Molecular cloning and characterization of an almond
9-hydroperoxide lyase, a new CYP74 targeted to
lipid bodies

Giovanni Mita1, Angela Quarta1, Pasqua Fasano1, Angelo De Paolis1, Gian Pietro Di Sansebastiano2,
Carla Perrotta2, Rina Iannacone3, Eric Belfield4, Richard Hughes4, Nicolas Tsesmetzis4, Rod Casey4
and Angelo Santino1,†

1 Institute of Sciences of Food Production CNR Section of Lecce, via Monteroni, I-73100 Italy
2 Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università di Lecce, via Monteroni,
I-73100, Lecce, Italy
3 Metapontum Agrobios, S.S. Jonica 106, Km 448.2, I-75010, Metaponto (MT), Italy
4 John Innes Centre, Norwich Research Park, Colney, Norwich NR3 7UH, UK

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Abstract

Oxylipin metabolism represents one of many defence mechanisms employed by plants. It begins with the oxygenation of polyunsaturated fatty acids by lipoxygenases to form fatty acid hydroperoxides that are substrates for several enzymes, including specialized cytochrome P450s known as CYP74s. The targeting of a new CYP74, a 9-hydroperoxide lyase (HPL) from almonds, to the endomembrane system and lipid bodies, both as enzyme activity in almond seeds and as GFP fusions transiently expressed in tobacco protoplasts, is described. Such association of a CYP74 with lipid bodies has not been reported previously. Also described are the properties of a 9-HPL gene, the developmental regulation of its expression, the production and characterization of recombinant 9-HPL in Escherichia coli, and the developmental correlation between gene expression, enzyme activity, and the appearance of volatile C9 aldehydes from HPL action.

Key words: Almond, hydroperoxide lyase, lipid body, oxylipins, seed development.

Introduction

The pathway of oxylipin metabolism is a key element of plant defence and development. The products of the pathway (which include octadecanoids, jasmonates, 6- or 9-carbon aldehydes, oxoacids, and divinyl ether fatty acids; Feussner and Wasternack, 2002) are not only biologically active, both in defence signalling and as direct anti-microbial agents, but some are also significant in determining the organoleptic characteristics of food, through their flavour and aroma properties (Casey and Hughes, 2004).

The defence role of oxylipins is considerably influenced by the specificity and compartmentation of the enzymes on the pathway. The production of jasmonate in leaves in response to wounding or other stimuli, for instance, begins with lipoxygenase (LOX) activity, which specifically inserts oxygen at the 13 position of α-linolenic acid, to produce a 13-hydroperoxide that is converted into oxophytodienoic acid (OPDA) through the action of allene oxide synthase (AOS) and cyclase (AOC) (Feussner and Wasternack, 2002). All three activities—LOX, AOS, and AOC—are chloroplastic (Blé and Joyard, 1996; Ziegler et al., 2000; Froehlich et al., 2001; Feussner and Wasternack, 2002). OPDA is reduced and converted, via...
β-oxidation, into biologically active jasmonate, the last step taking place in a separate subcellular compartment, the peroxisome (Graham and Eastmond, 2002). An alternative route of metabolism of 13-hydroperoxides proceeds through hydroperoxide lyase (HPL), which is structurally related to AOS (both being members of the CYP74 subgroup of cytochrome P450s; Song et al., 1993; Feussner and Wasternack, 2002) and some forms of HPL are localized to chloroplasts (Froehlich et al., 2001). HPL converts fatty acid hydroperoxides into aldehydes and oxoacids; in the case of 13-hydroperoxides and 13-HPL the products are 6-carbon aldehydes that are believed to have a signalling function (Bate and Rothstein, 1998) and also play a direct role in plant defence (Croft et al., 1993). Interestingly, AOS and HPL, which use this common 13-hydroperoxide substrate to produce different classes of biologically active oxylipins within chloroplasts, appear to be routed to different envelope membranes by different targeting pathways (Froehlich et al., 2001), which has implications for the differential regulation of the two branches of oxylipin metabolism.

Although the location of the early part of the pathway to jasmonate and aldehydes is established as plastidial, a plethora of other locations for LOX have been recognized. These include cytosolic, mitochondrial, vacuolar, and particulate forms (Vernooy-Gerritsen et al., 1984; Tranbarger et al., 1991; Matsui et al., 1992; Braidot et al., 2004), with the particulate forms being further subdivided into those present in microsomal membranes (Feussner and Kindl, 1994), plasma membranes (Nellen et al., 1995), and lipid bodies (Feussner and Kindl, 1992; Georgalaki et al., 1998). By contrast, there is relatively little information on the subcellular distribution of AOS and HPL. There are several reports of their localization in chloroplasts of leaves (Feussner and Wasternack, 2002), but they are restricted to those that metabolize 13-hydroperoxides. Similarly, there is only limited information on the pathway of 9-hydroperoxide metabolism (Fig. 1). Feussner and Wasternack (2002) suggested that the cytosol might be primarily the location of 9-hydroperoxide-derived compounds.

