 and CYP77A4, CYP77B1 is also able to catalyze FA oxidation, we therefore deciphered the phylogenetic relationship of *CYP77A* and *CYP77B* subfamilies. Homologs for both *CYP77Bs* and *CYP77As* were searched in Phytozome 12 databases (http://www.phytozome.org) regrouping genomes from 64 species in all major evolutionary clusters of the *Viridiplantae*, including green algae, mosses and flowering plants. Predicted amino acid sequences from putative homologs were used to build a Maximum Likelihood phylogenic tree (Fig 8A). Phylogenic analysis shows that for both *CYP77A* and *CYP77B* subfamilies, members could be found in all angiosperms, while absent from lycophytes, mosses or green algae. Homologs for both *CYP77A* and *CYP77B* subfamilies could be found in *Amborella trichopoda*, belonging to a primitive lineage of flowering plants, indicating that the subfamilies separation is ancient and occurred prior or concomitant with angiosperm evolution. Interestingly, homologs of *CYP77B1* could be found in all tested angiosperm species, suggesting a strict conservation in this lineage (Figure 8B). Furthermore, mostly one, otherwise very few *CYP77B* members could be found in each species. Strictly one member could be found in all available monocotyledon genomes. In some taxons however, particularly the ones that had undergone recent whole-genome duplication, few duplicates could be found (Figure 8B). This result suggests that *CYP77B* subfamily evolved under purifying selection supporting a role in key biological functions. Interestingly, the analysis of homolog genes expression for which data are available on ePlants (http://bar.utoronto.ca) (tomato's homolog (*Solyc02g080330*), Poplar's homolog (*Potri.008G025500*), potato (*PGSC0003DMG400002078*)), show a conserved preferential expression in the flowers similar to the pattern we describe here for *CYP77B1*. Altogether these data show that *CYP77B1* is specific to angiosperms and putatively strictly conserved in this lineage, including known expression patterns. All these data strongly points toward an important function supported by the *CYP77Bs* in angiosperms and reinforce the view that *CYP77B1* does not support the same function as *CYP77As*.

4 DISCUSSION:

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Previous investigation from our laboratory enabled us to characterize two members of the CYP77A subfamily in *Arabidopsis thaliana*: CYP77A4 and CYP77A6. Our work showed that both participate to FA metabolism. In a biochemical approach, heterologous production of CYP77A4 in a yeast allowed us to demonstrate its capacity to catalyse *in vitro* epoxidation of unsaturated FA [21]. Recently, Kawade et al., (2018) [27] described a developmental defect in *cyp77a4 Arabidopsis thaliana* embryos. Based on toluidine blue permeability test, authors demonstrated importance of CYP77A4 in cuticle production in embryos. Involvement of CYP77A6 in *Arabidopsis thaliana* cutin synthesis has also clearly been demonstrated [30]. To pursue our investigation on the CYP77 family we focused on CYP77B1, indeed during investigation on the CYP77A subfamily, we noticed the presence of this single member of CYP77B subfamily (http://www-ibmp.u-strasbg.fr/~CYPedia/#CYP77) [36]. It appeared to be particularly interesting to investigate the function of this P450 because its unicity indicates an important function that precluded gene duplication. An approach based on an *in silico* analysis showed that although co-expression correlation relatively low compared with other co-expressed genes acting in a common pathway, with a Pearson correlation coefficient not exceeding 0.74, it was striking that 5 out of the top 8 co-regulated genes co-expressed with CYP77B1 have been functionally characterized as being involved in FA metabolism. This was in agreement with enzymatic activities of previously described CYP77s.

