Cloning, Functional Expression, and Characterization of CYP709C1, the First Sub-terminal Hydroxylase of Long Chain Fatty Acid in Plants

**INDUCTION BY CHEMICALS AND METHYL JASMONATE**

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We cloned and characterized CYP709C1, a new plant cytochrome P450 belonging to the P450 family, that so far has no identified function except for clustering with a fatty acid metabolizing clade of P450 enzymes. We showed here that CYP709C1 is capable of hydroxylating fatty acids at the ω-1 and ω-2 positions. This work was performed after recording and heterologous expression of a full-length cDNA isolated from a wheat cDNA library in an engineered yeast strain. Investigation on substrate specificity indicates that CYP709C1 metabolizes different fatty acids varying in their chain length (C12 to C18) and unsaturation. CYP709C1 is the first identified plant cytochrome P450 that can catalyze sub-terminal hydroxylation of C18 fatty acids. cis-9,10-Epoxysearic acid is metabolized with the highest efficiency, i.e. $K_{m(app)}$ of 8 μM and $V_{max(app)}$ of 328 nmol/min/nmol P450. This, together with the fact that wheat possesses a microsomal peroxygenase able to synthetize this compound from oleic acid, strongly suggests that it is a physiological substrate. Hydroxylated fatty acids are implicated in plant defense events. We postulated that CYP709C1 could be involved in plant defense by producing such compounds. This receives support from the observation that (i) sub-terminal hydroxylation of 9,10-epoxysearic acid is induced (15-fold after 3 h) in microsomes of wheat seedlings treated with the stress hormone methyl jasmonate and (ii) CYP709C1 is enhanced at the transcriptional level by this treatment. CYP709C1 transcript also accumulated after treatment with a combination of the safener naphthalic anhydride and phenobarbital. This indicates a possible detoxifying function for CYP709C1 that we discussed.

The products of the oxidative transformation of fatty acids are raising an increasing interest in all organisms because it appears that a very broad range of such derivatives can be generated, most of them having important biological activity (1–3). In mammals the products of the “arachidonic cascade” provide a good illustration of the diversity of structures and biological activities (1, 4).

In plants, a large number of fatty acid oxygenations are catalyzed by cytochrome P450 enzymes (2). The functions of the resulting products include different aspects of plant development, as well as wound and abiotic stress response and defense against insects and pathogens. Fatty acid hydroxylases belong to the cytochrome P450 superfamily, a highly diversified set of heme-containing proteins found in bacteria, fungi, plants, and animals (5). One can distinguish ω-hydroxylases that catalyze the hydroxylation of the terminal methyl of aliphatic acids (ω-position) and sub-terminal or in-chain hydroxylases that oxidize carbon in the aliphatic chain (ω-n position). So far the majority of the work has addressed the study of ω-hydroxylases. Biochemical studies led to the characterization of cytochrome P450-dependent ω-hydroxylases in microsomal preparations from pea and Vicia sativa (6–8). A strategy based on the use of radiolabeled suicide substrates allowed us to clone CYP94A1 from V. sativa (9). This enzyme may play a role in plant defense events. Indeed, recombinant CYP94A1 catalyzes the in vitro formation of 9,10,18-trihydroxysearic and 18-hydroxy-9,10-epoxysearic acids (10). These two compounds are the major C18 monomers of cutin, a part of the cuticle that protects plant against different stress (11). Furthermore they are also potent elicitors of defense mechanisms (12, 13). The hypothesis of CYP94A1 participation in a plant defense event received support from the observation that this enzyme is induced at the transcriptional level by the stress hormone methyl jasmonate (14). Plant fatty acid hydroxylases could also be involved in catabolism like members of the CYP4 family in animals (15, 16) or like CYP52A3 and CYP52A4, which enable Candida maltosa to use aliphatic hydrocarbon as a source of carbon and energy (17). In a previous work (18), we proposed that the CYP94A5 that we cloned from tobacco could be involved in rapidly turning over free fatty acids liberated during oxidative stress and that they are harmful to the cell. Indeed, CYP94A5 is able to catalyze the complete set of reactions oxidizing the terminal methyl to the corresponding carboxyl.

