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Molecular cloning and biochemical characterization of a lipoxygenase in almond (*Prunus dulcis*) seed

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We have characterized an almond (*Prunus dulcis*) lipoxygenase (LOX) that is expressed early in seed development. The presence of an active lipoxygenase was confirmed by western blot analysis and by measuring the enzymatic activity in microsomal and soluble protein samples purified from almond seeds at this stage of development. The almond lipoxygenase, which had a pH optimum around 6, was identified as a 9-LOX on the basis of the isomers of linoleic acid hydroperoxides produced in the enzymatic reaction.

A genomic clone containing a complete lipoxygenase gene was isolated from an almond DNA library. The 6776-bp sequence reported includes an open reading frame of 4667 bp encoding a putative polypeptide of 862 amino acids with a calculated molecular mass of 98.0 kDa and a predicted pI of 5.61. Almond seed lipoxygenase shows 71% identity with an *Arabidopsis* LOX1 gene and is closely related to tomato fruit and potato tuber lipoxygenases. The sequence of the active site was consistent with the isolated gene encoding a 9-LOX.

Keywords: almond; hydroperoxides; lipoxygenase; *Prunus dulcis*.

Lipoxygenases (LOX, EC 1.13.11.12) are ubiquitous non-heme iron containing dioxygenases that catalyse the hydroperoxidation of polyunsaturated fatty acids (PUFA) containing a *cis*,*cis*-1,4 pentadiene moiety. Hydroperoxidation products derived from LOX activity are rapidly converted into a number of compounds involved in plant defence, senescence, seed germination, plant growth and development. In particular, some final products of the LOX pathway such as traumatin, jasmonic acid and its methyl ester have been extensively studied for their regulatory functions [1]. PUFA hydroperoxides can be further converted to volatile aldehydes, alcohols and ketones through the action of other enzymes of the lipoxygenase pathway, e.g. hydroperoxide lyases and isomerases [1]. Some of these volatile compounds are thought to induce the synthesis of molecules, such as systemin, that are important in intercellular signaling during plant-pest and pathogen interactions [2].

Linoleic and linolenic acids, the most common substrates of plant LOXs, are important constituents of plant membrane phospholipids. The hydroperoxides derived from LOX activity can therefore influence physical and chemical features of biological membranes. This results in a loss of membrane integrity that is known to accompany several physiological events such as fruit ripening and senescence [3,4]. LOXs are also believed to play a role in lipid metabolism during seed germination [5,6].

LOXs are also of great interest in food science because hydroperoxides and free radicals resulting from LOX activity can have deleterious effects on nutritionally important compounds such as proteins, vitamins and essential PUFAs. LOXs also have a role in the production of volatile molecules that can positively or negatively influence the flavour and aroma of many plant products [7].

Almond is an important temperate crop whose seeds and derivatives are widely used in the agro-food industry. Almond seed accumulates large amounts of unsaturated fatty acids, including linoleic (about 20% of the total fatty acid content) and linolenic acids (about 0.5%) [8,9]. In the light of the abundance of PUFA in almond seed, it is interesting to investigate the role of lipoxygenases in seed metabolism. We have therefore studied the expression of lipoxygenases in almond seed and carried out a biochemical and molecular characterization of a specific seed lipoxygenase.

MATERIALS AND METHODS

Plant material

Almond seeds, *Prunus dulcis* (Mill.), D.A. Webb, cultivar Scorza verde, were harvested at 20, 30, 60, 90, 120 and 150 days after flowering (DAF) from the experimental field belonging to the Facoltà di Agraria, Valenzano (Bari, Italy). Seeds were shelled, frozen in liquid nitrogen and stored at −80 °C.

PCR amplification of almond LOX genomic probes

Two homologous probes (pGEM-LOX4, pGEM-LOX18) were obtained by PCR experiments conducted with

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Abbreviations: DAF, days after flowering; HODE, 10,12-hydroxy octadecadienoic acid; LOX, lipoxygenase; PUFA, polyunsaturated fatty acids; RP-HPLC, reverse-phase HPLC; SP-HPLC, straight-phase HPLC.

Enzymes: lipoxygenase (LOX, EC 1.13.11.12).

Note: the nucleotide sequence reported is in the EMBL database under the accession number AJ404331.

