ω-Hydroxylation of epoxy- and hydroxy-fatty acids by CYP94A1: possible involvement in plant defence

F. Pinot†, M. Skrabs†, V. Compagnon*, J.-P. Salaün*, I. Benveniste*, L. Schreibert and F. Durst*
*IBMP-CNRS UPR406 Dept. d’Enzymologie Cellulaire et Moléculaire, 28 rue Goethe 67083 Strasbourg, France, and †Lehrstuhl für Botanik II, Julius Von Platz 3, D-97082 Würzburg, Germany

Abstract
The C₁₈ fatty acid derivatives 9,10-epoxystearic acid and 9,10-dihydroxystearic acid were hydroxylated on the terminal methyl by microsomes of yeast expressing CYP94A1 cloned from Vicia sativa. The reactions did not occur in incubations of microsomes from yeast transformed with a void plasmid or in the absence of NADPH. After incubation of a synthetic racemic mixture of 9,10-epoxystearic acid, the chirality of the residual epoxide was shifted to 66:34 in favour of the 9S,10R enantiomer. Both the 9S,10R and 9R,10S enantiomers were incubated separately. We determined respective $K_m$ and $V_{\text{max}}$ values of 1.2 ± 0.1 μM and 19.2 ± 0.3 nmol/min per nmol of cytochrome P450 for the 9R,10S enantiomer and of 5.9 ± 0.1 μM and 20.2 ± 1.0 nmol/min per nmol of cytochrome P450 for the 9S,10R enantiomer. This demonstrated that CYP94A1 is enantioselective for the 9R,10S, which is preferentially formed in V. sativa microsomes. Cutin analysis of V. sativa seedlings revealed that it is mainly constituted of derivatives of palmitic acid, a C₁₈ fatty acid. Our results suggest that CYP94A1 might play a minor role in cutin synthesis and could be involved in plant defence. Indeed, 18-hydroxy-9,10-epoxystearic acid and 9,10,18-trihydroxystearic acid have been described as potential messengers in plant–pathogen interactions.

Key words: cytochrome P450, epoxide, messenger.

Introduction
We previously showed that, in Vicia sativa microsomes, oleic acid is subjected to a cascade of reaction involving three different enzymes: an epoxygenase, an epoxide hydrolase and a cytochrome P450-dependent ω-hydroxylase [1]. Using oleic acid as a starting material, these enzymes are able to produce in vitro the major C₁₈ cutin monomers. Inhibition studies suggested the presence of distinct fatty acid ω-hydroxylases [2]. This was confirmed recently by the cloning of CYP94A1 [3] and CYP94A2 [4]. When expressed in yeast, CYP94A1 catalyses the ω-hydroxylation of saturated and unsaturated fatty acids with chain lengths ranging from C₁₆ to C₁₈ [3]. Treatment of etiolated V. sativa seedlings by the plant hormone methyl jasmonate led to an accumulation of transcripts coding for CYP94A1 and, concomitantly, to a stimulation of microsomal fatty acid ω-hydroxylase activity [5].

Experimental
CYP94A1 was expressed in Saccharomyces cerevisiae as described in [3]. Enzymic activities were determined by following the rate of metabolite formation by TLC [1]. Chiral analyses were performed by using pure synthetic 9R,10S-epoxystearate methyl ester as a standard. Briefly, radiolabelled enantiomers of 9,10-epoxystearic acid were separated on HPLC (Waters, equipped with two 510 pumps, and a U6K injector from Waters) using a chiral column (Column Chiralcel OB, 4.6 × 250 mm; J. J. Baker Chemical Co.).
Enantiomers were resolved using an isocratic solvent, hexane/isopropanol/acetic acid (997:2:1, by vol), at a flow of 0.8 ml·min⁻¹. Isolation and analysis of cutin were performed as described in [6].

**Results and discussion**

Incubation of 9,10-epoxystearic acid in the presence of NADPH with microsomes of yeast expressing CYP94A1 led to the formation of one metabolite identified by GC–MS as 18-hydroxy-9,10-epoxystearic acid (Figure 1). The requirement for NADPH, together with the fact that it was not produced in incubation with yeast microsomes transformed with a void plasmid, demonstrated clearly the involvement of CYP94A1. Similarly, with 9,10-dihydroxystearic acid, CYP94A1 produced only one metabolite identified as the corresponding ω-hydroxyl 9,10,18-trihydroxystearic acid (Figure 1).