Some fatty acid derivatives hydroxylated on ω-1 position involved in plant resistance have been described. Volicitin, which is an ω-1 hydroxy of linoleic acid coupled to l-glutamine, has a key role in plant resistance against herbivores (19, 20). Sub-terminal hydroxylation of jasmonates may be fundamental for reactions in the regulation of the signaling pathway for plant defense (21, 22). However, little information is available so far concerning sub-terminal hydroxylases despite the fact that they have been shown to be present in microsomes of different plants (23, 24). About 1 decade ago, we showed the presence in microsomes in wheat of a cytochrome P450-dependent system involved in sub-terminal hydroxylation of oleic and lauric acids (25). Biochemical studies suggested the presence of at least two cytochromes P450, and one of these cytochromes P450 was responsible for the resistance of wheat to diclofop (26).
Here we describe the cloning of a new plant cytochrome P450 classified as CYP709C1. Functional expression in an engineered yeast allowed us to show that CYP709C1 metabolizes fatty acids, and among all the fatty acids tested, cis-9,10-epoxystearic acid produced by a peroxygenase is hydroxylated at positions ω-1 and ω-2 with the highest efficiency. This is the first report of a sub-terminal hydroxylase of long chain fatty acid cloned in plant. CYP709C1 is up-regulated at the transcriptional level by a treatment of wheat seedlings with a combination of the safener naphthalic acid anhydride with phenobarbital or by the stress hormone methyl jasmonate. Its physiological meaning remains to be established, and we discuss its possible implication in the plant defense and detoxifying process.

MATERIALS AND METHODS

Chemicals—Radiolabeled [1-14C]lauric acid (45 Ci/mol) was from CEA (Gif sur Yvette, France). [1-14C]Myristic acid (55 Ci/mol), [1-14C]palmitic acid (54 Ci/mol), [1-14C]oleic acid (50 Ci/mol), [1-14C]linoleic acid (58 Ci/mol), and [1-14C]linolenic acid (52 Ci/mol) were from PerkinElmer Life Sciences. Racemic sample of cis-9,10-[1-14C]epoxystearic acid was synthesized from [1-14C]oleic acid using m-chloroperoxybenzoic acid.

The silylating reagent N,O-bistrimethylsilylfluoracetamide containing 1% of trimethylchlorosilane was from Pierce. NADPH was from Sigma. Thin layer plates (Silica Gel G60 F254; 0.25 mm) were from Merck.

Probe Identification and Full-length cDNA Cloning—An 810-nucleotide-long P450 fragment was identified by PCR on the wheat α-ZipLox, NotI-Sall arms (Invitrogen)-oriented library (27). We used the primer RTAVGCRAXXKTYCHCCVAKRCAH, which is the reverse primer of the one described by Meijer et al. (28), with the primer T7 of the right border of our induced library. The PCR was carried out as described in Meijer et al. (28), and the different PCR products were cloned into pGEM-T vector (Promega) and sequenced. The putative P450 sequence was identified by a BLASTX of the NCBI plant sequence data base. This incomplete P450 fragment was 32P-labeled to screen 250,000 plating-forming units of the induced library with the Ready-to-Go DNA labeling beads (Amersham Biosciences). A complete cDNA sequence was identified (GenBank accession number AY641449) and was named CYP709C1.

Enzyme Activities—All radiolabeled substrates were dissolved in ethan-4-ol that was evaporated before the addition of microsomes into the glass tube. Resolubilization of the substrates was confirmed by measuring the radioactivity of the incubation media.