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almond genomic DNA as template, the primers 5′-CTATGATTATGATGTCTACAATGATTTGGG-3′ and 5′-GCAAATTCTTCATCAGTCATCCATGCAGAC-3′, and the following amplification conditions: 94 °C for 5 min (1 cycle); 94 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min (25 cycles) 72 °C for 5 min (1 cycle). The amplified fragments were treated with Klenow enzyme and cloned in the *Sma*I site of pGEM 3Zf (–) plasmid vector (Promega).

Construction and screening of an almond genomic library

Genomic DNA was extracted from young seedlings as follows. Five grams of tissue were ground to a powder in liquid nitrogen, transferred to a tube containing 25 mL of extraction buffer (0.1 M Tris/HCl, pH 8; 50 mM EDTA; 0.5 M NaCl; 0.2% 2-mercaptoethanol; 1% polyvinylpolypyrrolidone 40 000; 2% SDS) and heated at 65 °C for 20 min. After the addition of potassium acetate to a final concentration of 1.7 M, the sample was transferred on ice (5 min) and centrifuged at 15 000 ***g*** for 15 min. DNA was precipitated with 2-propanol, resuspended and partially digested with *Eco*RI. After *Eco*RI digestion, high molecular weight DNA was fractionated on a sucrose gradient and used to prepare a genomic library in lambda EMBL 4 (Stratagene) according to the manufacturer's instructions. More than 2×10^5 phages were transferred to Hybond N nylon membrane (Amersham) according to [10]. Prehybridization of the membranes was carried out at 65 °C in 3 × NaCl/Cit, 0.1× Denhardt's solution, 0.5% SDS for 2 h. Hybridization was carried out overnight under the same conditions after the addition of a [α-^{32}P]dCTP labelled pGEM-LOX4 probe, using a random prime oligonucleotide kit (Pharmacia). Filters were washed twice for 30 min in 2 × NaCl/Cit, 0.5% SDS at 65 °C and the stringency gradually increased to 0.1 × NaCl/Cit, 0.5% SDS, 65 °C for 30 min. Positive plaques were picked and further subjected to two additional rounds of screening under the same conditions. Fifteen positive plaques were isolated using the pGEM-LOX4 probe. Nucleotide sequences of genomic clones were determined on subcloned *Eco*RI fragments in pBluescript SK$^+$ (Stratagene). Automated dideoxy sequencing was performed by primer extension analyses on an ABI 377 sequencer (PE Applied Biosystems), using the sequencing facility of the Laboratorio Genoma Vegetale, ENEA, Rome.

RNA extraction and northern blot analysis

Total RNA was extracted from almond seeds at different developmental stages as follows. Seeds were ground to a powder in liquid nitrogen, transferred to a tube containing one volume of extraction buffer (0.1 M Tris/HCl, pH 8; 10 mM EDTA; 0.1 M LiCl; 1% SDS) preheated at 80 °C. After the addition of one volume of Tris-saturated phenol (preheated at 80 °C) and chloroform : isoamyl alcohol (24 : 1) samples were mixed and centrifuged. The aqueous phase was precipitated overnight on ice with one volume of 4 M LiCl. After centrifugation, the pellet was resuspended in water and precipitated with 0.3 M sodium acetate pH 5.2 and 2.5 vol. of absolute ethanol. RNA was resuspended and precipitated twice with 2 M NaCl and two volumes of absolute ethanol.

Five micrograms of total RNA was fractionated on a 1.2% formaldehyde gel according to [10] and transferred to Hybond N membrane. Membranes were baked at 80 °C for 2h and hybridized as described by [11] with [α-^{32}P]dCTP pGEM-LOX4 and pGEM-LOX18 probes labelled as described above. Filters were washed in 0.1 × NaCl/Cit, 0.1% SDS at 65 °C and exposed to preflashed X-ray film at −70 °C.

Protein extraction and lipoxygenase activity

Lipid bodies were isolated using the method described by [12]. After the removal of lipid bodies, crude cytosol was centrifuged at 100 000 ***g*** for 30 min and the supernatant (soluble protein fraction) was separated from the microsomal pellet. Microsomes were resuspended in 100 mM sodium carbonate and the pH adjusted to 7.5 before determining LOX activity.