Heterologous protein production is a key step for the characterization of P450 allowing identification of potential substrates and evaluation of their metabolism by an enzyme isolated from other P450s present in the same organism. We therefore produced CYP77B1 in the yeast *WAT11* [31] engineered for P450 expression. However, we did not detect any enzymatic activity toward the FA model substrate lauric acid (C12:0). Because of their membrane-bound characteristic, a critical point for P450 functional expression is their anchoring in the membrane. Another important point, linked to heterologous expression, is the codon bias which becomes critical during high gene expression [38]. To circumvent these two potential problems, we replaced the 300 first nucleotides (5') of *CYP77B1* by a recoded sequence for optimized yeast expression, including the N-ter of BdCYP73A92 which allowed increase of production when introduced in the *WAT11* strain yeast [37]. This strategy allowed to demonstrate the capacities of CYP77B1 to oxidize our model substrate, lauric acid, thus suggesting that the lack of activity first observed in experiments with the original gene, likely resulted from a problem of expression or a problem of protein folding an binding to the ER membranes. Another possibility to explain the lack of detectable activity with the native N-ter anchor could be a targeting of the protein not to ER as expected but to plasma membrane as previously observed with CYP450 heterologously expressed in yeast [39].

Positions attacked on lauric acid (carbons 9, 10 and 11) are in the vicinity of unsaturation in the physiological FA C18:2 (linoleic acid) abundant in plants. We then tested capacities of CYP77B1 to make C18:2 epoxide derivatives by oxidation of its unsaturations. We knew from previous investigations that diols resulting from epoxides hydrolysis gives a much clearer fragmentation pattern

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than epoxides when analyzed in mass spectrometry [21, 33]. We therefore added AtEH1, a soluble epoxide hydrolase [28] in incubation media of microsomes of yeast expressing CYP77B1 and C18:2. This allowed us to identify a diol resulting from hydrolysis of 12,13-epoxy-octadec-*cis*-9-enoic generated by CYP77B1. Several lines of evidence suggest that epoxidation of C18:2 is a physiological function of CYP77B1. Indeed, *CYP77B1* is co-regulated with 1) genes coding for FA desaturases responsible for production of unsaturated FA and 2) a gene coding for an epoxide hydrolase which converts epoxides to diols (http://www-ibmp.u-strasbg.fr/~CYPedia/CYP77B1/CoExpr_CYP77B1_Organs.html) [36].

Epoxides of FA may represent up to 60% of cutin monomers [40, 41] and even if not major, epoxides and their diol derivatives have been described in *Arabidopsis thaliana* cutin [28, 42]. If genome or RNA full sequencing allowed precise numbering and sorting of P450s in some plant species, the physiological roles of the majority of them remain to be established. In an attempt to unravel the role of CYP77B1 in plant development we focused on a potential role in cutin biosynthesis. Indeed, in addition to the presence of epoxides and diols in *Arabidopsis thaliana* cutin, two members of the CYP77 family, CYP77A4 and CYP77A6 mentioned above, participate to cutin synthesis [27, 30], furthermore *CYP77B1* is coregulated with the epoxide hydrolase *AtEH1* (*At3g05600*) also involved in cutin synthesis [28]. However, when we analyzed cutin of loss of function mutants *cyp77b1*, we did not detect any difference compared to cutin of wild type plants suggesting that its epoxy FA product has another role than cutin component. As mentioned above, CYP77A4 is also able to catalyze FA epoxidation [21] but the lack of particular cutin phenotype of *cyp77b1* is unlikely to be the consequence of a functional complementation by *CYP77A4*. Indeed, microarray analysis indicated that *CYP77A4* is expressed primarily in early-stage embryos (bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi), furthermore Kawade et al., (2018) [27] confirmed that its involvement in cuticle matrix synthesis is limited within embryos. However, we can not ignore the possibility that a still unidentified enzyme can help to restore cutin wild type phenotype in *cyp77b1 Arabidopsis thaliana*.

In the context of polymer synthesis, it is noteworthy that a fraction of the cuticle named cutan, highly recalcitrant to depolymerization and representing up to 40-60% of cutin mass has been described in several plant species [43, 44]. Chemical composition and structure of the cutan are poorly documented, it is highly unsoluble and non-deesterifiable [45]. Villena et al. [44] proposed that it is made of polymethylenic chains and free carboxylic groups linked by ether bonds. Tegelaar et al. [46] hypothetized that it is built with polymethylenic and polysaccharide moieties linked via non-hydrolyzable bonds. It is also speculated that it is made of derivatives of FA epoxides thought to be linked by ether bonds highly resistant to depolymerization, originating from ring-opening of the epoxide. Additional experiments would be necessary to investigate on a possible role for CYP77B1 in the production of epoxides dedicated to cutan formation.