Enzymatic activities of CYP709C1 or wheat microsomes were determined by following the formation rate of metabolites. The standard assay (0.1 ml) contained 20 mM sodium phosphate (pH 7.4), 1 mM NADPH, plus a regenerating system (consisting of a final concentration of 6.7 mM Glc-6-P and 0.4 units of Glc-6-P-dehydrogenase) and radio-labeled substrate (100 μM). The reaction was initiated by the addition of NADPH and was stopped by the addition of 20 μl of acetonitrile (0.2% acetic acid). The reaction products were resolved by HPLC4 or TLC as described below. Kinetic studies were performed for 7 min at 27 °C with

4 The abbreviations used are: HPLC, high pressure liquid chromatography; RP-HPLC, reverse phase-HPLC; GC/MS, gas chromatography/mass spectrometry; TMSi, trimethylsilyl. 
0.7 and 0.1 pmol of CYP709C1 for lauric acid and 9,10-epoxystearic acid, respectively. Determination of \( K_m \) and \( V_{max} \) values was based on six data points ranging from 3 to 150 \( \mu M \) for lauric acid and from 3 to 30 \( \mu M \) for 9,10-epoxystearic acid. Peroxygenase activity of wheat microsomes was measured as described previously using 100 \( \mu M \) of cumene hydroperoxide as cofactor (32).

**Chromatographic Methods**—Incubation media were directly spotted on TLC plates. For separation of hydroxy fatty acids from residual substrate (lauric and 9,10-epoxystearic acids), TLC were developed with a mixture of diethyl ether/light petroleum (boiling point, 40–60 °C)/formic acid (70:30:1 or 50:50:1 v/v/v, for lauric and 9,10-epoxystearic acids, respectively). For separation of 9,10-epoxystearic acid from residual oleic acid in peroxxygenase measurement, TLC were developed with a mixture of diethyl ether/hexane/formic acid (50:50:1 v/v/v). The plates were scanned with a thin layer scanner (Berthold LB 2723). The area corresponding to the metabolites was scraped into counting vials and quantified by liquid scintillation, or they were eluted from the silica with 10 ml of the mixture diethyl ether/hexane (50:50, v/v), which was removed by evaporation. They were then subjected to GC/MS analysis.

For HPLC analysis reaction products were directly injected after incubations. The metabolites were resolved by RP-HPLC (Waters, equipped with two 600 pumps and a Packard 500 TR series radiodetector) on a 5-\( \mu m \) UltraspHERE C18 column (150 \( \times \) 4.6 mm, Beckman Instruments, France) using isocratic solvent at a flow of 1 ml/min. A mixture of acetonitrile/water/acetic acid (40:60:0.2, v/v/v) was used to elute 18-, 17-, and 16-hydroxy-9,10-epoxystearic acids. A linear gradient (0–100%) of 80% acetonitrile in aqueous acetic acid was used to elute residual 9,10-epoxystearic acid.

**Chiral Analysis**—Chiral analyses were performed as described previously by using optically pure synthetic (9R,10S)-epoxystearate methyl ester as a standard (33). To determine the chirality of the residual epoxide after incubation with CYP709C1, the epoxide and the hydroxylated product were separated on TLC. The area corresponding to the epoxide was scraped, and the epoxide was eluted from the silica. The residual epoxide was dissolved in hexane (40 \( \mu l \)) after methylation with diazomethane and analyzed by HPLC (Waters equipped with two 600 pumps and a Packard 500 TR series radiodetector). Both enantiomers were resolved using a chiral column (Chiracel OB (4.6 \( \times \) 250 mm) J. T. Baker Inc.) with an isotropic solvent hexane/propan-2-ol/acetic acid (99.7:0.2:0.1, v/v/v) at a flow rate of 0.8 ml/min. Under the present conditions of analysis, methyl esters of (9S,10R)- and (9R,10S)-epoxystearic acids have retention times of 31 and 40 min, respectively.

**GC/MS Analysis**—Metabolites generated during the incubations of lauric, oleic, and 9,10-epoxystearic acids with CYP709C1 or with wheat microsomes were eluted from silica with 10 ml of diethyl ether/hexane (50:50, v/v), methylated with diazomethane, and silylated with a mixture of pyridine and \((\text{N,O})_{2}\)-bistrimethylsilyl trifluoroacetamide containing 1% (v/v) trimethylchlorosilane (1:1, v/v).

GC/MS analysis was 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 \( \mu m \) (HP-5MS). The gas chromatograph was combined with a quadrupole mass selective detector (Agilent 5973N). Mass spectra were recorded at 70 eV and analyzed as in Eglington et al. (34). Yeast has only three cytochromes P450; they are expressed at a negligible rate under our growth conditions (30). None of these is able to oxidize fatty acids, ensuring us that the metabolism studied in this work is because of the recombinant enzyme. Furthermore, WAT11 overexpresses a plant P450 reductase that allows excellent electron transfer and probably increases the stability of the plant P450 in the yeast endoplasmic reticulum.

