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Plant Cytochrome CYP74 Family: Biochemical Features, Endocellular Localisation, Activation Mechanism in Plant Defence and Improvements for Industrial Applications

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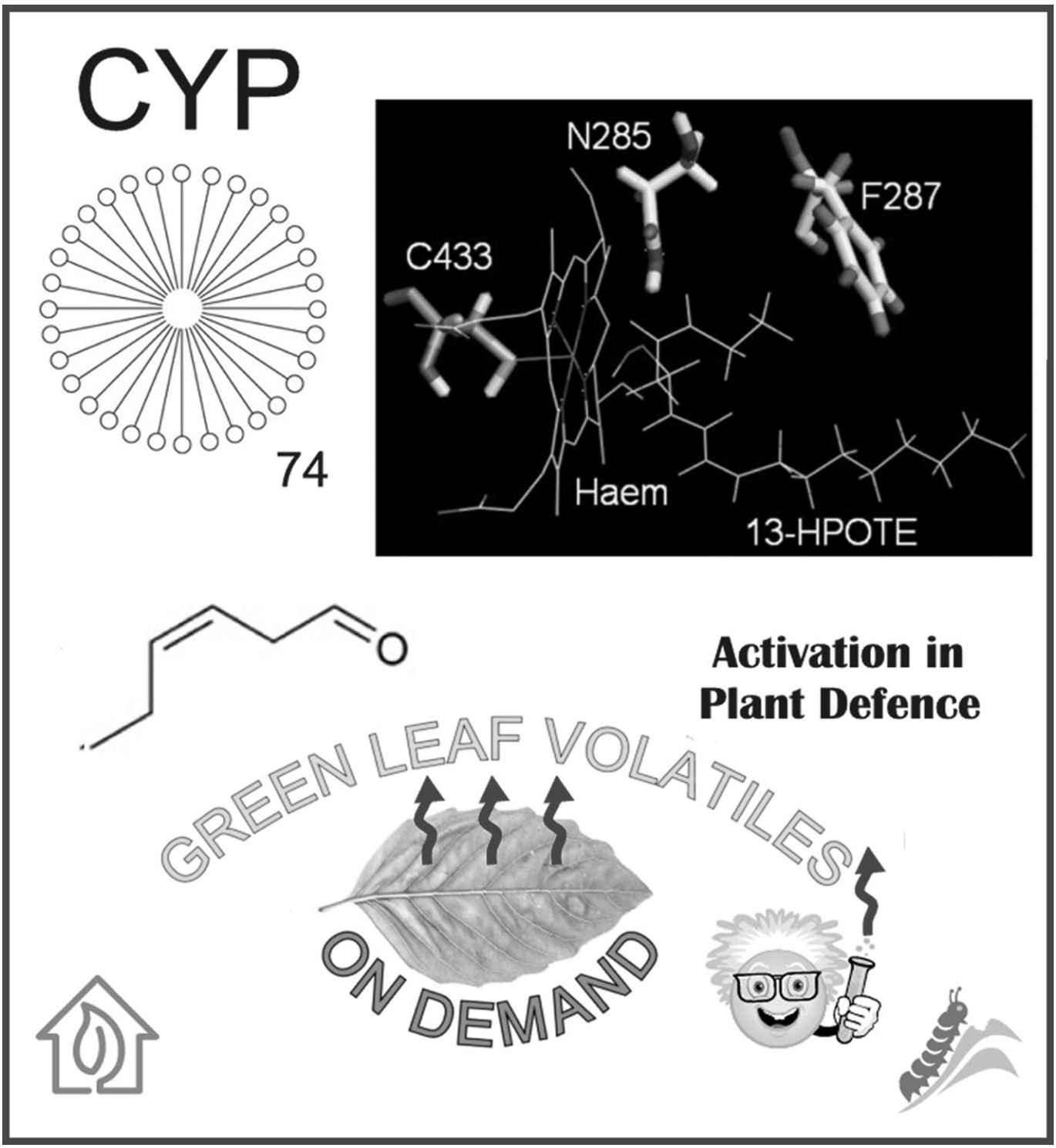

In order to respond to their rapidly changing environments, plants have evolved complex signalling pathways, which enable tight control over stress responses. Recent work has shed new light on one of these pathways that involves the different classes of plant oxylipins that are produced through the CYP74 pathway. These phytochemicals play an important role in plant defence, and can act as direct antimicrobials or as signalling molecules that inducing the expression of defence genes. The fine-tuning regulation of defence responses, which depends on the precise cross-talk among different signalling pathways, has important consequences for plant fitness and is a new, challenging area of research. In this review we focus on new data relating to the physiological significance of different phyto-oxylipins and related enzymes. Moreover, recent advances in the biotechnological production of oxylipins are also discussed.

1. Introduction

Plants have evolved complex signalling pathways (such as those based on oxylipins, salicylic acid, auxin, ethylene, and abscissic acid) to adapt themselves to rapidly changing environments. Complex interactions among these pathways permit tight control between development and response to stresses.[1–3] Upon the perception of a particular stress signal, a plant activates an extensive reprogramming of gene transcription that leads finally to enhanced resistance to a challenging pest or pathogen.

Oxylipin metabolism represents one of the main defence mechanisms employed by plants. It begins with the oxygenation of a polyunsaturated fatty acid (PUFA) by lipoxygenase (LOX), to form a fatty acid hydroperoxide. Because oxygen insertion catalysed by LOXs is position-specific, 9- or 13-hydroperoxides are produced from PUFAs such as linoleic or linolenic acids, the most common substrates of plant LOXs. PUFA hydroperoxides can be further metabolised by LOXs or by other enzymes located downstream in the pathway, including allene oxide synthase (AOS), hydroperoxide lyase (HPL), divinyl ether synthase (DES), peroxygenase and epoxy alcohol synthase. At the end, an array of different oxylipins, which includes jasmonates, aldehydes, ketols, epoxides and divinyl ethers, each with specific biological functions, are produced. Levels of oxylipins are low under normal physiological conditions but increase rapidly in response to mechanical wounding, herbivore and pathogen attack and other environmental or developmental inputs.

The cloning of several AOSs, HPLs and DESs from different plant species revealed that they are closely related members of the cytochrome P450 family and form a group, named CYP74, that is specialised in the metabolism of hydroperoxides. CYP74 enzymes are very different from other P450 enzymes in that they have an atypical reaction mechanism that requires neither oxygen nor an NADPH-reductase, and as a consequence have extraordinarily high turnover numbers.[4–7]

In this review we focus on new data relating to the physiological significance of different phyto-oxylipins and related enzymes. Moreover, recent advances in the biotechnological production of oxylipins are also discussed.

2. CYP74 Pathway for Phyto-oxylipin Biosynthesis

The relative product specificities of different CYP74 members that act on 13- or 9-hydroperoxides substantiated the hypothesis that plant oxylipin metabolism is organised into the following three main branches (Scheme 1).