Previous work demonstrated that oleic acid was epoxidized when incubated with *V. sativa* microsomes [1]. In the present work, we subjected the epoxide formed to chiral HPLC analysis. The epoxide presents an enantiomeric excess in favour of the 9R,10S enantiomer. In order to determine if CYP94A1 is enantioselective, we first analysed the chirality of the residual epoxide after incubation. It was no longer racemic but was a 66:34 mixture in favour of 9S,10R. We also determined the kinetics of ω-hydroxylation of pure enantiomers. We recorded $V_{max}/K_m$ values of 16 and 3.42 ml/min per nmol of cytochrome P450 for 9R,10S and 9S,10R respectively. We concluded that CYP94A1 was enantioselective for the 9R,10S formed preferentially in *V. sativa* microsomes.

We isolated and analysed epicotyl cutin of 4-day-old seedlings. The C₁₆ monomers represented more than 80% of the total monomers. Figure 2 shows that 9,16-dihydroxypalmitic and

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**Figure 1**

Metabolism of oleic acid and its derivatives in *V. sativa* microsomes: involvement of CYP94A1

![Diagram](https://via.placeholder.com/150)

- Oleic acid
- Epoxigenase
- 9,10-epoxystearic acid (9R,10S,10R 90/10)
- CYP94A1 enantioselective for 9R,10S
- Epoxide hydrolase enantioselective for 9R,10S
- 18-hydroxy-9,10-epoxystearic acid
- 9,10-dihydroxystearic acid
- Epoxide hydrolase
- CYP94A1
- 9,10,18-trihydroxystearic acid
10,16-dihydroxypalmitic acids were the most highly represented, together representing 68% of the total monomers.

Our results demonstrate that CYP94A1 is able to hydroxylate regioselectively the terminal methyl of 9,10-epoxystearic acid and of the corresponding diol. The resulting products have been described as major cutin monomers in plants [7]. However, we did not detect them in the cutin of V. sativa seedlings. This suggests that, in the context of cutin synthesis, CYP94A1 plays a minor role, if any. By producing 18-hydroxy-9,10-epoxystearic and 9,10,18-trihydroxysearic acids, CYP94A1 is likely to be involved in plant-defence events. Indeed, these two compounds have been shown to act as endogenous molecules for the induction of plant resistance in different models of pathogen-challenged plants [8–10]. The hypothesis of CYP94A1 involvement in plant-defence events is supported by data showing that this enzyme is strongly induced in seedlings treated with the plant hormone methyl jasmonate [5].

References
Influence of (9Z)-12-hydroxy-9-dodecenoic acid and methyl jasmonate on plant protein phosphorylation

I. A. Tarchevsky', F. G. Karimova, A. N. Grechkin and N. U. Moukhametchina
Kazan Institute of Biochemistry and Biophysics, Russian Academy of Sciences, P.O. Box 30, Kazan, 420503, Russia

Abstract
The products of the lipoxygenase pathway, methyl jasmonic acid (MeJA) and (9Z)-12-hydroxy-9-dodecenoic acid (HDA), hardly changed the relative level of phosphorylated polypeptides (RLPPs) during 2 h of incubation: 15 and 17 kDa RLPPs were enhanced by HDA, but decreased by MeJA. RLPPs of 73 and 82 kDa were increased by both compounds. MeJA and HDA treatment induced specific and unspecific effects in some RLPPs. It was shown that HDA and MeJA increased protein kinase activity in the presence of 1 µM cAMP.

Introduction
The activation of the lipoxygenase signal system under biotic and abiotic stressors induces the formation of different types of oxylipin, including jasmonic acid and methyl jasmonic acid (MeJA). It has been shown that the main product of linoleate oxidation in pea leaf homogenate is (9Z)-12-hydroxy-9-dodecenoic acid (HDA), which possesses high physiological activity [1]. The important components of all known signalling systems in cells are protein kinases, which catalyse protein phosphorylation for switching the cellular pathways in response to changing circumstances. There is not enough information about jasmonic acid and MeJA, especially regarding HDA action on protein kinase activity and protein phosphorylation. The aim of this work was to study the effect of MeJA and HDA treatment on protein phosphorylation.

Key words: lipoxygenase pathway, methyl jasmonic acid, pea, Pisum sativum.
Abbreviations used: MeJA, methyl jasmonic acid; HDA, (9Z)-12-hydroxy-9-dodecenoic acid; RLPPs, relative level of phosphorylated polypeptides.
*To whom correspondence should be addressed (e-mail tarchevsky@sci.kcn.ru).