LOX activity was determined polarographically at 25 °C with a Clark O_2 electrode using linoleic acid as substrate. 30 mM linoleic stock solution was prepared according to [13]. The reaction mixture (final volume 1.0 mL) contained 0.3 mM linoleic acid in 100 mM sodium phosphate buffer, pH 6. For pH optimum determination 100 mM sodium acetate was used for pH range of 4–5.5, 100 mM sodium phosphate buffer for pH range of 6–7.5 and 100 mM sodium borate for pH range of 8–9.5. The reaction mixture was preincubated for two minutes before the addition of enzyme solution (0.6 mL). Decrease in O_2 concentration was followed for three minutes and the enzyme activity was calculated from the initial rate of O_2 uptake. One unit of LOX activity corresponds to the uptake of 1 μmol of O_2 per min.

SDS/PAGE and western blot analysis

Total protein (10 μg) extracted from seeds harvested at different developmental stages were subjected to SDS/PAGE and transferred to nitrocellulose membrane (Amersham). Western blot analysis was performed using the ECL protocol (Amersham) and a 1 : 5000 dilution of the pea LOX antibody [14].

Identification of lipoxygenase products

Two hundred micrograms of proteins from soluble and microsomal fractions were incubated for 1 h in 1 mL of 0.1 M sodium phosphate buffer pH 6 containing 0.3 mM linoleic acid. Reaction products were reduced with sodium borohydride, extracted with chloroform/methanol (2 : 1, v/v), dried, resuspended in methanol : water : acetic acid (85 : 15 : 0.1) and separated by reverse-phase-HPLC (RP-HPLC) as described by [6]. RP-HPLC analysis was carried out using a Beckman System Gold apparatus equipped with a 126 solvent module and a 168 detector. RP-HPLC of hydroxy-10,12-octadecadienoic acid (HODE) was performed with a C_{18} Ultrasphere column (Beckman, 0.46 × 25 cm) with a solvent system of methanol : water : acetic acid (85 : 15 : 0.1) at a flow rate of 1 mL·min^{-1}. The absorbances at 234 nm and 210 nm were recorded simultaneously. Analysis of HODE isomers was carried out with a Silica Ultrasphere column (Beckman, 0.46 × 25 cm) equilibrated with *n*-hexane/2-propanol/acetic acid

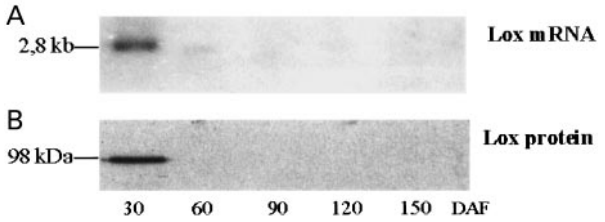

Fig. 1. Lipoxygenase expression during seed development. Relative amounts of lipoxygenase mRNA and protein in developing almond seeds. (A) 5 μg of total RNA was separated on a 1.2% formaldehyde-agarose gel, transferred to nylon membranes, hybridized with [α-^{32}P]dCTP pGEM-LOX4 and washed at high stringency (0.1 × NaCl/Cit, 0.1% SDS at 65 °C). (B) 20 μg of total protein separated on SDS/PAGE, transferred to nitrocellulose and LOX detected with pea-LOX antibodies. Numbers indicate developmental stage as days after flowering (DAF).

(100 : 2 : 0.1) at a flow rate of 1 mL·min^{-1}. Authentic standards of 9- and 13-HODE were purchased from ICN.

RESULTS

Isolation of homologous probes

In order to isolate almond lipoxygenase sequences to use as homologous probes, we prepared two primers, previously described by [15], that border the 937-bp conserved region between nucleotides 2103 and 3039 of the soybean LOX3 gene [16]. These primers were used in PCR experiments with almond genomic DNA as template. After amplification, two fragments of about 750 bp and 1200 bp were obtained, but only the 750-bp fragment hybridized with a potato LOX probe [17]. This fragment was cloned into pGEM-3Zf(−). Sequence analysis of plasmid DNA obtained from several transformants revealed the presence of two different inserts of 796 bp (pGEM-LOX4) and 719 bp (pGEM-LOX18) which shared an identity of 67%. pGEM-LOX4 showed the highest identity (73%) to a potato leaf LOX [18], whereas pGEM-LOX18 was 63% identical