**Metabolism of Lauric Acid by CYP709C1**—After incubation of lauric acid (C12:0) with microsomes of yeast expressing CYP709C1, incubation media were directly analyzed by TLC. Fig. 1 shows radiochromatograms obtained after incubation in the absence (Fig. 1A) or in the presence (Fig. 1B) of NADPH. Two polar metabolites were formed in the presence of NADPH (Fig. 1B, peaks 1 and 2). They were not produced in the incubation of boiled microsomes or in the incubation with microsomes of yeast transformed with a void plasmid (Fig. 1C). Taken together, these results demonstrate the involvement of CYP709C1 in their formation. We determined an optimum pH of 7.2. Metabolites of peaks 1 and 2 were purified, derivatized, and analyzed by GC/MS. Mass spectrum of the metabolite 1 of lauric acid after derivatization (Fig. 2A) showed ions at \( m/z \) (relative intensity %) 73 (43%) ((CH\(_3\))\(_2\)Si\(^+\)), 75 (25%) ((CH\(_3\))\(_3\)Si\(^+\) = O), 117 (base peak 100%), 146 (4%) (CH\(_2\) = C\(^+\)(OSi(CH\(_3\))\(_2\))–OCH\(_3\)), 139 (10%) (CH\(_2\) = C\(^+\)(OSi(CH\(_3\))\(_2\))–OCH\(_3\)), 201 (0.5%), 215 (2%), 255 (11%) (M – 47) (loss of methanol from the (M – 15) fragment), 271 (3%) (M – 31) (loss of OCH\(_3\) from the methyl ester), 287 (5%) (M – 15) (loss of...
a methyl from the TMSi group). This fragmentation pattern is characteristic of the derivative of 11-hydroxylauric acid (M/H11005 302 g/mol) (Fig. 2A). Mass spectrum of the second metabolite of lauric acid after derivatization (Fig. 2B) showed ions at m/z (relative intensity %) 73 (75%) ((CH3)3Si-O), 75 (33%) ((CH3)2Si-OH), 131 (base peak 100%), 146 (5%) (CH3=O+(OSi(CH3)3-OCH3)), 159 (8%) (CH3-O=O+(OSi(CH3)3-CH=CH2)), 201 (5%), 215 (1%), 255 (10%) (M − 47) (loss of methanol from the (M − 15) fragment), 271 (4%) (M − 31) (loss of OCH3 from the methyl ester), 273 (43%), 287 (2%) (M − 15) (loss of a methyl from the TMSi group). This fragmentation pattern is characteristic of the derivative of 10-hydroxylauric acid (Fig. 2B). These metabolites were not formed in incubation with boiled microsomes (not shown) or in incubation with microsomes of yeast transformed with a void plasmid (Fig. 1C).

We performed kinetic studies to determine K_{m(app)} and V_{max(app)} values of 158 ± 6 μM and 132 ± 3 nmol/min/nmol P450, respectively.

Previous biochemical studies suggested that a cytochrome P450 fatty acid hydroxylase was responsible for the resistance of wheat to diclofop (26). Therefore, we incubated this herbicide with microsomes of transformed yeast, but we did not observe any metabolism of diclofop in these incubations.

_Epoxidation of Oleic Acid in Wheat Microsomes_—We incubated oleic acid (C18:1) with wheat microsomes in the presence of cumene hydroperoxide, and the incubation medium was directly analyzed by TLC. Radiochromatogram of Fig. 3 shows that one metabolite was formed (Fig. 3A, peak 1). It was not formed in incubation with boiled microsomes (Fig. 3B). After purification, this product was methylated with diazomethane and subjected to GC/MS analysis. We also methylated and analyzed by GC/MS authentic 9,10-epoxystearic acid. The
mass spectrum obtained for the metabolite purified from peak 1 was identical to that of authentic 9,10-epoxystearate methyl ester (M = 312 g/mol) showing ions at \( m/z \) (relative intensity %) 155 (100%) (base peak, loss of (CH\(_2\)_7COOCH\(_3\)), 199 (14%) (loss of (CH\(_2\)_7CH\(_3\)), 281 (2%) (M – 31, loss of OCH\(_3\)), and 294 (1%) (M – 18, loss of H\(_2\)O). This mass spectrum is also identical to mass spectrum of methylated 9,10-epoxystearic acid produced by partially purified peroxygenase from soybean (32). This shows that wheat possesses a microsomal peroxygenase able to synthesize 9,10-epoxystearic acid using oleic acid as substrate.