Biochemical and cytological evidence that a new 9-HPL from almond seeds is localized to lipid bodies, which has implications for the biological significance of oxylipins derived from 9-hydroperoxides, is presented here. The specificity of the 9-HPL, which shows 63% sequence identity to melon and cucumber HPLs, has been confirmed by expression in Escherichia coli and analysis of the properties of the recombinant enzyme. The expression of the almond 9-HPL gene is specific for the early stages of almond seed development. Its expression correlates well with the developmental progression of enzyme activity and the production of 9-carbon volatile aldehydes.

**Materials and methods**

**Plant material**

Seeds of almond, *Prunus dulcis* cv. Scorza Verde, were harvested at 20, 30, 60, and 90 d after flowering (DAF, stages I–IV) from the experimental field of the Istituto Sperimentale Frutticoltura, Caserta, Italy. Seeds were shelled, frozen in liquid nitrogen, and stored at −80 °C.

![Fig. 1. 9-Lipoxygenase pathway for the metabolism of α-linolenic acid. 9-LOX, 9-lipoxygenase; 9-HPOT, 9(S)-hydroperoxy-(10E,12Z,15Z)-octadecatrienoic acid; AOS, allene oxide synthase; HPL, hydroperoxide lyase; DES, divinyl ether synthase; 10-OPDA, 10-oxo-phytodienoic acid; 9, 10-EOT, 9,10-epoxy octadecatrienoic acid.](image-url)
Different tissues (cotyledons, leaves, and roots) collected from 2-week-old almond plantlets were used for expression and HPL activity studies. Flowers were collected from adult plants. All these samples were stored at −80 °C until use.

**Protein extraction and enzymatic activities in almond seed**

Lipid bodies were isolated by two-layer flotation, as reported by Lin and Huang (1984), with the exception that total protein extracts from almond seeds at different developmental stages were centrifuged at 100 000 g for 30 min. After centrifugation the following fractions were recovered: the lipid bodies fraction from the top of the sucrose gradient; the soluble protein fraction (100 000 g supernatant); and the microsomal fraction (100 000 g pellet). The microsomal fraction was resuspended in 100 mM sodium carbonate and the pH adjusted to 7.0 before assaying HPL activity. Lipid bodies were further purified by two sequential washings with 2.0 M NaCl and 0.05 % Tween 20.

HPL activity was assayed in 50 mM sodium phosphate buffer, pH 6.5, containing 20 μM 9(S)-hydroperoxy-(10E,12Z)-octadecadienoic acid (9-HPOD), or any other substrate, by measuring the decrease at 234 nm due to the cleavage of the conjugated diene structures of the substrate. One unit of HPL activity (U) corresponds to the amount of enzyme which can convert 1 nmol of substrate per minute.

HPL activity was also tested with an NADH-coupled method for detection of aldehyde reaction products (Vick, 1991). Polyunsaturated fatty acids (PUFA) hydroperoxides were purchased from Larodan (Malmö, Sweden).

**Identification of HPL products**

About 500 μg of Triton-solubilized almond micromece protein or 100 μg of partially purified recombinant HPL were incubated with 2 ml of 100 mM sodium phosphate buffer, pH 6.5, containing 40 μM 9-HPOD (or any other substrates at the same concentration), for 30 min at 20 °C in a stirred 20 ml reaction tube closed with a septum. Headspace compounds were trapped by solid phase micro-extraction (SPME) (75 μm polydimethylsiloxane fibre; Supelco) and desorbed at 230 °C for 3 min in the injection port of a GC-MS (Hewlett-Packard, equipped with a HP6890 GC and a HP5973 MS) column (HP Innowax polyethylene glycol (PEG) capillary column 30 m-0.25 mm). The temperature programme used was as follows: 40 °C for 5 min; 40–200 °C in 20 min; 200–250 °C in 5 min; 250 °C for 2 min. Identification of products was by retention time compared with authentic standards and from mass spectra of the standards and the HPL products.

The endogenous volatiles present in almond seeds at different developmental stages were characterized as above, starting from 5 g of seeds which were ground to a fine powder in liquid nitrogen, sealed, and incubated first at 37 °C for 30 min and then at 55 °C for 10 min.