2.1 The AOS branch

This branch of the LOX pathway is involved in the synthesis of jasmonates from 13-hydroperoxyoctadecatrienoic acid (13-HPOT), and includes important signal molecules involved in stress responses and development, such as jasmonic acid (JA), methyl jasmonate (MJ) and its precursor 12-oxo-phytodienoic acid (12-OPDA). They are produced from an unstable allene oxide, which is in turn converted into 12-OPDA by the enzyme allene oxide cyclase (AOC). JA is obtained from the reduction of 12-OPDA and three further chain-shortening steps (Scheme 1).[7–9] In the absence of AOC, allene oxide can spontaneously hydrolyse to form α- and γ-ketols or can undergo a non-enzymatic cyclisation.[10] JA can be further modified by conjugation with amino acids such as isoleucine, leucine, valine, alanine etc. to yield the corresponding jasmonoyl derivatives (that is, jasmonoyl-Ile (JA-Ile), jasmonoyl-Leu (JA-Leu) etc.), methylated to form the volatile methyl jasmonate, decarboxylated at C-1 into (*Z*)-jasmone, another volatile derivative, hydroxylated at C-12 to form tuberonic acid, which can subsequently be sulfonated or glycosylated, or finally can be reduced at C-6 into cucurbit acid.[9,11–14] An important point has to do with the biological activities of the different JA-related compounds. It is commonly believed that only some of these molecules are able to activate a physiological response by binding to specific receptors, whereas the others are inactive

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compounds that function as precursors or end-products of the JA signalling deactivation process.

Since the first report on the cloning of an AOS from flax, *Linum usitatissimum*,[15] more than twenty more AOSs from plant species have been reported. Most of them show strict specificity for 13-hydroperoxides, with the exception of the barley AOS, which is able to metabolise both 9- and 13-hydroperoxides and was identified as a 9/13-AOS.[16]

Angelo Santino graduated in 1988 in Biological Science at the University of Lecce, defending a thesis on yeast genetics. In 1990-1991, he was fellow at the Istituto Biosintesi Vegetali, CNR, Milan, and in 1992 at the Institute of Natural Resources, Chatam Maritime, UK. Since 1997 Angelo Santino obtained a full position as scientist at the Institute of Science of Food Production, Unit of Lecce. His main research interests deal with the physiological significance of phytoxylipins and the biochemistry of cytochromes P450 belonging to CYP74 subfamily.

Richard Hughes obtained his PhD in Biochemistry from Imperial College and after a postdoc at the University of Sussex moved to the John Innes Centre in 1992 where he has worked ever since (apart from a brief period at the Institute of Food Research in 2002). He has worked on various research projects, mainly in the laboratory of Rod Casey studying enzymes of oxylipin metabolism in plants. As a result of this experience, Richard developed a more general interest in chemical diversity and enzymes of natural product metabolism with an emphasis on structural biology. In 2007 he moved to the laboratory of Anne Osbourn studying enzymes of avenacin biosynthesis in the oat plant where his interest in structural biology flourished. In 2008 he moved to the new laboratory of Mark Banfield, where he is implementing a medium-throughput protein production system for the structural biology of relevant plant and microbial proteins.

Stefania De Domenico graduated in 2004 in Biological Science at the University of Lecce, defending a thesis on plant molecular biology. She is currently attending her PhD, working on the intracellular localisation and sorting of enzymes involved in the oxylipins pathway and the link between the oxylipins and abiotic stress adaptation in legumes.

AOS enzymes specific for 9-hydroperoxides have so far been reported from only a few species, which include tomato, potato and *Petunia inflata*.[17–19] The potato 9-AOS gene is expressed in subterranean organs; it has been proposed that α-ketols, the products of 9-AOS catalysis, have a role in the root defence mechanism and/or in the regulation of sugar metabolism.[18] *Petunia inflata* 9-AOS is highly expressed during petal senescence and it has been proposed that it has a possible role in programmed cell death.[19]

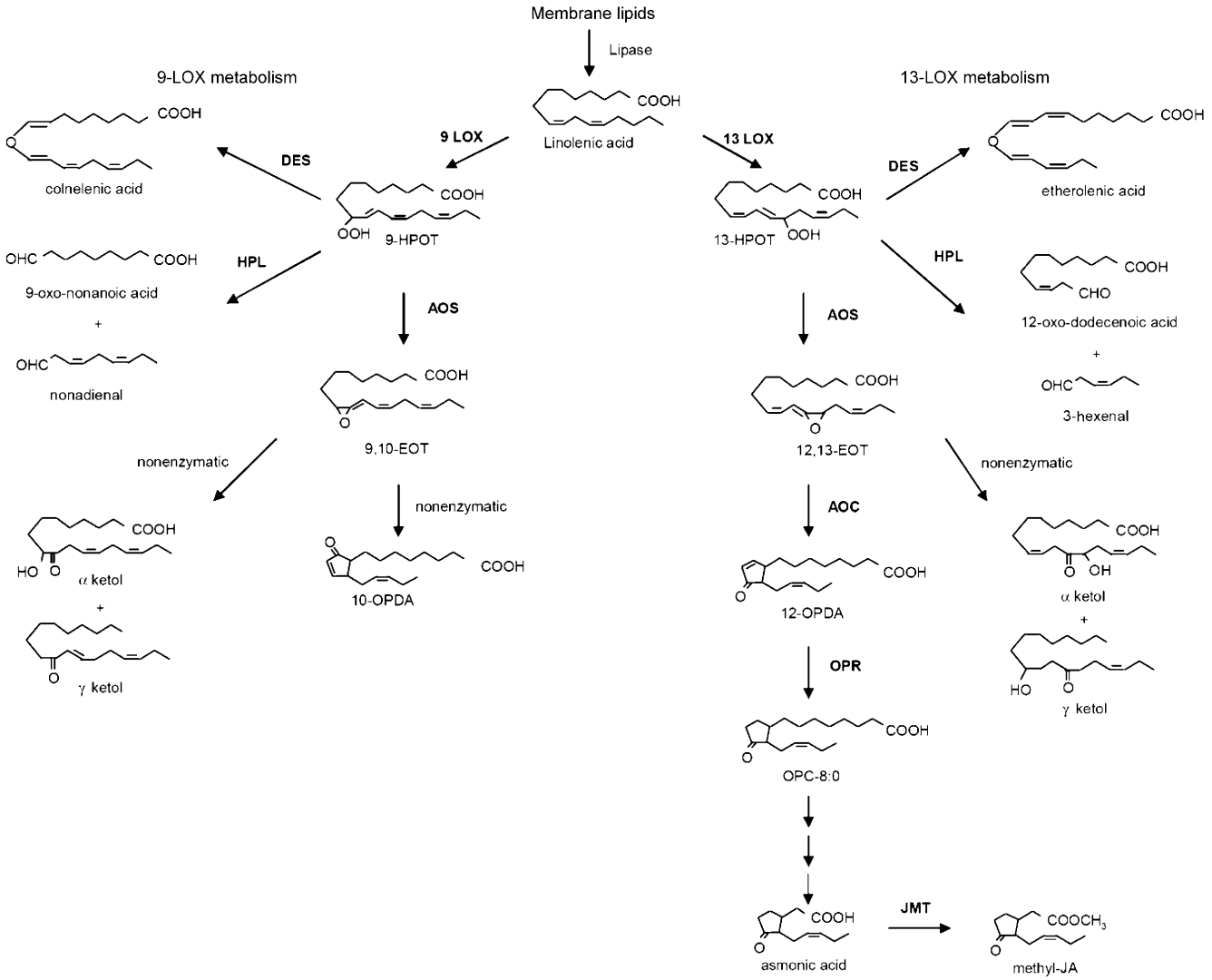
The widespread occurrence of enzymes involved in JA biosynthesis in the plant kingdom reflects the highly conserved mechanism by which plants prime defence responses towards challenging insects and pathogens.