Our phylogenetic studies suggest that CYP77B1 has evolved independently of CYP77As members from their apparition with the angiosperms. The two subfamilies are both putatively strictly

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conserved in this lineage supporting the fact they evolved to sustain two independent functions, in accordance with our results.

In plants, a few examples of fatty acid epoxides have been described. Cahoon et al., (2002) [24] characterized CYP726A1 from *Euphorbia lagascae* and showed its capabilities to catalyse epoxidation of linoleic acid into 12,13-epoxyoctadec-*cis*-9-enoic acid (vernolic acid) when linoleic is part of a phosphatidyl choline moiety. The resulting epoxide is then stored in seed oil [47] where it can represent up to 90% of the FAs in some *Euphorbiaceae*. In this context it can be noticed that *CYP77B1* is co-regulated with *At4g22490* coding for a potential lipid transfer protein involved in seed storage (http://www-ibmp.u-strasbg.fr/~CYPedia/#CYP77). FA and FA derivatives metabolism is a major player in the plant defense network and there are observations that give to CYP77B1 a potential role to play in this network. It is noteworthy that vernolic acid produced by CYP77B1 gives resistance to rice against *Pyricularia oryzae* by inhibiting spore germination and mycelium growth [25]. Vernolic acid can be converted to the corresponding vicinal diol by AtEH1 [28]. Hydroxylation of this diol by a cytochrome P450 specific of FA ω-1 position that we previously described in *Arabidopsis thaliana* microsomes [48] would lead to the formation of 12,13,17-trihydroxy-octadec-*cis*-9-enoic an antifungal compound [49]. When vernolic acid is metabolized by CYP77A4, a diepoxide is generated [21]. In animals, diepoxides are converted into tetrahydrofuran (THF) after hydrolysis by epoxides hydrolases [50]. THF formation has not been much documented in plant but few investigation report cytotoxicity of THF containing compounds [51]. Cyclisation of C18:2 diepoxide after hydrolysis by an epoxide hydrolase remains to be demonstrated in plants but it could correspond to the formation of a novel class of phytooxylipins [2].

Knowledge of the localization of an enzyme can be of great help for its biological role understanding. RT-qPCR analysis revealed that the highest expression for *CYP77B1* was located in flowers. More precisely, *CYP77B1* promoter-driven GUS expression indicated strong reporter activities in stamens and in pistil. Interestingly Yue et al., [52] isolated *Phcyp77B1* in Petunia, homolog of *CYP77B1* described here and also measured a higher expression in flower compared to vegetative tissues. Investigation on plant lipid composition has mainly focused on the oil from their seeds (reviewed in [53]). However non-seed tissues such as flowers also synthesize and store lipids although their function in these tissues needs to be discovered [54]. Only few data are available on lipids and their content in flowers. C18:2 can represent up to 57% of FA in flowers of different plants [55] and has been shown to represent ca. 25% of FA in triacylglycerol as well as in polar lipids in *Arabidopsis thaliana* flower [55]. In the floral context, analysis performed with *Nicotiana* and other plants demonstrated that distribution of oxidized FA (C18:1 and C18:2) was of primary importance for determination of self-compatibility during pollination process [56].

Two distinct types of plant enzymes unrelated to CYP450 carrying FA epoxidation activity have been described. The first one is a peroxygenase and uses hydroperoxides as cofactors to catalyze FA epoxidation [57, 58]. The second one, a non-heme di-iron enzyme named “desaturase-like” enzyme was

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described by Lee et al., [59]. This shows that FA epoxidation in plant can be catalyzed by evolutionarily divergent enzymes and suggests a key role of these epoxides and their derivatives. More work will be necessary to decipher the role of CYP77B1. It might be relevant to test for a participation in plant-pathogen interaction occurring in flower. Indeed, C18:2 is a major FA from Arabidopsis flower buds, but no epoxides or epoxides derivatives of this FA and have been described in flower buds lipids when Arabidopsis plants are quiescent [55]. These oxidized forms of C18:2 participate to plant resistance after epoxidation [25] or further metabolism and oxidation [49]. It would be of particular interest to look for an increase of oxidized forms of C18 :2 in flowed buds of infected WT Arabidopsis and compare with levels in infected *cyp77b1*.