**Construction and screening of the almond genomic library**

Almond (cv. Scorza Verde) genomic DNA was extracted from young seedlings, and used for the construction of a genomic library as described previously (Mita et al., 2001). The library was screened using a cucumber HPL cDNA (a kind gift of K Matsui, Yamaguchi University, Japan) as heterologous probe. The cucumber HPL cDNA was first digested with SacI and PstI; the 1600 bp cDNA was purified on an agarose gel and digoxigenin labelled according to the manufacturer’s instructions (Roche). The filters were washed at a final stringency of 0.2×SSC, 65 °C, and chemiluminescent detection was carried out using CDP-STAR as substrate according to the manufacturer’s protocol (Roche).

**Cloning and expression of the almond HPL in E. coli**

**EcoRI** and **XhoI** restriction sites (undigested) were introduced at the 5′ and 3′ ends of the almond HPL gene using the following primers: 5′-GGATCCGAATTCATGTCCTTTTCTCTTCTCTTC-3′ (full-length gene), 5′-GGATCCGAATTCATGTCAACCAAA-CAACCTTCC-3′ (without N-terminal sequence) and the antisense strand: 5′-TCTATGCTCGAGTCAGGCGCTTGGTCAATG-3′. The HPL gene was cloned in the pET24 and pET28 vectors (Novagen). For expression of HPL, cells were grown to an A600 0.8-1.1 then induced with 1 mM IPTG overnight at 21 °C.

**RNA extraction and real-time reverse transcription (RT) PCR analysis**

Total RNA was isolated from 100 mg of almond seeds at different developmental stages using the RNeasy plant mini-kit (Qiagen) following the manufacturer’s instructions. cDNA was prepared using 2 μg of total RNA, oligo-(dT)₁₅ primer, and the superscript II polymerase (Invitrogen) according to the manufacturer’s instructions. cDNA was used as template in real-time PCR experiments with oligonucleotides designed on the basis of the HPL genomic sequence using the Beacon designer 2.0 software (Bio-Rad) and the iCycler iQ Multicolor Real Time PCR detection system (Bio-Rad) with the Quantitect probe PCR kit (Qiagen). Primers and TaqMan probe were as follows:

**HPL** sense primer: 5′-CTACCGCGGTCTGCGCTTATC-3′; 
**HPL** antisense: 5′-GGGGTTCCAAGTTGAGAAACATG-3′; 
**HPL** TaqMan: 5′-FAM-CCCTCGAGGCCCACACGGACCA-C3′; 
**HPL** Actin sense primer: 5′-ATGGTTCAAGTGGAAAGACCATG-3′; 
**HPL** Actin antisense: 5′-TCTATGCTCGAGTCAGGCGCTTGGTCAATG-3′; 
**Actin** TaqMan: 5′-FAM-ACCATCAACCAGAATTCCACAA-CCA-C3′.

**Construct preparation**

Three chimeric proteins were generated, inserting appropriate restriction sites by PCR, to fuse GFP (Di Sansebastiano et al., 2001) and **HPL** sequences. The **SP-GFP** gene was obtained by fusing the DNA sequence coding for the first 19 N-terminal amino acids from **HPL** to the GFP gene through a **NheI** site. The restriction site was inserted in the **HPL** sequence using the primer **HPL2** (5′-TGGCCAGGCTGACATGTCAGAAAGGTTGG-3′). The **SP-GFP** gene was obtained by fusion of **SP-GFP** to the remaining portion of **HPL** through the restriction site **BglII** of **GFP**. The **HPL-GFP** gene was obtained by fusing the entire DNA sequence coding of **HPL** to the GFP gene through a **NheI** restriction site. The restriction site was inserted by mutating the **HPL** stop codon, using the primer **HPL3** (5′-GGGCTAGCTGAGGCTTGGTCAATG-3′). The **Oleosin-GFP** gene was obtained by fusing the entire DNA encoding the almond oleosin (accession number X78118) to the GFP gene through a **NheI** restriction site. All chimeric genes were cloned into the transient expression vector pGy (Di Sansebastiano et al., 2001) as...
Nile blue A staining was carried out as reported by Wahlroos et al. Lipid staining FITC (505–530 nm) while chlorophyll epifluorescence was detected confocal laser-microscope. GFP was detected with the filter set for fluorescence microscopy in their culture medium at different times. Protoplasts transiently expressing chimeric GFPs were observed by confocal laser scanning microscopy.