The isolation and characterisation of a series of different mutants impaired in specific steps of JA biosynthesis led to a better understanding of the role of jasmonates in several physiological processes including development, reproduction and senescence, as well as response to biotic and abiotic stresses.[8, 9, 12, 16, 20–22]

In the context of plant defence mechanisms against insects and pathogens, JAs are considered a key component even though ODPA is also required for the full activation of the response. Therefore, JA and OPDA may act co-ordinately.[23, 24]

Among the different mutants involved in JA signal perception, *coi1* (coronative insensitive) is one of the best characterised.[9, 12, 22] COI1 has a pivotal role in JA signalling and encodes a F-box protein. This feature of the COI1 protein led to the hypothesis that ubiquitination of specific target protein by the E3 ubiquitin ligase SCF^{coi1} (where SCF indicates Skp/Cullin/F-box) is associated with JA signalling. The targets of SCF^{coi1} were recently identified in *Arabidopsis thaliana* as the repressors belonging to the jasmonate ZIM domain (JAZ) protein family.[25, 26] It was proposed that repressors of the JAZ family—that is, JAZ1 and JAZ3—are able to prevent transcription of JA responsive genes by binding to the MYC2 transcription factor. The interaction of COI1 with JAZ members is promoted in a highly specific manner by JA-Ile and related JA conjugates (JA-Val, JA-Leu, JA-Ala, in decreasing order), but not by JA, MJ or OPDA.[27] Therefore, JA-conjugates are so far the only JA derivatives known to be active at the molecular level. However, recent results carried out on *Nicotiana attenuata* mutants impaired in the biosynthesis of JA conjugates (the *irjar4/6* mutant) or the whole JAs (the *aslox3* mutant) indicated that JA-Ile plays an important role in plant resistance to insects, but JA-Ile application cannot fully restore resistance in *aslox3* plants.[28] Moreover, microarray analysis confirmed these results, because some defence genes—that is, protease inhibitor, phenyl ammonia lyase, polyphenol oxidase and α-dioxygenase—are induced in a similar way by JA-Ile and JA, whereas other oxylipin-signalling genes—that is, AOS and OPR3—together with genes involved in photosynthesis are specifically regulated by JA.[28]

These results demonstrate that JA or related compounds play important and distinct roles in comparison with JA conjugates in eliciting defences against herbivores. JA, MJ and OPDA could promote the interaction of COI1 with other JAZ proteins with ligand binding specificities different from those



Scheme 1. Lipoxygenase pathway for the metabolism of α-linolenic acid. LOX: lipoxygenase. HPOT: (*S*)-hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid. AOS: allene oxide synthase. HPL: hydroperoxide lyase. DES: divinyl ether synthase. OPR: oxophytodienoate reductase. OPDA: oxo-phytodienoic acid. EOT: epoxy octadecatrienoic acid. OPC: 8-[3-oxo-2-*cis*-{(*Z*)-pent-2-enyl}cyclopentyl]octanoic acid (OPC-8:0).

of JAZ1 and JAZ3: that is, JAZXs proteins that specifically repress the expression of genes involved in JA biosynthesis. Alternatively, it has been proposed that non-conjugated JAs could regulate gene expression through some unidentified COI1 substrates or by mediating SCF^{coi1} interaction with non-JAZ proteins[28–30]

2.2 The DES branch

This branch of the LOX pathway catalyses the conversion of hydroperoxides into divinyl ether fatty acids (Scheme 1). As in the case of 9-AOSs, DESs specific for 9-hydroperoxides have been cloned from tomato, potato and tobacco, and the recombinant enzymes, once expressed in heterologous hosts, were shown to produce colneleic and colnelenic acids from 9-HPOD and 9-HPOT, respectively.[31–33] The presence of a DES able to convert 13-HPOT into etherolenic acid in garlic bulbs and *Ranunculus* spp. leaves has been reported.[34,35] Recently, a cDNA encoding a 9/13 DES in garlic was reported.[36] DES transcripts are induced during plant/pathogen interactions. It was proposed that DES participates in local rather than systemic defence responses because the expression of DES is elicited only in the vicinities of infected tobacco tissues.[33] Notably, together with a direct antimicrobial action, colneleic and colnelenic acid were also reported to affect root growth and cause the loss of apical dominance and a concomitant increase in the number of lateral and adventitious roots in the model plant *A. thaliana*.[37] Microarray analysis has also demonstrated that exogenously supplied colnelenic acid (in the absence of pathogen) has major effects on gene expression in *A. thaliana*, despite the absence of a DES gene or detectable DES protein or activity in this plant. Nevertheless, this plant clearly has retained the ability to recognise this compound, but it is not known if colnelenic acid is acting simply as a xenobiotic (or abiotic elicitor) or, by some undetermined mechanism, has a role in defence signalling (Belfield et al., unpublished results).

2.3 The HPL branch

Finally, hydroperoxide lyases (HPLs) catalyse the cleavage of hydroperoxides into short-chain aldehydes and ω-oxo fatty acids (Scheme 1). Since the first report dealing with the cloning of a HPL gene from green bell pepper (*Capsicum annum*),[38] more than 30 complete cDNAs from 15 different plant species have been reported. Biochemical characterisation of recombinant HPLs revealed that, as in the case of AOSs, some HPLs show a strict specificity for 13-hydroperoxides with consequent production of (*Z*)-hex-3-enal and (*Z*)-12-oxododec-9-enoic acid from 13-HPOT.

As well as 13-HPLs, 9/13-HPLs have also been reported. They were initially thought to be restricted to the *Cucurbitaceae* family,[39,40] but their occurrence in other plant species such as *Medicago* spp., rice and almond was later reported.[41–43] These 9/13-HPLs are able to use both 9- and 13-hydroperoxides. Starting from 9-HPOT, (*E*,*Z*)- nona-2,6-dienal and C_9-oxo-acids are synthesised. Unlike 13-HPLs, expression of which is restricted to green tissues, 9/13-HPLs are also expressed in underground tissues, as in the cases of a rice HPL (OsHPL1) and of the *Medicago truncatula* 9/13-HPL (MtHPLF), the cDNA of which was isolated from mRNA extracted from four-week-old *Rhizobium meliloti*-inoculated roots and nodules.[41,42] High levels of HPL activity were also described in cucumber roots.[39] However, the presence of high levels of HPL transcripts/enzymatic activity do not always parallel with the detection of volatile aldehydes in this tissue. They were detected in melon[40] but not in rice roots;[42] this indicates that substrate availability is an important control point in the biosynthesis of specific oxylipins in different plant tissues.