Author contributions:
E.P. and F.P. conceived, planned and designed the research. E.P. conducted cloning, heterologous expression, localization studies and enzymology. F.B. isolated the loss-of-function mutants, prepared and analyzed cutin. E.G. performed the phylogenic analysis. J-E. B. prepared and produced all graphical art. F.P. wrote the manuscript which was subsequently edited by E.G., F.B. and J-E. B.

Conflict of interest
None

ACKNOWLEDGEMENTS: E.P. was supported by a doctoral fellowship from the Ministère de l'Enseignement Supérieur et de la Recherche. Technical expertise of Dr. H. Renault for the N-Ter swapping of CYP77B1 is acknowledged. The scientific and stimulating discussions with Dr. A. Berr were appreciated.

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FIGURES LEGENDS:

Figure 1: Protein hydrophobicity pattern analysis. The program TOPCONS (https://topcons.net/) was used to compare the prediction of protein membrane topology of CYP77A4 (*At5g04660*), CYP77A6 (*At3g10570*) and CYP77B1 (*At1g11600*).

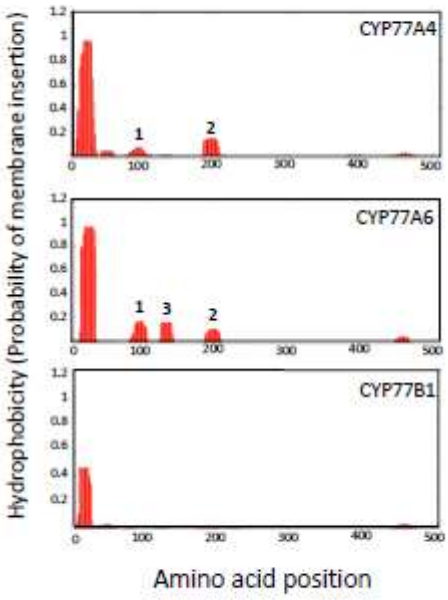
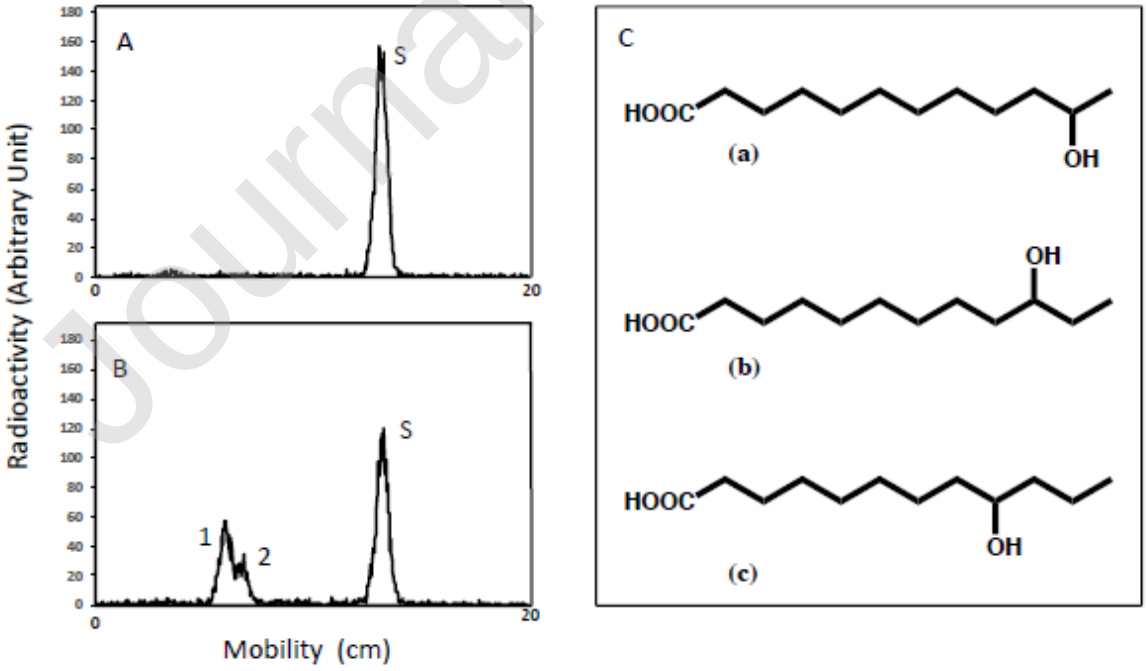