**Metabolism of cis-9,10-Epoxystearic Acid by CYP709C1**—We incubated 9,10-epoxystearic acid with microsomes expressing CYP709C1 to test if it was substrate. No metabolite were formed in absence of NADPH (Fig. 4A). Addition of this cofactor to the incubation medium led to the formation of two metabolites (Fig. 4B, peaks 1 and 2). They were purified and analyzed by GC/MS. Mass spectrum of the metabolite 1 of cis-9,10-epoxystearic acid after derivatization (Fig. 5A) showed ions at \( m/z \) (relative intensity %) 73 (58%) ([(CH\(_3\))\(_3\)Si])\(^+\), 75 (38%) ([(CH\(_3\))\(_2\)Si]O\(^+\)), 117 (base peak 100%), 146 (3%) (CH\(_3\)=C\(^+\)(OSi(CH\(_3\))\(_3\)=OCH\(_3\)), 159 (5%) (CH\(_3\)=O\(^+\)C\(^+\)(OSi(CH\(_3\))\(_3\))CH=CH\(_2\)), 199 (8%), 213 (5%), 243 (3%), and 353 (0.5%) (M – 47) (loss of methanol from the (M – 15) fragment), 369 (0.5%) (M – 31) (loss of OCH\(_3\) from the methyl ester), and 385 (1%) (M – 15) (loss of a methyl from the TMSi group) (M = 400 g/mol). Holloway (35) performed fragmentation of the same derivative of synthetic 18-hydroxy-9,10-epoxystearic acid.
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As expected, Holloway (35) observed ions at m/z 199, 213, and 243 resulting from cleavage on both sides of the epoxide. He also observed ions at m/z 171, 185, and 215 coming from ions 199, 213, and 243, respectively (loss of 28). Mass spectrum of metabolite 1 also showed ions at m/z 171 (4%), 185 (3%), and 215 (3%). Its fragmentation pattern is characteristic of the derivative of 17-hydroxy-9,10-epoxystearic acid after derivatization (Fig. 5). The ions at m/z 171, 185, and 215 (loss of 28) are formed 15 min at 27 °C in the absence (A) or in the presence (B) of NADPH. The reaction was stopped by the addition of 20 μL of acetonitrile (containing 0.2% of acetic acid), and the incubation was directly subjected to RP-HPLC analysis.

Concentration of 100 μM with microsomes of yeast expressing CYP709C1. All of them were metabolized, and Fig. 6 shows that it is the 9,10-epoxystearic acid that is turned over with the highest efficiency. As mentioned above, wheat possesses a microsomal peroxidase able to epoxidize oleic acid. We purified 9,10-epoxystearic acid produced in the incubation of wheat microsomes with oleic acid and cumene hydroperoxide, and we determined its chirality by using a chiral column. The epoxide was not racemic and presented an enantiomeric excess in favor of the 9R,10S form: 9R,10S/9S,10R 70/30 (not shown). To test if CYP709C1 was enantioselective for this form, we incubated our substrate 9,10-epoxystearic acid, which is racemic, and we analyzed the chirality of the residual epoxide. The residual epoxide was still racemic (Fig. 7), showing that CYP709C1 was not enantioselective. The 9,10-epoxystearic acid that we used in this work has the cis configuration because it was made from oleic acid. Experiments of competition between cis and trans stereoisomers allowed us to show that CYP709C1 also recognized the trans stereoisomer.