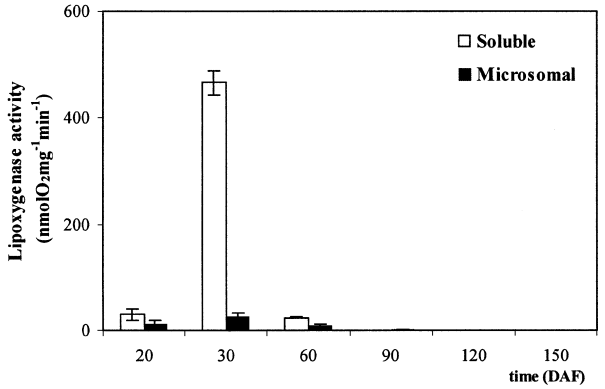

Fig. 2. Lipoxygenase activity during seed development. Changes in lipoxygenase activity in developing almond seeds harvested at different days after flowering (DAF). Enzyme activity was measured by polarography at pH 6 in the soluble and microsomal fractions. The mean and standard deviations of at least four independent experiments are shown.

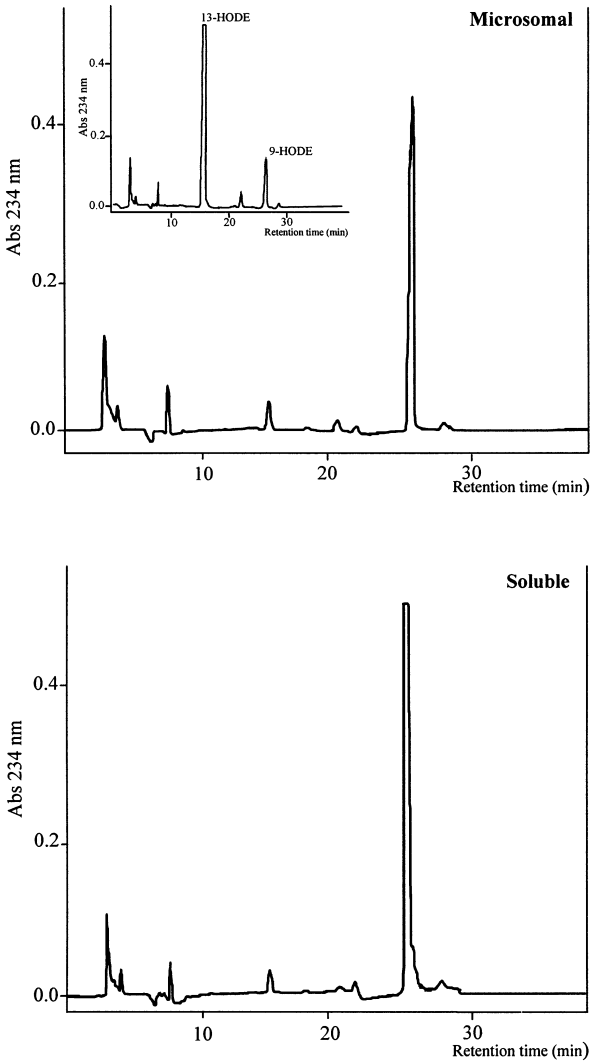

Fig. 3. HPLC analysis of hydroxy fatty acids produced by soluble and microsomal LOX from almond seeds. 200 μg of proteins from soluble and microsomal fractions were incubated for 1 h in 1 mL of 0.1 M sodium phosphate buffer pH 6 containing 0.3 mM linoleic acid. After reduction with sodium borohydride, reaction products were extracted with chloroform/methanol (2 : 1, v/v), dried, resuspended in methanol : water : acetic acid (85 : 15 : 0.1) and separated by RP-HPLC as described in Materials and methods. The peak adsorbing at 234 nm was collected and further analyzed by SP-HPLC to separate hydroxy isomers of linoleic acid. Inset shows the retention time of the isomers produced by soybean LOX1 compared with authentic standards of 9- and 13-hydroxy linoleic acid.

to *A. thaliana* lipoxygenase 1 gene [19]. These preliminary results indicated that pGEM-LOX4 and pGEM-LOX18 were partial sequences derived from two different lipoxygenase genes.