Figure 2: Radiochromatographic resolution by TLC of metabolites generated in incubations of lauric acid with microsomes of yeast expressing modified CYP77B1. A, microsomes (400µg of protein) were incubated with 100 µM [1-^{14}C]Lauric acid at 27°C for 10 min. B, experiment performed as in A, but 100 µM of NADPH were added. Peak S, Lauric acid, Peaks 1 and 2, metabolites. C, Structures of metabolites from peaks 1 and 2: (a) 11-hydroxylauric acid, (b) 10-hydroxylauric acid, (c) 9-hydroxylauric acid.



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Figure 3: Chromatographic resolution by GC/MS of metabolites generated in incubations of linoleic acid with microsomes of yeast expressing modified CYP77B1 and AtEH1. Microsomes (400µg of protein) were incubated during 10 min in absence (A) or in presence (B) of NADPH with 100 µM [1-^{14}C]linoleic acid. (C) was performed as in (B) with microsomes from yeast transformed with a void plasmid. Then cytosol (400µg of protein) of yeast expressing AtEH1 were added and incubation lasted another 10 min. After extraction and derivatization, metabolites were subjected to GC/MS analysis as described in Experimental Procedures. Peak 1: 12,13-dihydroxy-octadec-*cis*-9-enoic acid methylated on the carboxyl and trimethylsilylated on the hydroxyls; Peak X: Not determined.

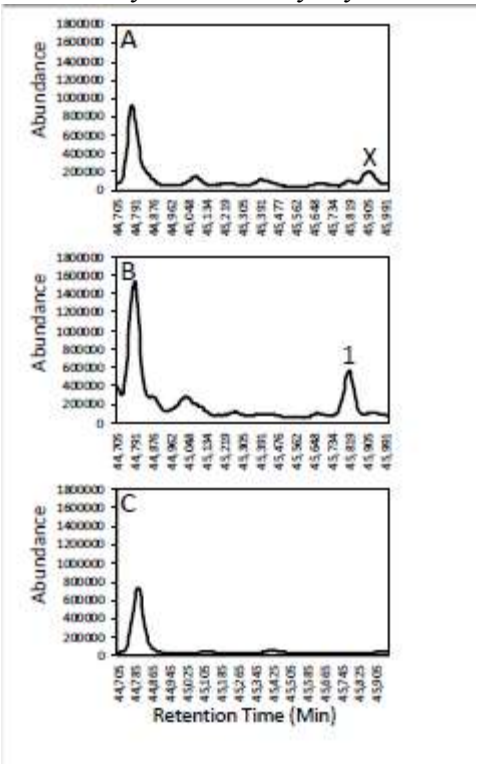


Figure 4: Mass spectra and drawings representing fragmentation scenarios for 12,13-dihydroxy-octadec-*cis*-9-enoic acid methylated on carboxyl and silylated on hydroxyls.