Nicotiana tabacum cv. SR1 protoplasts were isolated from leaves. Tobacco protoplast preparation and transformation was carried out following the protocol described by Nagy and Maliga (1976), cultured, and transformed by PEG-mediated direct gene transfer essentially as described elsewhere (Negrutiu et al., 1987; Freydl et al., 1995). Ten micrograms of plasmids were used for the transformation of 600 000 protoplasts. After 2 h, protoplasts were rinsed to remove the PEG, resuspended in 2 ml culture medium and incubated at 26 °C in the dark.

The amplified product was cloned into a modified pGreenII0029 plant expression vector (www.pgreen.ac.uk) upstream of the YFP coding sequence. Expression was driven by a double 35S promoter and 35S terminator.

Confocal laser scanning microscopy

Protoplasts transiently expressing chimeric GFPs were observed by fluorescence microscopy in their culture medium at different times after transformation. They were examined using a LSM Pascal Zeiss confocal laser-microscope. GFP was detected with the filter set for FITC (505–530 nm) while chlorophyll epifluorescence was detected with the filter set for TRITC (>650 nm).

Lipid staining

Nile blue A staining was carried out as reported by Wahlroos et al. (2003). Protoplasts were observed 10 min after dye addition to the medium without any washing step.

Results

HPL activity during almond seed development

It has been shown previously that 9-LOXs are expressed and active at early developmental stages of almond seeds before lipid body deposition begins (Mita et al., 2001). In order to establish if LOX activity parallels any HPL activity, the activity of the latter was studied throughout almond seed development. The results indicated that HPL activity was mainly associated with detergent-solubilized microsomes (100 000 g pellet) and with purified lipid bodies, which accumulate abundantly in almond seed from developmental stage IV. By contrast, no activity was found in the cytosolic protein fraction. In the microsomal fraction, HPL activity peaked in stage II (30 DAF; Fig. 2A) and declined in later stages, but activity was consistently detected in mature almond seeds. Similar levels of HPL activity were detected in microsomes and in lipid bodies from mature almond seeds (90 DAF, stage IV; Fig. 2A). For lipid body isolation no chaotropic agents such as urea were used so as to preserve the enzymatic activities and, therefore, contamination of endoplasmic reticulum (ER)-localized enzymes could not be excluded. For this reason, the activity of NADH cytochrome c reductase (a known ER marker) was assayed both in microsomal and lipid body fractions. Under the present experimental conditions, the activity of NADH cytochrome c reductase was minimal in the almond lipid body fraction (the specific activity detected in lipid bodies and microsomal fractions was about 2 and 50 U mg⁻¹, respectively) and was totally removed when lipid bodies were washed with Tween 20 at a final concentration of 0.05% (data not shown). By contrast, the HPL activity was similar in both fractions (Fig. 2A) and was not removed by detergent treatment.

The presence of C6 and C9 aldehydes in almond seeds collected at different developmental stages was monitored by GC-MS. Both C6 and C9 aldehydes were detected in early developmental stages (stages I and II). After this stage, no volatile compounds of the LOX pathway were detectable under the present experimental conditions (data not shown), even though HPL activity is detected at the later stages (Fig. 2A).

The almond HPL from developing seeds has an optimum pH between pH 6.5 and 7 and showed a strict substrate specificity, with the rate of breakdown of 13-HPOT [13(S)-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid] and 13-HPOD [13(S)-hydroperoxy-(9Z,11E)-octadecadienoic acid] being only 5% of that observed for 9-HPOD, the preferred substrate (Fig. 2B). 9-HPOD [9(S)-hydroperoxy-(10E,12Z,15Z)-octadecatrienoic acid], is also a good substrate for almond HPL (Fig. 2B). HPL activity was also detected in germinating seeds but, unlike HPL from developing seed, this isoform showed a high specificity for 13-hydroperoxides and no activity was found toward 9-HPOD or 9-HPOT (Fig. 2B).

The production of C9 volatile aldehydes from almond seeds was monitored by GC-MS analysis, using the microsomal fractions purified from developing almond seed. This analysis confirmed that (2E)-nonenal is the main product from 9-HPOD (Fig. 2C). This was identified by reference to the retention time of the purified standard and from mass spectral fragmentation data [m/z 139 (M⁺-H), 122 (M⁺-H₂O), 111 (M⁺-CHO)]. It is not clear if (2E)-nonenal derives from the isomerization of (3Z)-nonenal or if it is an immediate product of the almond HPL.

Noordermeer et al. (1999) reported the presence of only (2E)-isomers from crude microsomal preparations of alfalfa seedlings, which they attributed to the presence of a 3Z:2E-enal isomerase. It is possible that a similar enzymatic activity is present in microsomal preparations from almond seeds.
Under the present experimental conditions, no volatile products were detected with 13-HPOD and 13-HPOT as substrates, probably because they were beneath the levels of detection.