Lipoxygenase gene expression during seed development

Northern analysis of almond seed showed that RNA corresponding to pGEM-LOX4 was maximal at 30 DAF and declined rapidly during seed development; in seeds harvested at 60 DAF only a faint signal was observed and

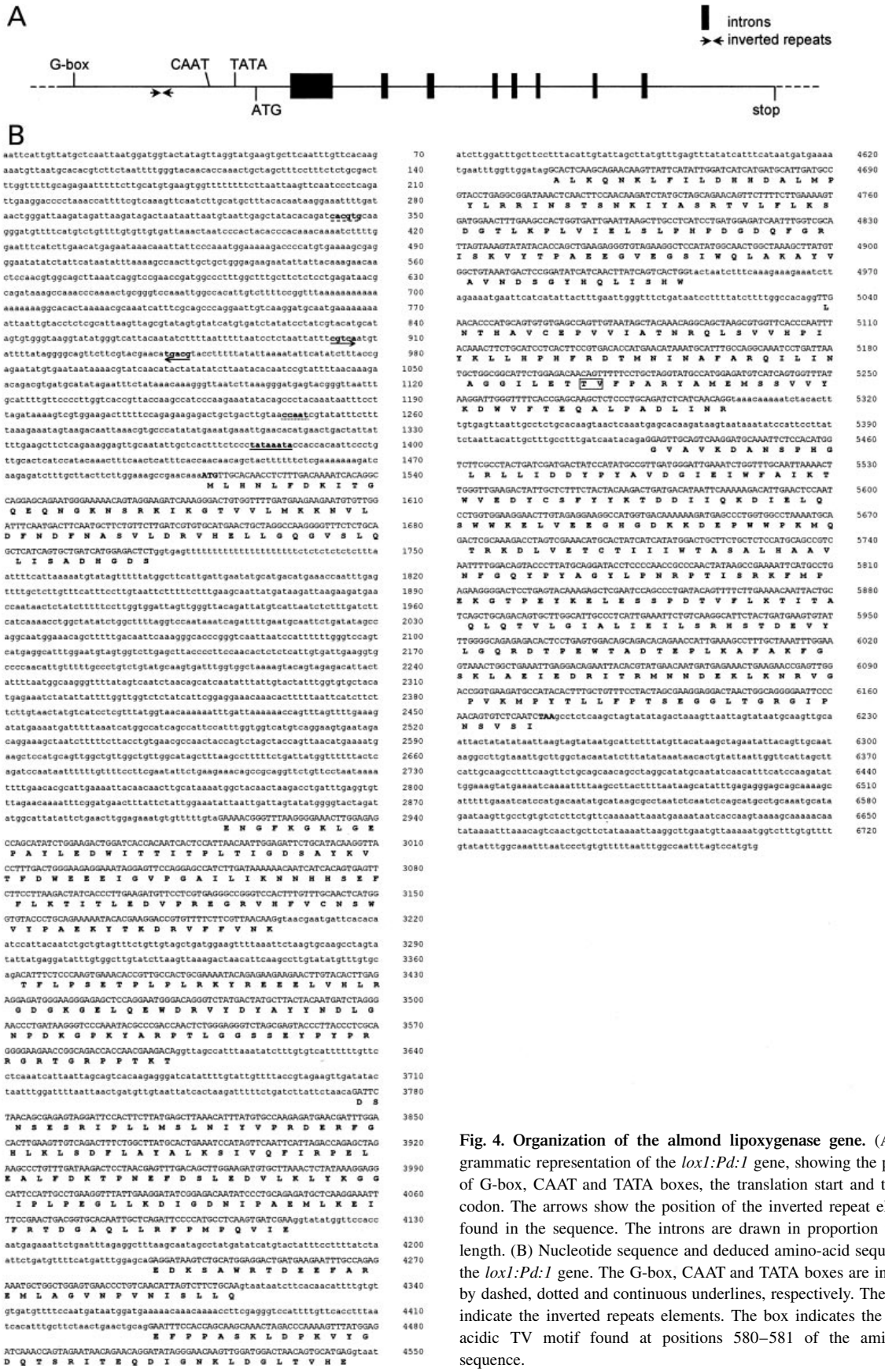

Fig. 4. Organization of the almond lipoxygenase gene. (A) Diagrammatic representation of the *lox1:Pd:1* gene, showing the position of G-box, CAAT and TATA boxes, the translation start and the stop codon. The arrows show the position of the inverted repeat elements found in the sequence. The introns are drawn in proportion to their length. (B) Nucleotide sequence and deduced amino-acid sequence of the *lox1:Pd:1* gene. The G-box, CAAT and TATA boxes are indicated by dashed, dotted and continuous underlines, respectively. The arrows indicate the inverted repeats elements. The box indicates the amino-acidic TV motif found at positions 580–581 of the amino-acid sequence.