The volatile aldehydes produced by LOX metabolism exert a wide range of actions in relation to the plant defence strategy. C6 aldehydes, alcohols and esters are important constituents of the green leaf volatiles (GLVs) and are rapidly released by plants in response to mechanical damage or herbivore attack.[6] GLVs, together with other volatile compounds—that is, MeJA and methyl salicylate—have been implicated in airborne long-distance signalling.[44] In particular, GLVs have been associated with induced resistance in intact plants. Corn seedlings exposed to GLVs from neighbouring plants produced larger amounts of JA and sesquiterpene in response to mechanical wounding or insect attack than plants not exposed to GLVs.[45] Similarly, (*Z*)-hex-3-enyl acetate, an important component of GLV, was able to boost extrafloral nectar secretion in lima bean, a typical JA-mediated defence response.[46] A similar response was also induced in the same species in response to beetle feeding, but only when air was moving freely between leaves; thus, this points to the importance of airborne signalling in plant defence strategy.[47] GLVs can rapidly diffuse in all parts of the plant, thus overcoming common restrictions of signals moving through the vascular system.[44] They can either function as inducers of the full defence mechanism or, at much lower doses, they can prime the plant to respond more rapidly or more effectively to a subsequent attack.[48,49]

The relative contributions of airborne and vascular signalling, both important in the fine tuning of systemic resistance, are likely to vary according to the diversity of plant anatomy and according to the interacting pathogens/pests. Recently, Chehab and co-workers reported that HPL-derived oxylipins do not give *A. thaliana* any protection towards aphids and herbivore insects.[50] These results might be indicative that plants have evolved different and species-specific subsets of volatile compounds responsible for priming.

Together with their role in direct defence, recent evidence has pointed to an important role of GLVs in indirect plant defence.[49,51,52] Indeed, GLVs produced in proximity to plant tissues damaged by insect pests are able to attract and help insect natural enemies in finding their specific targets.

Finally, volatile aldehydes have been also reported to function as direct antimicrobials.[53,54]

3. Tissue Specificity and Endocellular Localisation

3.1. 13-LOX metabolism

The defence role of oxylipins is considerably influenced by the specificities and compartmentation of the enzymes in the pathway (Figure 1). The first part of the jasmonate pathway, for instance, which requires the sequential action of LOXs, AOSs and AOCs, takes place in the plastids. OPDA is finally converted into the biologically active jasmonate in a separate subcellular compartment—the peroxisome.[5,7–9] Recently, the ATP-binding cassette (ABC) transporter COMATOSE was demonstrated to be involved in ATP-dependent transport of OPDA or its CoA-esterified form into the peroxisomes of *A. thaliana* leaves.[55] Together with this active transport, an alternative passive mechanism for OPDA import in this organelle has also been hypothesised, because the *cts* null mutant (defective in the ABC transporter COMATOSE) showed low but measurable levels of JA together with higher levels of α- and γ-ketols than the control plant.[55]

An alternative route of metabolism of 13-hydroperoxides proceeds through 13-HPLs (CYP74B), which show the same plastidial localisation as 13-AOSs that belong to the CYP74A subfamily.[5,7]

Therefore, CYP74 A and B enzymes compete for the same substrate, and 13-hydroperoxide availability is an important control point in the biosynthesis of different classes of oxylipins. This was confirmed in *A. thaliana*, potato and tomato, in which the co-suppression of specific LOX isoforms resulted in dramatic reductions in specific oxylipins.[56–58] These results may reflect a possible compartmentation of substrates and/or CYP74 enzymes within the plastids, as also pointed out by Froehlich and co-workers, who reported different localisations inside the chloroplast for tomato AOS and HPL, AOS being targeted to the inner membrane and HPL to the outer one.[59]

However, recent results indicated a similar localisation for potato LOX H1 and LOX H3, both being localised in the stroma and thylakoid membranes. Similarly to LOXs, tomato AOS and HPL showed a close association at the level of the grana thylakoids. Notably, AOC was also found close to the thylakoids, although it was more weakly bound to the membrane than AOS

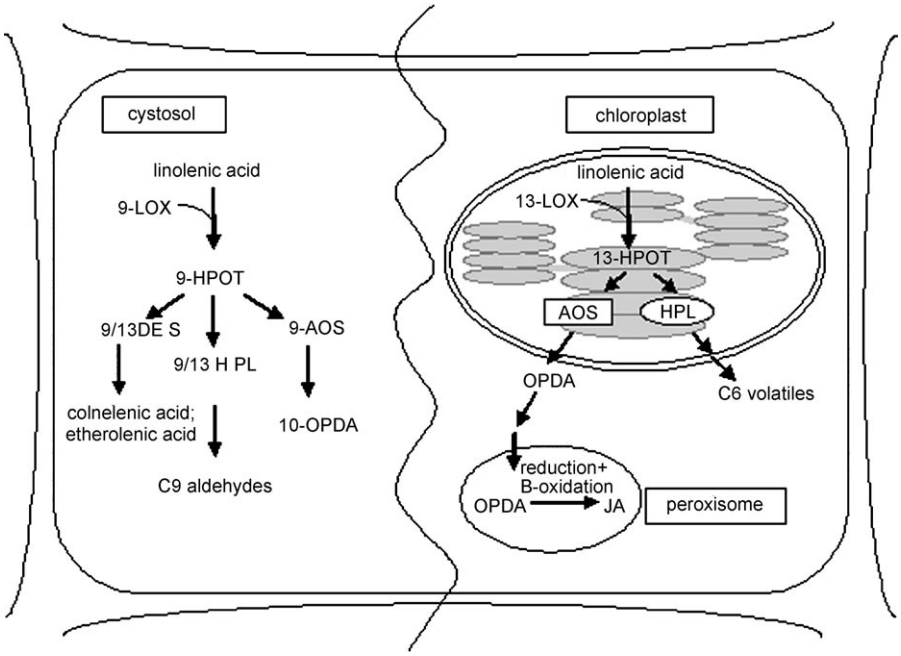


Figure 1. Schematic representation of endocellular compartmentation of 9- and 13-LOX metabolism. LOX: lipoxygenase. HPOT: (*S*)-hydroperoxy-(10*E*,12*Z*,15*Z*)-octadecatrienoic acid. AOS: allene oxide synthase. HPL: hydroperoxide lyase. DES: divinyl ether synthase. OPDA: oxo-phytodienoic acid.

and HPL. Therefore, all the enzymes required for OPDA biosynthesis appear to be closely connected.[60] It is noteworthy that *A. thaliana* AOS was also reported to be associated with plastoglobules, together with the chloroplast inner envelope membrane.[61]

3.2. 9-LOX metabolism

Unlike in the case of 13-LOX metabolism, there is little information on the subcellular localisation of 9-LOXs and of those CYP74s—CYP74 C and D enzymes—that metabolise the 9-hydroperoxides that result from its activity (Figure 1). Even though a cytosolic localisation appears to be clearly established for 9-LOXs,[18,33] different localisations were reported for the other enzymes located downstream in the pathway. A cytosolic localisation was recently reported for tobacco DES belonging to the CYP74D subgroup.[33] As far as the 9-AOS ascribed to CYP74C is concerned, immunolocalisation with specific antibodies revealed that potato 9-AOS was detected in amyloplasts and leucoplasts of cells from subterranean organs.[18] Different localisation was reported for *Petunia inflata* 9-AOS, another member of the CYP74C group, which was localised in the tonoplast when expressed as a GFP-tagged chimera in transgenic tobacco plants.[19]

In the case of 9/13-HPLs associated with CYP74C, a microsomal localisation was shown for the first time for a 9-HPL from almonds.[43] Interestingly, together with this localisation, the protein was also found associated with lipid bodies when transiently expressed in tobacco protoplasts as a GFP-tagged chimera.[43] A similar localisation was found for the cucumber 9/13-HPL (A.S. et al., unpublished results). Like these proteins, the *Medicago truncatula* 9/13-HPL was also specifically associated with lipid bodies together with a cytosolic distribution.[62]

Both the two rice HPLs (OsHPL1 and OsHPL2) classified as members of the CYP74C subfamily showed plastidial localisation.[42] However, rice HPLs share a lower identity towards the other members of this subfamily and could therefore represent evolutionarily divergent members of the CYP74C subfamily.