Cloning and characterization of a putative almond HPL gene

An almond genomic library was prepared as described (Mita et al., 2001), and a positive clone was identified using a 9-HPL-encoding cDNA from cucumber (accession number AF229811; Matsui et al., 2000) as a probe. Sequence analysis revealed that it contains a complete gene encoding a new member of the cytochrome P450 CYP74 family. By aligning the predicted protein sequence with that of other HPLs, it is surmised that the gene comprises a 657 bp 5'-untranslated region, an intron-less open reading frame of 1452 bp, and a 202 bp 3'-untranslated region (Fig. 3A). The open reading frame encodes a putative polypeptide of 483 amino acids with a predicted molecular mass of 54.3 kDa and a pl of 6.53. The predicted amino acid sequence of the putative almond HPL revealed the presence of the four domains that are highly conserved in many cytochrome P450s and the cysteine residue (Cys437) involved in haem binding (Fig. 3A, B). The A, B, C, and D domains are highly conserved throughout all CYP74 subfamilies (Fig. 3B).

The amino acid sequence shows a range of identity with other members of the CYP74 family. The highest identity (about 63%) was towards 9-HPL from cucumber (AF229811; Matsui et al., 2000) and melon (AF081955; Tijet et al., 2001). High identity was also observed with the 9/13 Medicago truncatula HPLs (AJ316562 and AJ316563; 60.8% and 59%, respectively) and tomato 9AOS (56%) ascribed to the CYP74C family. A lower degree of identity was found towards other plant AOSs specific for 13-hydroperoxides (CYP74A, 50–56% identity), cytochrome P450s belonging to CYP74D [divinyl ether synthases (50–51%)] and CYP74B [13-HPL (41–42%)] subfamilies. The dendrogram in Fig. 3C confirms that the almond 9-HPL gene can be ascribed to the CYP74C subfamily, together with the other 9-HPLs so far identified, and was named PdCYP74C5.

Southern blot analysis using the PdCYP74C5 gene as a probe revealed a simple hybridization pattern (Fig. 3D), including a major EcoRI fragment of a size predicted from the gene sequence, indicating that 9-HPL may be present as a single copy in the almond genome. The faint signals detected could be related to other genes encoding 13-HPL or other members of CYP74 sub-families.

The almond CYP74C5 encodes a functional 9-HPL

The almond CYP74C5 gene does not contain introns, so the coding region of the gene was cloned in pET expression
from E. coli cells harbouring the almond HPL gene; no volatile compound was detected using cells harbouring the empty plasmid. From these results, it is concluded that the CYP74A5 gene encodes a functional 9-HPL.

The almond 9-HPL gene is expressed in developing almond seed

HPL expression was studied during almond seed development by real-time RT-PCR (actin was used as an endogenous control). As reported in Fig. 5A, HPL is expressed mainly at early seed developmental stages (I and II). HPL expression was also studied in different tissues: cotyledons.

Table 1. Substrate specificity of native and recombinant almond HPL

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<tr>
<th>Substrate</th>
<th>Native HPL</th>
<th>Recombinant HPL</th>
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<tr>
<td>9-HPOD</td>
<td>100</td>
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<td>9-HPOT</td>
<td>85</td>
<td>80</td>
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<tr>
<td>13-HPOD</td>
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<td>13-HPOT</td>
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Fig. 4. GC-MS analysis of C₉ aldehydes produced by the almond recombinant HPL. About 100 µg protein from lysate of E. coli harbouring the almond HPL gene was incubated for 30 min in 100 mM sodium phosphate buffer pH 6.5, containing 40 µM 9-HPOD (+). Head space compounds were trapped by SPME and analysed by GC-MS. Control reaction with E. coli lysate harbouring the empty plasmid was carried out in the same way (−).