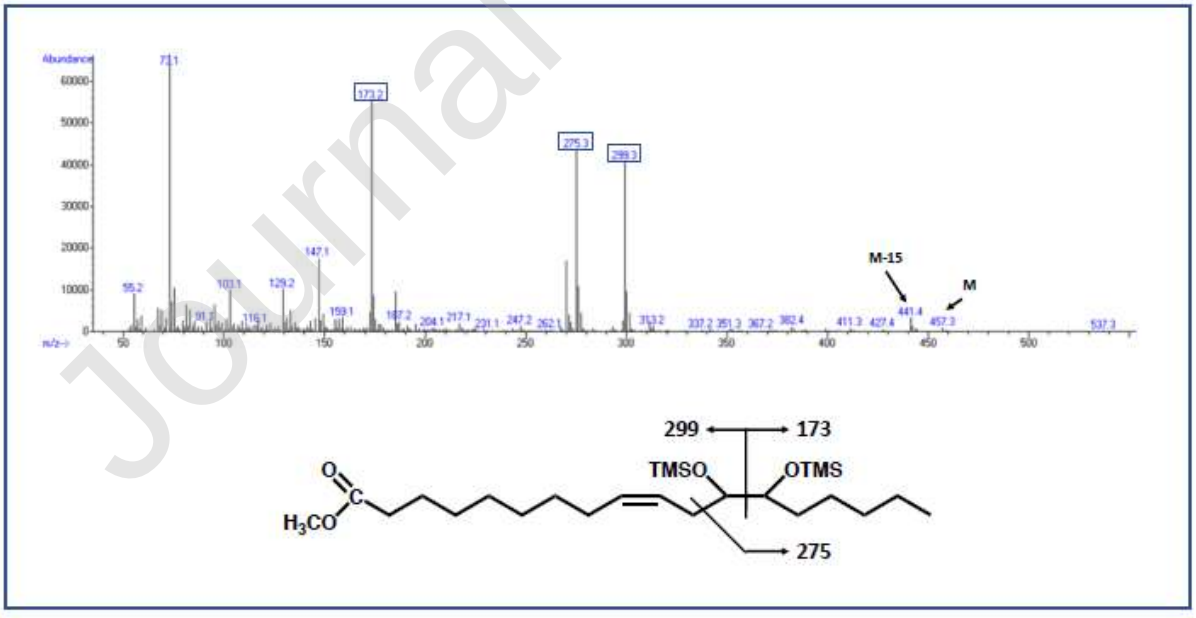


Figure 5: Scheme of 12,13-dihydroxy-octadec-*cis*-9-enoic acid formation *in vitro*. Epoxidation of linoleic acid (a) by CYP77B1, the resulting 12,13-epoxy-octadec-*cis*-9-enoic acid (b) is then hydrolysed into 12,13-dihydroxy-octadec-*cis*-9-enoic acid (c) by AtEH1.

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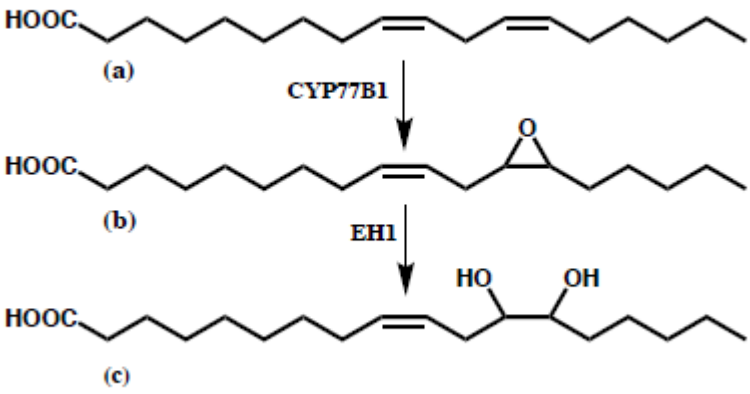


Figure 6: Quantitative RT-qPCR analysis of *CYP77B1* expression. For each organ, expression was determined by real time PCR using gene specific primers and normalized using EXP (*At4g26410*) and TIP41 (*At3g18780*) as reference genes. Values represent geometrical average of analysis performed in technical triplicates.

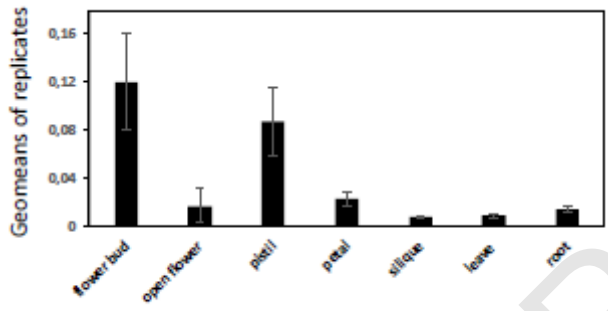


Figure 7: Histochemical GUS staining in flower tissues of *ProCYP77B1::*GUS plants. (A) Whole flower; (B) Pistil and stamens; (C) isolated stamens; (D) isolated pistil.