4. Biochemistry and Activation Mechanism of CYP74 Enzymes

The structural and kinetic properties of CYP74 enzymes were quite poorly understood until recently, when some interesting features of this class of enzymes were first uncovered.[41,63,64] This work suggests that the association between a protein monomer and a single detergent micelle, and not an oligomeric state, regulates the catalytic activity of an HPL from *Medicago truncatula* (barrel-medic) called CYP74C3. This represents a new mechanism for a membrane-associated P450 enzyme and may be a distinguishing feature of CYP74 enzymes that separates them from classical P450 enzymes, which require association with a reductase in order to carry out their full range of biological activities. It is suggested that CYP74 enzymes may simply act as peripheral membrane proteins that are rapidly activated after membrane attachment in vivo, presumably through some hydrophobic interaction that positively modifies the conformation of the protein around the substrate-binding site. This activation process would fit well with a mechanism by which the plant is able to regulate the extremely rapid release of volatile aldehydes that is observed soon after wounding or tissue disruption. It is noteworthy that CYP74C3 has been shown to be one of the fastest enzymes recorded in nature.[41] This probably suggests that HPL has been under selective pressure during evolution and has been optimised for its critical role in plant defence and development such that it is able to release, extremely quickly (without the liability of a P450 reductase partner), high local concentrations of the volatile aldehydes and produce the desired biological effect. The demonstration of a massive in vitro activation of CYP74C3 by lipid bodies, at a level comparable to that obtained with synthetic detergent micelles, together with in vivo localisation studies would support the biological relevance of this proposed mechanism.[62] The association of a CYP74 monomer with membrane (phospho-)lipid is more likely to be the active conformation; full activity of the enzyme is only present when it is physically attached to a membrane anchor. Although the

exact physicochemical characteristics of protein binding to membrane phospholipid and detergent micelle are subtly different, the fact remains that the surface hydrophobic nature of CYP74 enzymes clearly has some role to play in positioning the protein and providing the exact conformation for substrate binding and turnover. It may even serve to help localise CYP74 enzymes to a specific cellular compartment (where substrates are available) through membrane attachment. This mode of action may be responsible for maintaining associations of, for example, AOS and HPL on the inner or outer envelope membrane, respectively, of the chloroplast.[59]

It should be emphasised that it is not known if the water solubility (in the absence of detergent) of CYP74C3 is typical or atypical of CYP74 enzymes. It is also unknown whether or not the stability of CYP74C3 protein in the absence of detergent is unique; however, it is known that in all published examples, CYP74 enzymes require detergent for extraction of the proteins, both from plant tissues and from *E. coli* (when expressed heterologously). This clearly suggests that all CYP74 enzymes are membrane-bound. Moreover, all the CYP74 enzymes studied to date from various sources and laboratories have been purified in the presence of detergent and, to the best of our knowledge, there are no reported studies (other than for CYP74C3) on the effects of removing detergent from the detergent-containing enzymes. These observations suggest that, in vitro, detergent is an essential factor in maintaining the enzymatic activity of all CYP74 enzymes. This in turn would suggest that membrane association of CYP74 enzymes in vivo is required for the expression of functional protein.

CYP74 enzymes require the supply of substrates generated by the action of LOXs on PUFAs; however, it is unlikely that there is an in vitro and in vivo activation mechanism for LOXs similar to that discussed for CYP74 enzymes. LOX enzymes each have an N-terminal domain that could potentially act as a membrane-binding domain, but this would appear not to be its role because LOX enzymes are entirely water-soluble and do not require detergent for soluble extraction. The activation of LOXs in vivo would appear to be controlled more by the supply of PUFA substrates, rather than by any induced protein conformational change following membrane attachment. Interfacial phenomena in LOX catalysis have been widely reported, but these are due to the relative water-insolubility of PUFAs; the activation of LOXs by detergent micelles, to the best of our knowledge, has not been reported. The role of LOXs in CYP74 activation is more likely to be at the level of providing the necessary supply of hydroperoxide substrates, and in this respect the co-localisation (or not) in vivo of LOX and CYP74 enzymes would appear to be paramount in biological systems.

5. Primary Determinants of CYP74 Specificity: Mutagenesis, Modelling and Crystallography

More detailed structural and kinetic information on CYP74 enzymes has also been published very recently.[64,65] In silico structural analysis of CYP74C3 showed that it had strong similarities to the structural folds of the classical microsomal P450 enzyme from rabbits (CYP2C5). It was not only the secondary structure predictions that supported the analysis; it was also consistent with site-directed mutagenesis of the substrate-interacting residues. This allowed the development of a substrate-binding model of CYP74C3 (Figure 2A) that predicted

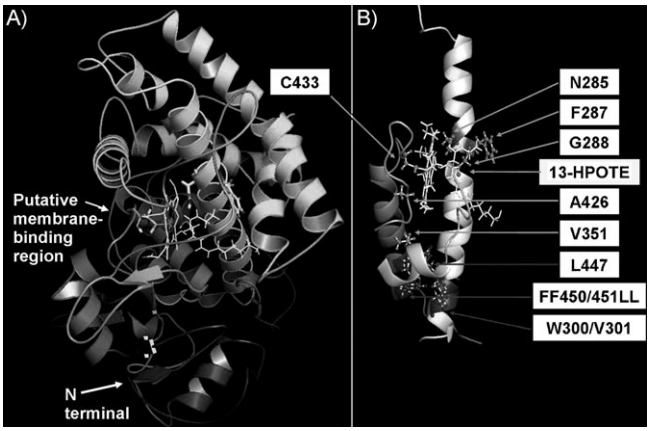


Figure 2. Homology modelling of the substrate-binding pocket of CYP74C3. A) Overall structure of CYP74C3, showing putative membrane-binding region required for enzyme activation. B) Proposed hydrophobic substrate-binding pocket of CYP74C3 with 13-HPOTE docked. Amino acid residues critical for catalysis and other neighbouring residues are shown.