Fig. 3. Molecular analysis of the almond HPL gene. (A) Nucleotide and deduced amino acid sequences of the almond HPL gene. The conserved domains (A, B, C, and D) are underlined. The conserved cysteine involved in the haem binding is indicated with an asterisk. Comparison of the highly conserved sequences (A, B, C, and D) among different members of the CYP74 family. Pd HPL, almond HPL (AJ578748); 9 HPL, melon HPL (AF081955); 9 AOS, tomato AOS3 (AF454634); DES, tobacco DES (AP070976); 13 AOS, A. thaliana AOS (Y12636); 13 HPL, A. thaliana HPL (AP087932). (C) Phylogenetic relatedness of almond HPL. The amino acid sequence of the almond HPL (PdHPL, AJ578748) was compared with other plant cytochrome P450s, belonging to the CYP74 family. The phylogenetic tree was obtained using the PHYLIP (Phylogeny Inference Package) program. AOS sequences were from Arabidopsis thaliana (AtAOS, Y12636), tomato (LaAOS, AJ271093; LeAOS2, AF230371; LeAOS3, AF454634), potato (StAOS1, AJ457080; StAOS2, AJ457081), tobacco (NuAOS, AJ295274), flax (LuAOS, U00428), guayule (PaAOS, X78166), rice (OsAOS1, Y1055775; OsAOS2, AB116527), barley (HaAOS1, AJ250864; HaAOS2, AJ251304), maize (ZmAOS, AJ488135), wheat (TeAOS, YA196004), melon (CmAOS, AF081954), Citrus sinensis (CitrusAOS, AJ243478). HPL sequences were from Medicago truncatula (MtHPL1, AJ316562; MtHPL2, AJ316563), Medicago sativa (MsHPL, AJ249245), Psidium guajava (PgHPL, AF239670), tobacco (NaHPL, AJ41400), bell pepper (CaHPL, U51674), potato (StHPL, AJ310520), tomato (LeHPL, AF230372), Arabidopsis thaliana (AtHPL, AP087932), cucumber (ChHPL, AF229811), melon (ChHPL, AF081955). DES sequences were from: potato (SiDES, AJ109941), tomato (LeDES, 317515), tobacco (NiDES, AP070976). For sequence LeCYP74C4 (AF461042) from tomato no function has been reported. (D) Southern hybridization of almond genomic DNA with a digoxigenin-labelled HPL probe. Genomic DNA (10 µg) was digested with EcoRI (E), BamHI (B), and HindIII (H) and hybridized with the almond HPL gene.
leaves, and roots from young plantlets and flowers from adult plants. In cotyledons from young plantlets a similar expression as in stage I seeds was observed. In all the other tissues, low expression levels were observed. Taken together, the molecular and biochemical results (Figs 2B, 5) indicate that at least a 13-HPL and a 9-HPL are expressed and active in almond tissues.

The almond 9-HPL is targeted to the endomembrane system and to lipid bodies

The almond 9-HPL contains an unusual N-terminal sequence containing nine consecutive serines (Fig. 3A). TargetP and ChloroP prediction analyses (Emanuelsson et al., 2000) did not give a clear indication of possible endocellular localization of the putative almond HPL. TMPRED (prediction of transmembrane regions and orientation, an algorithm based on TMBase, a database of naturally occurring transmembrane proteins) identified two regions of potential transmembrane helices spanning positions 200–220 and 280–300 in the almond HPL.

To clarify the HPL subcellular localization, a set of GFP-tagged HPL gene fusions were prepared and analysed by confocal laser scanning microscopy after transient expression in tobacco protoplasts. Figure 6 shows the three different chimeric GFPs used: SP-GFP, SP-GFP-HPL, and HPL-GFP. Fluorescence patterns were compared with those obtained with an ER-targeted GFP (GFP-KDEL) and a secreted GFP (sec-GFP) reported previously (Di Sansebastiano et al., 2001). In the case of GFP-KDEL, the chimeric protein accumulated in the ER with a characteristic fluorescence distribution at the nuclear envelope, and no fluorescence was associated with other compartments (Fig. 7a).

The SP-GFP construct was obtained by fusing the first 19 amino acids of the almond HPL to GFP. The resulting chimera was transiently sorted through the ER, which was only transiently labelled by GFP fluorescence (Fig. 7b). SP-GFP sorting was very similar to sec-GFP sorting, where a bright labelling of the ER was also observed, but it faded with time and no accumulation was evident in other compartments (data not shown).

The SP-GFP-HPL construct was obtained by fusing the remaining portion of HPL to the previous chimeric gene (Fig. 6), leaving the C-terminus of the protein exposed as in the native form of the protein. This chimeric protein appeared to be localized to ER and to ER-associated discrete spherical bodies of about 2–5 μm (Fig. 7c, d). A similar fluorescence pattern was observed with the HPL-GFP construct, demonstrating that both the N- and C-terminus of the almond HPL are permissive sites for GFP-tagging (Fig. 7e, f).