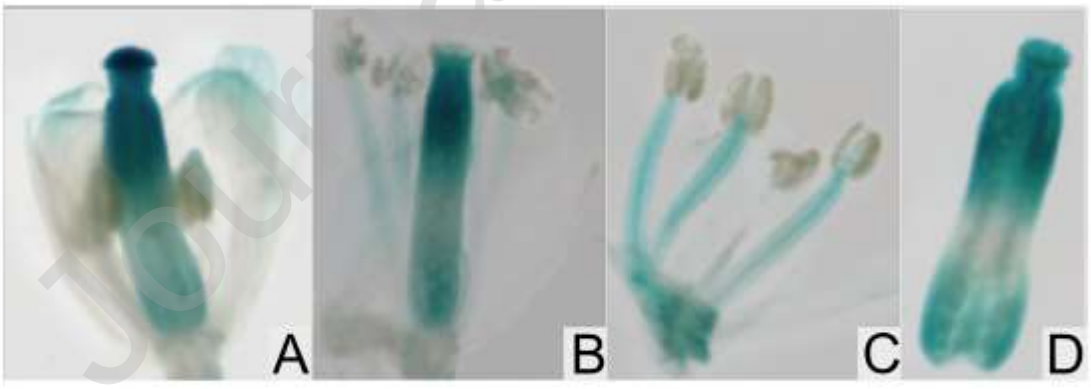
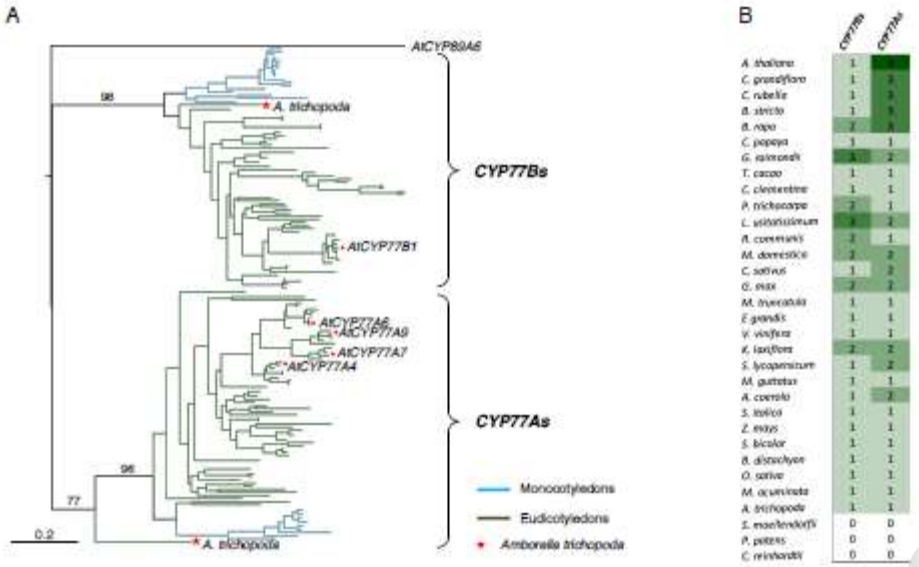


Figure 8: Phylogenetic analysis of *CYP77s* in Angiosperms. A, Maximum likelihood phylogenetic tree base on amino acid sequences of selected *CYP77*. Red circles indicate *Arabidopsis thaliana's* homologs. Red star indicates *Amborella trichopoda*'s homologs. Bootstrap values (500 iterations) above 70% are indicated on branches. *AtCYP89A6*, closest homolog of *CYP77B1* in *Arabidopsis thaliana*, is shown as reference. B, Numbers of *CYP77s* homologs found in 32 selected viridiplantae species selected based on our phylogenetic analysis.



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