three amino acid residues—N285, F287 and G288—located in the putative I-helix and that the distal haem pocket of CYP74C3 should be in close proximity to the preferred substrate 13-HPOTE (Figure 2B). These residues were judged to be in equivalent positions to those identified in SRS-4 of CYP2C5. The effects of the mutations suggest that subtle protein conformational changes in the putative substrate-binding pocket regulate the formation of a fully active monomer–micelle complex with low-spin haem iron and that structural communication exists between the substrate- and micelle-binding sites of CYP74C3. Conservation in CYP74 sequence alignments suggests that N285, F287 and G288 in CYP74C3, as well as the equivalent residues at positions in other CYP74 enzymes (see Figure S1 in the Supporting Information), are likely to be critical to catalysis. In support of this hypothesis, the residue G324 in *Arabidopsis thaliana* 9/13-AOS (classified as CYP74D4 according to the new proposed nomenclature for CYP74 enzymes based on analysis of specificity of recombinant enzymes[64]), equivalent to G288 in CYP74C3, has been shown to be a primary determinant of positional specificity. These data suggested that the overall structures of CYP74 enzymes were likely to be very similar to those described for classical P450 monooxygenase enzymes. Structural resolution of the first CYP74 enzyme (*Arabidopsis thaliana* 9/13AOS)—the first for any plant cytochrome P450 enzyme[66]—has confirmed that the overall structures of plant and mammalian P450s are indeed highly similar (including the presence of the I-helix), and the critical role of N285 in CYP74C3 catalysis[65] is also clear from the structure.[65] Structural analysis of *Arabidopsis thaliana* 9/13-AOS[66] has also confirmed the presence of a membrane-binding domain that was predicted from earlier biochemical[41,63] and modelling and mutagenesis[65] studies of both HPL and AOS.

This would appear to be the region that is essential in regulating the activation mechanism of CYP74 enzymes.[41,65] More crucially, however, the new structure has also provided evidence for the critical role of a single amino acid residue (F137 in *Arabidopsis thaliana* 9/13-AOS) and, hence, the simplicity of the evolutionary mechanism at work that determines CYP74 specificity in Nature. A single mutation at this bulky residue to Leu completely converted this AOS into one with HPL activity. This landmark paper provided, in unparalleled and elegant detail, the structural basis for the mechanism and evolution of CYP74 enzymes.

6. CYP74 Enzymes in Bacteria and Animals and the Evolutionary History of those from Plants

It was not previously possible to detect CYP74 in animals by homology-based polymerase chain reaction cloning techniques. However, through the combination of structural data for AOS from *A. thaliana* with bioinformatic and biochemical analyses, CYP74 enzymes in bacteria and animals have recently been discovered. These include those from plant growth-promoting rhizobacteria, AOSs in coral, and epoxyalcohol synthase in amphioxus. This is a remarkable development and clearly demonstrates the advances that can be made by using structural biology in combination with chemistry and biochemistry to study enzymes. Three unique motifs in CYP74 enzymes that can be used for probing genome databases have been identified. Most of these sequences show less than 30% sequence identity with the plant CYP74 family—less than the 40% identity required for formal classification as CYP74 enzymes under the current P450 nomenclature system. However, they all, with one exception, retain the characteristic nine-residue insertion (within the FxxGx_3CxG signature motif) in the proximal Cys ligand loop, the Cys residue of which is absolutely conserved (see Figure S1 in the Supporting Information for selected sequences). Interestingly, the sequence of almond (*Prunus dulcis*) AOS is the only CYP74 with a ten-residue insert (WSNG**RQMDDHPTAE**NKQC). In all other respects it shares all the unique features of the CYP74 family. Further investigation of recombinant almond AOS makes this a most interesting target for biochemical analysis and structural resolution.

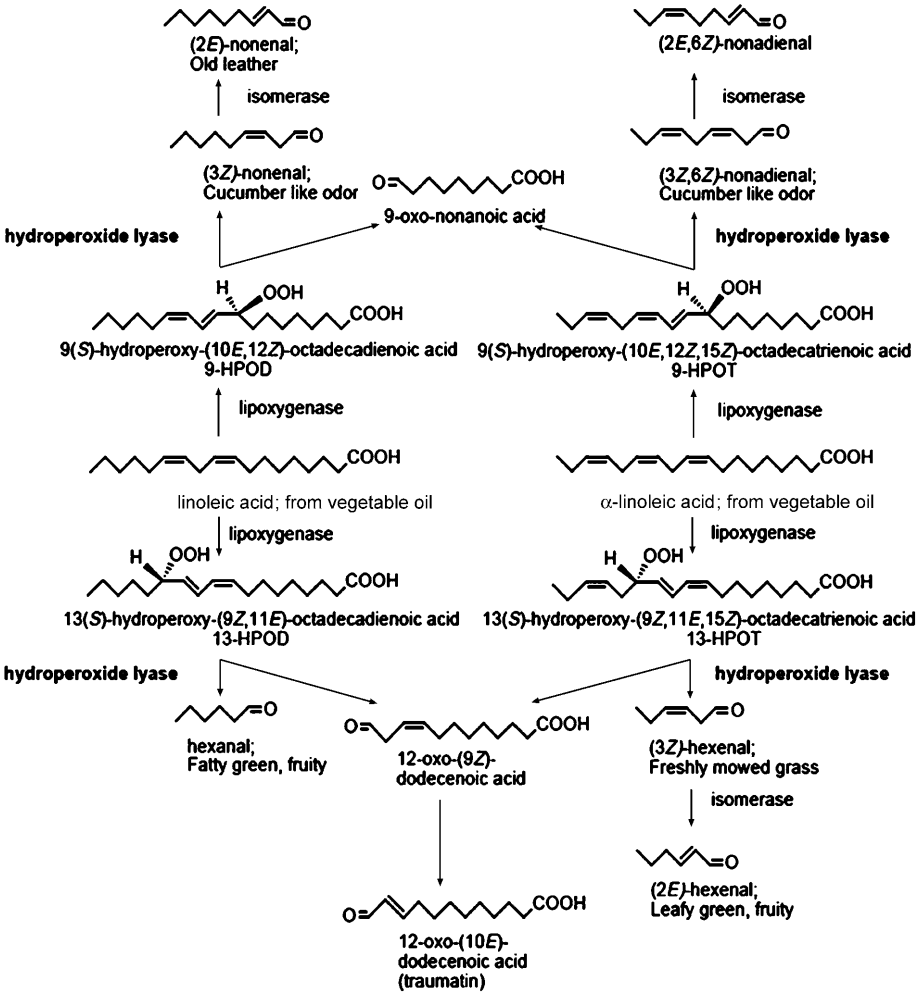
7. Biotechnological Uses of CYP74 P450 Enzymes and their Catalytic Products: New Developments

P450 enzymes are ubiquitous in nature and are essential to all life because they can metabolise a plethora of naturally occurring compounds. These can have both beneficial and adverse effects on the health of the organism. P450s from humans, and from organisms causing human disease, have been studied most extensively in order to investigate their roles in human health and to explain the effects of drugs. CYP74s have, until recently,[59] been described only in plants, and so have been less well studied, but are clearly of similar interest to plant health.