To confirm these results on the almond HPL localization two different controls were chosen: an oleosin-GFP chimeric protein was used as a positive control and Medicago truncatula HPL3-YFP (MtHPL-YFP) as a negative control. In the case of oleosin-GFP, the chimera was localized in spherical bodies of similar features to those with which the fluorescence of the almond HPL-GFP chimeric protein was associated (Fig. 7g). As expected, MtHPL targeted YFP to plastids (Fig. 7h).
Fluorescence patterns of representative chimeric proteins in tobacco protoplasts: (a) GFP-KDEL; (b) SP-GFP; (c) SP-GFP-HPL with the nuclear envelope labelled; (d) SP-GFP-HPL; (e) HPL-GFP; (f) HPL-GFP, a protoplast showing several oil bodies (indicated by the arrows); (g) oleosin-GFP with the GFP fluorescence localized in oil bodies (indicated by the arrows); (h) *M. truncatula* HPL-GFP with GFP fluorescence associated with plastids.
Both almond HPL-GFP and oleosin-GFP chimeric proteins identify peculiar compartments characteristic of the in vivo localization of these proteins. To characterize the nature of these spherical, optically dense compartments (Fig. 8a–c), tobacco protoplasts were stained with the neutral lipid-specific stain Nile blue A. These spherical bodies were selectively stained with Nile blue A, indicating that they can be identified as lipid bodies as suggested previously (Wahlroos et al., 2003). Moreover, fluorescence from Nile blue A staining and fluorescence from the HPL-GFP and oleosin-GFP fusion proteins precisely co-localized (Fig. 8d–f and Fig. 8l–n for HPL-GFP and oleosin-GFP, respectively). By contrast, no co-localization was found either with the endomembrane marker GFP-Chi (Fig. 8g–i), which was reported to accumulate in small vacuoles of similar size (Di Sansebastiano et al., 1998, 2001), or with MtHPL-YFP which was localized in the plastids (Fig. 8o–q).

Discussion

Oxylipin metabolism plays a key role in plant defence in several ways, and there are a number of aspects of the regulation of the pathway of oxylipin metabolism that impact on specific elements of the oxylipin-mediated defence process. These include: the specificities of the LOX that catalyse the production of 9-, 13-, or 9/13 hydroperoxides; the specificities of the CYP74s that subsequently metabolize the hydroperoxides; whether CYP74 metabolism of hydroperoxides is via AOS, HPL, or DES, which in turn can be regulated via substrate specificity or localization, or enzyme localization; and the compartmentation of products along the pathway. There is little information on the relative importance of each of these potential control points but there is some evidence for metabolic interaction between LOX and HPL from the work of Leon et al. (2002), who demonstrated that transgenic potato plants in which a specific 13-LOX had been depleted are not affected in terms of jasmonate accumulation via AOS, but are deficient in the production of HPL-derived aldehydes. This indicates that CYP74s interact with specific LOX isoforms in ways that have yet to be elucidated, but which may include compartmentation. Work on Nicotiana attenuata (Halitschke et al., 2004) strongly indicates a significant role for both metabolic compartmentation and substrate ‘crosstalk’ in oxylipin synthesis.

The cellular localization of plant HPLs and other enzymes ascribed to the CYP74 family is still largely unclear. At least some of them are targeted to the chloroplast. Froehlich et al. (2001) reported a different localization inside the chloroplast for tomato AOS and HPL, AOS being targeted to the inner, and HPL to the outer, membrane. Some CYP74 enzymes, e.g. tomato AOS and Arabidopsis HPL, contain a chloroplast transit peptide, whereas others, e.g. tomato, alfalfa, and melon HPL, do not (Bate et al., 1998; Froehlich et al., 2001; Noordermeer et al., 2001; Tijet et al., 2001). In the case of those CYP74 enzymes lacking a clear transit peptide, a microsomal localization has been proposed (Noordermeer et al., 2001).

There is little information on the subcellular localization of 9-LOX and those CYP74s that metabolize the 9-hydroperoxides that result from its activity and, consequently, the physiological role of the 9-hydroperoxide-derived oxylipins is less completely understood, although it is clear that the 9-LOX pathway plays an important role in plant defence. Previously characterized 9-HPLs from melon and cucumber, which show a less rigorous specificity for 9-hydroperoxides than almond seed 9-HPL (Matsui et al., 2000; Tijet et al., 2001), are of unknown location within the cell.

Results presented here show that, in developing almond seed, HPL activity becomes associated with microsomes and lipid bodies (Fig. 2A). To confirm this preliminary observation, a similar approach was adopted to that previously used to study oleosin trafficking in non-oil-storing tissues, such as tobacco leaves, by Wahlroos et al. (2003). These authors demonstrated that oleosin-GFP chimeric protein is synthesized in the ER and is later associated with spherical bodies of about 2–4 μm, identified as oil bodies. The present results indicate that HPL-GFP and SP-GFP-HPL chimeric proteins, when transiently expressed in tobacco protoplasts, are trafficked to discrete spherical bodies which show similar size and features (optical density and presence of neutral lipid selectively stained by Nile blue) to tobacco leaf lipid bodies. It is also shown that the SP (the first 19 amino acids of HPL) alone is sufficient to direct GFP transiently to the ER, but further segregation to the spherical bodies identified as genuine lipid bodies requires the rest of the HPL sequence.