Metabolism of cis-9,10-Epoxystearic Acid in Wheat Microsomes—Fig. 8 shows the radioactivity profiles obtained after incubation of 9,10-epoxystearic acid with microsomes from naphthalic anhydride and phenobarbital-treated wheat coleoptile. Two major metabolites (Fig. 8B, peaks 1 and 2) were generated only in presence of NADPH. They were not formed with boiled microsomes (not shown). Metabolites from peaks 1 and 2 were purified, derivatized, and subjected to GC/MS analysis. Their fragmentation patterns are identical to the ones of derivatized metabolites produced during incubation of 9,10-epoxystearic acid with microsomes of yeast expressing CYP709C1. Metabolites from peaks 1 and 2 were identified as 17- and 16-hydroxy-9,10-epoxystearic acids, respectively. The minor metabolite from peak 3 co-elutes in HPLC with 18-hydroxy-9,10-epoxystearic acid (not shown). Compared with control, treatment of wheat seedlings with naphthalic acid anhydride and phenobarbital increased the microsomal activity of 9,10-epoxystearic hydroxylation 150-fold to 45 pmol/min/g fresh weight. Northern blot analysis presented in Fig. 9 revealed that such treatment strongly induced accumulation of the transcript coding for CYP709C1. The highest induction was observed in the coleoptile. We also studied the effect of methyl jasmonate (500 μM) treatment of wheat seedling on 9,10-epoxystearic hydroxylation. As shown in Fig. 10, the activity was enhanced 15 times after 3 h of treatment, and the maximal activity was reached after 24 h of treatment (100-fold induction). Fig. 11 shows that even at the lowest concentration tested (20 μM), the activity was stimulated 2-fold compared with control (8 versus 4 pmol/min/mg protein in assay and control, respectively). Northern blot analysis showed that methyl jasmonate treatment also led to accumulation of the transcript coding for CYP709C1 (Fig. 12). The transcript accumulated in both root
and coleoptile, with higher levels in coleoptile, and the maximum was reached at 3 h in root and 6 h in coleoptile.

**DISCUSSION**

In an attempt to clone all cytochromes P450 from wheat involved in xenobiotic metabolism, we isolated a full-length cDNA. Sequence alignment showed that the deduced protein was a new member of the CYP709 family of cytochrome P450 named CYP709C1. In the phylogenetic tree of plant cytochromes P450 (36), CYP709 family belongs to the group non-A II, which contains the first cloned plant fatty acid ω-hydroxylases, CYP94A1 (9) and CYP86A1 (37). We transformed yeast with a recoded cDNA coding for CYP709C1 to get a better expression. By using microsomes of transformed yeast and the model substrate lauric acid, we showed that CYP709C1 is indeed a fatty acid hydroxylase. However, this new enzyme differs from CYP94A1 (9) and CYP86A1 (37) by its regiospecificity; it attacks exclusively ω-1 and ω-2 but not the ω position of fatty acids. We previously characterized CYP81B1 from *Helianthus tuberosus*, which also hydroxylates fatty acids in the chain (38). However, CYP81B1 does not recognize the most common C16 and C18 plant fatty acids. Furthermore, the position of hydroxylation depends on the fatty acid chain length. This suggests that fatty acids are not the physiological substrates of CYP81B1. On the contrary, CYP709C1 metabolizes common plant fatty acids, and the fact that the regiospecificity of CYP709C1 is independent of the aliphatic chain length suggests that sub-terminal hydroxylation is the real function of the enzyme.

Among all fatty acids tested, 9,10-epoxystearic acid is hydroxylated with the highest efficiency. The low $K_{\text{m(app)}}$ value (8 μM) determined together with the fact that this fatty acid is produced by a peroxygenase localized in the microsomes strongly suggest that it is a physiological substrate of CYP709C1. The physiological role of this enzyme remains to be established. A participation in cutin synthesis is not conceivable, and indeed it does not attack the ω-position, and to our knowledge ω-1 and ω-2 hydroxy fatty acids have not been reported in cutin so far. Polyhydroxy C18 fatty acids have been shown to be responsible for plant resistance against pathogens (39–41). An epoxide hydrolyase that transforms 9,10-epoxystearic acid to the corresponding diol has been described in wheat (42). Hydrolysis by this enzyme of 16- or 17-hydroxy-9,10-epoxystearic acids produced by CYP709C1 would lead to the formation of trihydroxy C18 fatty acids with chemical structures close to the structure of compounds mentioned above. This could give a role in plant defense to CYP709C1. The strong and rapid induction at the transcriptional level by the stress hormone methyl jasmonate corroborates this hypothesis. Induction was detectable at the lowest concentration tested (20 μM), which is physiological in wounded plant (43). Most interestingly, recent work describes the strong induction at the transcriptional level of CYP709C3, a member from the same family after fungal infection by *Fusarium graminearum* (44).