Biotechnological applications of mammalian P450 enzymes are numerous, as any patent search of "P450" would indicate. Biotechnological applications of CYP74 enzymes are, however, considerably more limited, due in part to the complete absence, until very recently (2008), of structural information for P450s of plant origin. Another key factor that has greatly limited the scope for biotechnological applications is the apparent lack of availability of stable CYP74 enzyme preparations in significant amounts. This is especially relevant for HPLs, which have maintained their commercial interest for many years (commercial interest in AOSs has remained very limited). CYP74 enzymes cannot be extracted from plants because they are present at extremely low levels, and as a consequence, heterologous expression technologies have been adopted to provide sources of recombinant CYP74 enzymes. CYP74 enzymes would also appear to be generally quite unstable to purification—the same applies to the products of CYP74 catalysis (oxylipins). These cannot usually be synthesised chemically, and for those that can be, it would be deemed preferable if they were synthesised through the same biological reaction that occurs naturally. At present, the commercial use of oxylipins is limited because they are produced naturally in extremely low amounts and some are quite unstable during storage. (*Z*)-Hex-3-enal production is a possible exception. It is produced and marketed by a number of companies—often without the requirement for an enzyme, a process that is perceived as somewhat "unnatural". The myriad of specific product isomers that are enzymatically produced (and which could potentially be trapped) are a rich source of structural diversity for the flavour and fragrance industries in particular. Cheap sources of, for example, vegetable oils rich in linoleic and linolenic acids and the enzyme LOX (in soybean flour) have been used to generate the substrates for CYP74 enzymes. Most interest has been generated in the use of recombinant HPLs for the industrial production of C6 and C9 volatile aldehydes, which are important constituents of the "fresh green" odour and the flavours and fragrances of many fruits and vegetables. HPL activity is responsible for the smell of freshly mowed grass (in essence a response of the plant to mechanical damage) or the characteristic aromas of melon and cucumber. Understandably, these natural compounds are of high commercial value. HPLs have broad substrate specificity and so also have great potential for catalysing the production of a diverse range of novel odorous compounds, either alone or in combination with other naturally occurring enzymatic processes (Scheme 2).

7.1 New procedures: stable, dried CYP74 enzymes

Current recombinant HPL preparations suffer from purification and stability problems; this has meant that relatively fresh crude extracts have had to be used, and these inevitably suffer from production, stability and storage problems. Current methods of HPL production are expensive and time-consuming and have not produced stabilised, pure, enzymes at high concentrations. Recently, though, new and reliable protocols for the production of milligram, and potentially gram, quantities of purified recombinant CYP74 enzymes have been developed



Scheme 2. Versatility in CYP74C3 biocatalysis relevant to the flavour and fragrances industry. The scheme illustrates the potential for the production of different volatile compounds with the aid of CYP74C3. Vegetable oils act as sources of linoleic and linolenic acids, which are converted into their corresponding hydroperoxides by the action of lipoxygenase and thus provide the substrates for CYP74C3. Typical enzymatic and nonenzymatic products of HPL reactions with fatty acid hydroperoxides are shown. These are usually classified according to three criteria: "fatty green fruity", "leafy green fruity" and "freshly mowed grass". The specific structures of volatile products identified as similar to the smell of old leather and cucumber are illustrated; the aromas of watermelons and apples are also perceived as products of the HPL reaction.

and patented. Examples of such enzymes have included an HPL from *Medicago truncatula* (barrel-medic), called CYP74C3 and an AOS from *Arabidopsis thaliana* (thale cress), called CYP74D4, but the procedures are most likely to be applicable to all members of the CYP74 enzyme family.

Patent-protected procedures have also been developed for the stabilisation of these purified CYP74 enzymes in a dried, detergent-free state. Extensive stability and enzyme activity trials have been carried out with various CYP74 enzymes to identify conditions for storage and stabilisation of CYP74 enzymes in an optimised state. UV/Visible spectra of detergent-free preparations of CYP74C3, CYP74D4 and CYP74B1 (Pepper 13-HPL) were determined before and after freeze-drying, as well as before and after drying in a speedvac. In addition, the UV/Vis spectra of preparations of CYP74C3, CYP74D4 and CYP74B1 in Emulphogene detergent before and after freeze-drying, as well as before and after drying in a speedvac, were determined. The UV/Visible spectra of preparations of CYP74C3, CYP74D4 and CYP74B1 in the detergent Triton X-100 before and after freeze-drying were further determined. Activity measurements of these preparations with the preferred substrate 13-HPOTE were used to determine the percentage losses of active protein resulting from freeze-drying or drying in a speedvac, and the results are summarised in Table 1.

A comparison of Figure 3 A (stability of CYP74 enzymes stored dried in the absence of detergent and then reconstituted with a detergent-containing buffer and tested), with Figure 3 B–E (storage of CYP74 enzymes in solution with or without detergent, either at 4 °C or at −20 °C) demonstrates very clearly that the CYP74 enzymes retain essentially 100% of their activity over an extended period of time, up to 15 weeks at a minimum, when stored dried in the absence of detergent, in contrast with the very rapid drop-offs in activity seen when stored in solution, with or without detergent, either at 4 °C or at −20 °C. UV/Visible spectroscopy of the three different CYP74 enzymes before and after drying demonstrated that there was a close correlation between the absorption of the proteins de-

Table 1. Effect of detergents and storage processes on the stability of CYP74 proteins expressed as percentage losses of active protein. The CYP74 enzymes were classified according to proposed new nomenclature.[64]

	Treatment					
CYP74	Freeze-drying			Speedvac drying		
enzyme	Detergent-free	Emulphogene (0.3%, *w/v*)	Triton X-100 (0.2%, *w/v*)	Detergent-free	Emulphogene (0.3%, *w/v*)	Triton X-100 (0.2%, *w/v*)
CYP74C3	5.1	73.2	59.0	23.1	63.2	n.d.
CYP74D4	2.3	58.1	41.3	17.6	83.1	n.d.
CYP74B1	9.3	89.6	74.4	1.3	78.8	n.d.