no hybridization was obtained in RNA samples from later developmental stages (Fig. 1A). No hybridization was observed in seed samples when pGEM-LOX18 probe was used (data not shown). These results suggest that the two amplified probes correspond to two different lipoxygenase genes that display different expression patterns.

pGEM-LOX4 and pGEM-LOX18 were also used as probes in northern experiments with RNA extracted from roots, leaves and cotyledons obtained from young almond seedlings (14–18 days after germination). A hybridization signal of the expected molecular weight for LOX RNA was observed in roots with both pGEM-LOX4 and pGEM-LOX18 probes; a faint signal was also detectable in cotyledon samples (data not shown).

Lipoxygenase accumulation and enzyme activity in developing seeds

We used pea LOX antibodies [14] to investigate the presence, in almond seeds, of polypeptides in the molecular weight range expected for lipoxygenases. Using these antibodies a polypeptide of about 98 kDa was recognized in total protein extracts obtained from 30 DAF seeds (Fig. 1B). A faint signal was observed at 60 DAF after a prolonged exposure and no reaction was detected in later developmental stages.

We also assayed LOX activity in microsomal membranes and the soluble protein fraction from seeds. In order to reduce the influence of the endogenous lipid fraction on lipoxygenase activity, we removed the lipid bodies that abundantly accumulate in almond seeds from about 75 DAF. The enzymatic activity was initially analyzed at different pH values (pH 5–9) and the highest activity was obtained at pH 6 for both samples (data not shown). Under these experimental conditions it was possible to detect LOX activity both in microsomal and soluble fractions, although the soluble fraction retained most of the activity (Fig. 2). Lox activity was detected in all the developmental stages analyzed and was maximal in seeds at 30 DAF. After this stage it declined throughout seed development. A small amount of LOX activity was also detected in the lipid bodies (data not shown), but it is unclear if it derives from the activity of a different LOX isoform synthesized late in development and targeted to this subcellular compartment, or if the same lipoxygenase found in the soluble fraction and microsomes is able to associate with lipid body membranes.

In order to better characterize the microsomal and soluble LOX, we analyzed the reaction products obtained using linoleic acid as substrate and microsomal and soluble fractions purified from seeds at 30 DAF as sources of LOX. After reduction with sodium borohydride, reaction products were first separated by RP-HPLC and the peak adsorbing at 234 nm collected and further analyzed by SP-HPLC to separate HODE isomers. Figure 3 shows that 9-HODE is the main product of almond lipoxygenase as deduced by the elution profiles of soybean lipoxygenase type I (Fig. 3, inset) and authentic standards of 9- and 13-HODE. The same elution profile was observed with samples from seeds at 60 and 90 DAF, although the peak areas were reduced as a consequence of the lower LOX activity in these samples (data not shown). From these results we can hypothesize that the lipoxygenase activity detected in developing almond seeds derives from a 9-lipoxygenase.