CYP94A1 ω-hydroxylates 9,10-epoxystearic acid with a strong enantioselectivity for the 9R,10S enantiomer (10). By contrast, CYP709C1 does not exhibit any enantioselectivity and recognizes both cis and trans forms of the epoxide. This might just reflect the less hindered conformation of the substrate in the active site of CYP709C1 compared with CYP94A1. Indeed, to occur, the less thermodynamically favored hydroxylation of a terminal methyl requires strong steric constraints to retain the substrate in a tight position that allows the terminal methyl to approach the ferryl-oxo species (45, 46).

In the context of plant resistance, it is interesting to note that ω-1 hydroxy derivatives of fatty acids have been described. In response to elicitors present in the oral secretion of herbivores, plants produce and
secrete volatile chemicals that attract natural enemies of the herbivore. Volicitin is responsible for the majority of elicitor activity in the oral secretion of caterpillar species (19). Volicitin is an ω-1 hydroxy of linolenic acid coupled to l-glutamine. Paré et al. (20), suggested that hydroxylation of linolenic acid occurred within the caterpillar, but it is conceivable that products of reactions catalyzed by CYP709C1 have eliciting properties or are a precursor of molecules with eliciting properties. Also studies on the metabolism of dihydrojasmonic and jasmonic acids showed that they are hydroxylated on the ω and ω-1 positions (21, 22). The ω-1 hydroxylation of a pollen suppressant has been described in wheat (47). The hydroxylation enzymes involved in these different reactions have not yet been characterized.

In animals, arachidonic acid (C20:4), the major fatty acid, is transformed to different metabolites through the arachidonic cascade composed of prostaglandin synthase, lipoxigenases, and cytochromes P450-dependent ω and ω-1 hydroxylases (48). The regulation of the hydroxylases by peroxisome proliferator-activated receptor-α suggests that these reactions contributed to fatty acid homeostasis (48). However, recent works attributed important physiological properties to the ω-1 hydroxy of C20:4 (19-hydroxyeicosatetraenoic acid) produced by the murine CYP2J9 (49) and the human CYP2U1 (50). The murine CYP2J9 is highly expressed in cerebellar Purkinje cells and synthesizes exclusively 19-hydroxyeicosatetraenoic acid, which can inhibit ionic channels involved in triggering neurotransmitter release (49). This compound is also involved in the regulation of the renal Na⁺,K⁺-ATPase (51) and has been reported to be a vasodilator (52). Therefore, hydroxylation of C20:4 cannot only be considered as catabolism. To our knowledge, no biological activities have been attributed to ω-1 or ω-2 hydroxy fatty acids in plant, and such a finding would give potential roles to CYP709C1.

We demonstrated previously that fatty acid hydroxylation is induced in microsomes of wheat treated with naphthalic acid anhydride and phenobarbital (25). Our results suggested the presence of distinct cytochrome P450-dependent hydroxylases in wheat. The finding of a mixture of 16-, 17-, and 18-hydroxy-9,10-epoxyeicaric acid produced by wheat microsomes, when CYP709C1 does not hydroxylate the last methyl, confirms the existence of at least two hydroxylating systems in wheat. The finding of a mixtures of wheat microsomes of wheat treated with naphthalic acid anhydride and nobarbital is the prototype for a large number of chemical inducers of cytochrome P450 (mainly the CYP2 family) and other enzymes involved in plant resistance. This should help to investigate the participation of members of the CYP709 family in the plant-pathogen interaction and in the detoxifying process.

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