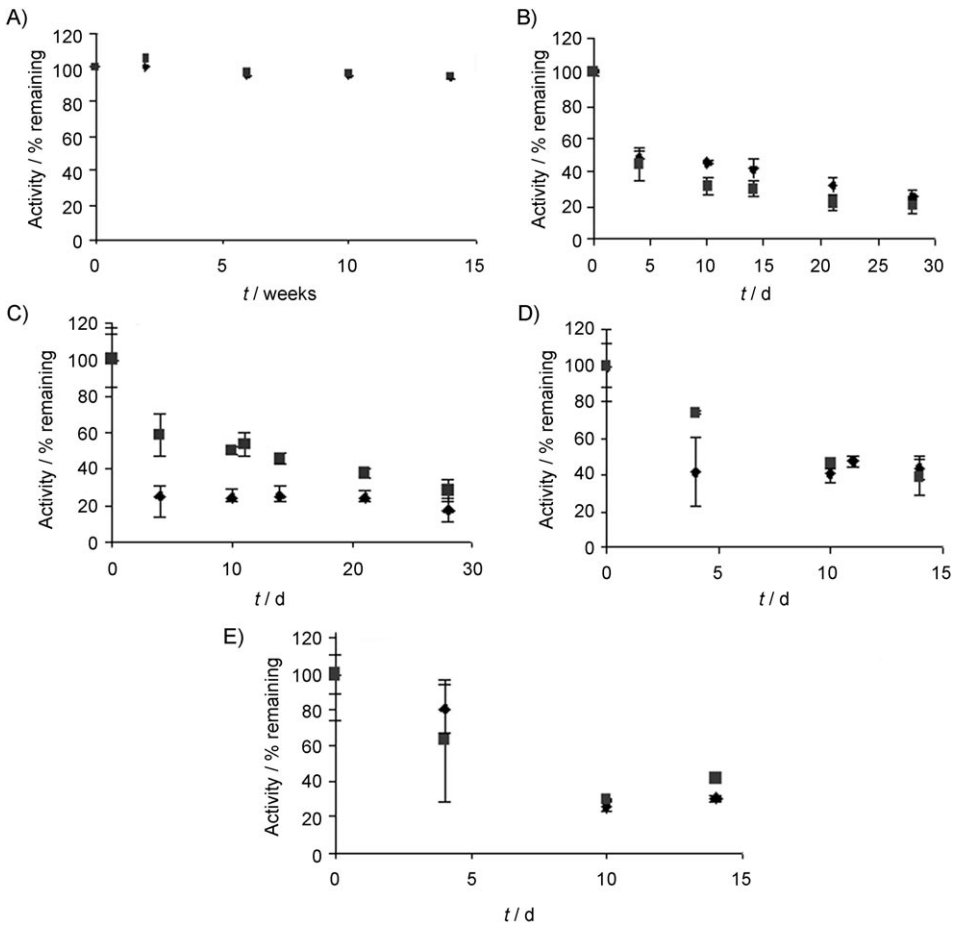


Figure 3. Stability of activities of CYP74C3 (*Medicago truncatula* 9/13-HPL) and CYP74D4 (*Arabidopsis thaliana* 9/13-AOS): effect of freeze-drying, storage temperature and detergent. CYP74 enzymes (■: AOS, ◆: HPL) stored in a dried detergent-free composition as compared with the same enzymes stored in detergent-free or detergent-containing solution at 4 °C and −20 °C. A) Dried detergent-free composition. B) Detergent-free solution at 4 °C. C) Detergent-containing solution at 4 °C. D) Detergent-free solution at −20 °C. E) Detergent-containing solution at −20 °C. Stability tests were conducted on dried (detergent-free) CYP74 enzyme reconstituted with phosphate buffer containing Emulphogene (5 mM), in comparison with the stabilities of the same enzymes stored in solution at 4 °C or at −20 °C with Emulphogene (5 mM) and glycerol (20%).

termined at 391 nm (due to the intact haem in the correct spin state) and the measurable enzyme activity. The presence of detergent during freeze-drying is extremely detrimental to the storage and activities of the proteins. Our evidence both from activity measurements and from UV/Visible spectroscopy for three different CYP74 enzymes suggests that the haem conformation of these proteins is disrupted in the presence of detergent during the drying process, due either to apparent unfolding or denaturation events, or to adverse and irreversible changes in protein conformation. Loss of haem from the proteins was also apparent in some instances. The ability to store CYP74 enzymes in the absence of detergent and to retain the haem in the original conformation on hydration of the dried product with water is the key development.

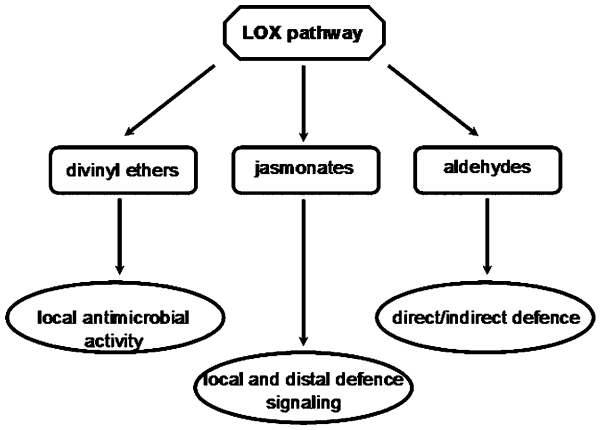
The new disruptive technology is predicted to outperform current procedures with available sources of CYP74 enzymes and may also permit the development of other, as yet unexplored and perhaps more refined, applications in which the production of "fresh green" compounds by "green chemistry" has value. Potential applications may also include their use as aromatics for the home or as natural food flavourings: essentially, any situation in which the rapid replacement on demand of an unpleasant smell with a natural "fresh green" aroma would have value.

The technology is currently being evaluated under licence through contact with Plant Biosciences Limited (Norwich, UK). Other interested parties may receive a sample of stabilised enzyme on request after signing a confidentiality agreement. It is hoped that the new technology will be adopted in industry for processes in current use and that the future will see more diverse applications for CYP74 enzymes in the food, chemical and other industries in a manner similar to that adopted for P450 enzymes of mammalian origin and/or of clinical relevance.

8. Conclusion and Perspectives

Recent work has shed new light on the physiological roles of different classes of plant oxylipins. These phytochemicals play an important role in plant defence, acting either as direct antimicrobials or as signalling molecules inducing the expression of defence genes in proximal/distal tissues (Scheme 3). Plants are often exposed to different combinations of attackers and it is likely that they have evolved coordinated defence mechanisms. The fine-tuning regulation of defence responses, which depends on the precise cross-talk among different signalling pathways, has important consequences for plant fitness and is a new challenging area of research.[52,61,62,67,68]

Our knowledge on the molecular structure and activation mechanism of CYP74 enzymes has increased rapidly in recent years. The sheer quantity and quality of the new information that has been gained from HPL/AOS mutagenesis and modelling programmes[65] and, more recently, from structural resolution of *A. thaliana* AOS,[66] has clearly illustrated the power of structural biology to uncover the molecular mechanisms that drive oxylipin metabolism. Structural resolution of new HPLs or AOSs and selected mutant variants remains a priority area. The new information on AOS structure is highly valuable, but the structure of a natural HPL (not a mutated AOS) remains elusive.



Scheme 3. Effects of oxylipins produced by different CYP74 enzymes in plant defence. Model of the different roles in defence mechanism proposed for the oxylipins produced by the three main branches of the plant lipoxygenase pathway.

Structural insights into the dynamics of the mechanism of CYP74 (HPL and AOS) activation and details of the role played by the membrane-binding domain in the regulation of this mechanism would be of fundamental interest to both plant and structural biologists. Progress will almost certainly require the adoption of medium-throughput cloning and expression procedures to identify clones that express high yields of soluble protein—protein that is either suitable for the production of crystals that diffract to high resolution, or can be used for the determination of the structure of the protein in solution.

As far as the future of biotechnological applications for CYP74 enzymes is concerned, many lessons can be learnt from the experiences in the mammalian P450 community. Successful P450 patent applications have largely developed, and will continue to develop, from incentive to use the valuable information coming out of high-throughput structural biology laboratories, such as the identification of drug-binding targets like the warfarin-bound P450 (Astex Therapeutics, UK).[69] Similar concerted efforts will unquestionably lead to the development of new, more refined, procedures and applications for CYP74 enzymes.

Keywords: cytochromes · lyases · natural flavors · oxylipins · plant defense

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