The nature of these organelles has yet to be clarified. Murphy (2001) reported that the stresses induced during the digestion of leaf discs by cellulases and other hydrolases...
to make protoplasts are known to result in the conversion of non-chloroplastic lipids into triacylglycerols, which are then believed to accumulate as cytosolic lipid bodies. However, the presence of lipid bodies was also reported in tobacco leaf cells (Wahlroos et al., 2003) and, even though the molecular organization of lipid bodies observed in tobacco protoplasts and leaf cells is currently unknown, their selective staining with Nile blue A indicates that they are strictly related to seed oil bodies. Therefore, the GFP-targeting data reported here substantiate the conclusion, from density gradient centrifugation (see earlier), that some elements of the sequence or structure of almond seed 9-HPL are able to direct it to lipid bodies in almond seeds.

The factors that govern the targeting of the almond 9-HPL to lipid bodies are not clear. There are a number of hydrophobic tracts within the almond 9-HPL sequence (amino acids 11–20, 64–81, 202–216) that may play a role in targeting to the ER and oil bodies. A role for these 9-HPL sequences in lipid body targeting will, however, remain speculative in the absence of appropriate structure/function assays.

The sequence of the almond 9-HPL is 63% identical to those of cucumber and melon 9-HPLs, ascribed to the CYP74C family, and slightly less so (56% identity) to the 9-AOS from tomato (Itoh et al., 2002). Although it is considered to be a member of the CYP74C family (for CYP74 nomenclature, see Feussner and Wasternack, 2002), it differs significantly from other members and, in that sense, is a new CYP74C sequence. It also differs in its substrate specificity; whereas melon and cucumber show a preference, but not absolute specificity, for 9-hydroperoxides, with 13-hydroperoxides also acting as good substrates (Hornostaj and Robinson, 1999; Matsu et al., 2000; Tijet et al., 2001), the almond 9-HPL scarcely metabolizes 13-hydroperoxides and shows strict 9-specificity. In this context, the almond HPL looks more similar to a HPL partially purified from pears (Kim and Grosch, 1981). Both almond and pear belong to the Rosaceae family. Current understanding of the factors that determine HPL specificity is very limited, by contrast with those of the LOXs (Feussner and Wasternack, 2002).

At present the physiological role of HPLs in developing seed is unclear. Their activity seems to coincide with rapid remodelling and reorganization of membranes and newly synthesized lipid bodies, and it may be that LOX and HPL are involved in the regulation of cell growth and cell division processes occurring during the early stages of seed development. A similar function has been proposed for potato LOX1 in the control of tuber growth (Kolomiets et al., 2001). Almond and potato LOXs share a similar product specificity and about 71% identity. At present it is unknown if the products of 9-HPL catalysis can function as signal molecules and induce the expression of other genes, as already proposed for C6 aldehydes (Bate and Rothstein, 1998). Modulation of 9-HPL expression should help to clarify this.

This report describes a CYP74 associated with lipid bodies. It is assumed that the lipid body 9-HPL (which differs appreciably from, and has a tighter substrate specificity than, previously described 9-HPLs from melon and cucumber) catalyses the conversion of 9-hydroperoxides, produced by the LOX isoform expressed at early stages of seed development, into C₉ aldehydes and oxoacids. The analysis of volatile compounds in developing almond seeds supports this view and confirms a good developmental correlation between LOX activity, HPL gene expression and activity, and the formation of C₉ volatiles. 9-HPL is present throughout seed development, but its substrate may be available only at the early stages of seed development because 9-LOX activity is limited to these stages (Mita et al., 2001; Santino et al., 2005); its products should, however, be readily available to 9-HPL which is localized to microsomes and on the surface of lipid bodies. Substrate availability could therefore play a role in the control of oxylipin biosynthesis, and the crucial role of LOXs in controlling oxylipin biosynthesis has to be considered in programmes aimed at manipulating the expression of genes located downstream in the pathway.

In summary, a gene has been characterized for a new member of the CYP74C family of cytochrome P450s, its 9-HPL product has been expressed in E. coli, and its substrate specificity examined, and 9-HPL has been shown to be localized in lipid bodies, which is an unprecedented observation for any CYP74.

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