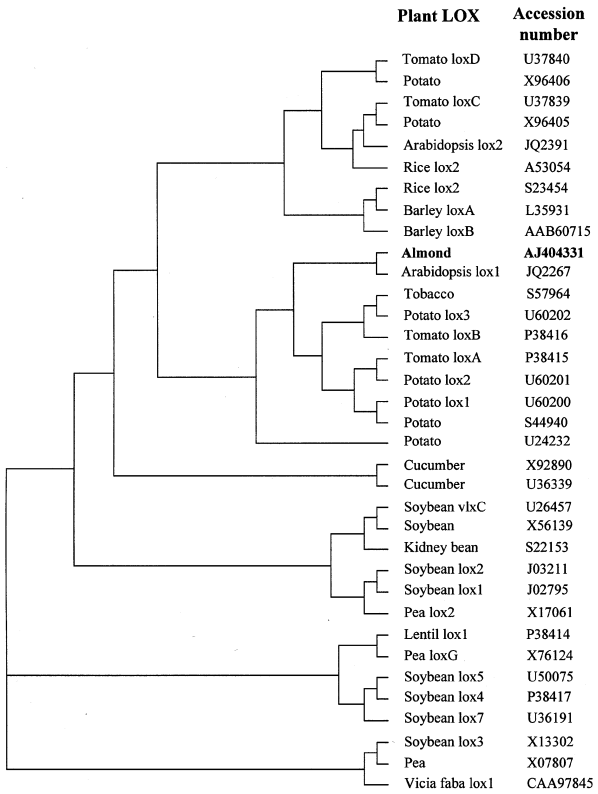

Fig. 5. Evolutionary relatedness of *lox1:Pd:1* gene. Comparison of the almond LOX sequence with the deduced amino-acid sequences of different plant LOXs. The phylogenetic tree was compiled using PHYLIP (Phylogeny Inference Package, version 3.5c) program.

Organization of an almond lipoxygenase gene

A genomic DNA library was constructed in lambda EMBL 4 and screened with pGEM-LOX4. Six out of 15 positive clones were further analyzed by subcloning and sequencing, providing a complete lipoxygenase gene sequence. Sequence comparison revealed that the pGEM-LOX4 probe exactly matches the almond LOX gene from base 3469 to base 4265.

The 6776-bp sequence consists of a 1508-bp 5′ untranslated sequence, an open reading frame contained in a 4667-bp and 598-bp 3′ untranslated sequence. The open reading frame encodes a putative polypeptide of 862 amino acids with a molecular mass of 98.0 kDa and a predicted pI of 5.61. The almond LOX gene comprises nine exons separated by eight introns (Fig. 4), containing consensus donor and acceptor splice sites. Several highly conserved regions are also detectable, among them two domains thought to be important for substrate (amino acids 360–376) and oxygen (amino acids 709–722) binding [1,20]. Moreover, the five conserved amino-acid residues essential for active site iron atom binding (His523, His528, His714, Asn718 and Ile862) are present [21]. Another highly conserved region comprises the eight amino acids GIPNSVSI at the C-terminus [1].

The 5′ untranslated region contains a TATA box (TATAAATA) located at −131 bp from the ATG translation start codon. We also found a CCAAT consensus sequence at −144 bp upstream of the TATA box. A G-box (CACGTG) was identified at −1030 bp. The presence of G-box sequences has been associated with genes regulated by abscissic acid and methyl jasmonate [22] and it is well known that some lipoxygenases are inducible by jasmonic acid and its methyl ester. We also observed the presence of the TGACG element located at −436 bp upstream of the TATA box and its inverted repeat (at −475 bp). This motif was showed to confer methyl jasmonate responsiveness in a barley LOX1 promoter [23].

Almond LOX shows various degree of identity with other plant LOX. The highest identity (71%), at the amino-acid level, was found with *Arabidopsis* LOX1 [19]. The dendrogram shown in Fig. 5 confirms that almond LOX is closely related to that from *Arabidopsis* and that both are grouped in the same cluster with tomato fruit, tobacco leaf and potato tuber LOX [3,25,26].

The importance of a conserved threonine/valine (TV) motif in plant 9-LOXs for determining product specificity has recently been demonstrated [27]. The almond LOX also contains this motif in the expected position (580–581), in agreement with the observed enzymatic activity.

DISCUSSION

Almond is an important crop species belonging to the genus *Prunus* which is known to comprise species characterized by a small genome size [28] although, to our knowledge, the exact size of almond genome has not yet been reported. The small genome size renders this species particularly attractive for gene isolation for molecular studies. The low complexity of the almond genome already reported for the storage protein genes [29] could help to clarify the role of LOX in this species.

The almond LOX characterized in this work appears to be closely related to *Arabidopsis* (*Crucifereae*), tomato, tobacco and potato lipoxygenases (*Solanaceae*) [3,19,25,26], and seems to be more distant from legume LOXs [16,30–33] even though the protein cross-reacted with antibodies against pea seed lipoxygenase. To our knowledge this is the first LOX gene so far reported in the *Rosaceae*, which comprises many fruit crops, and it could be interesting to verify if other LOXs of the same family show the same phylogenetic relationships.

The almond LOX gene is expressed only in early stages of seed development, with maximum accumulation of LOX (mRNA and protein) at 30 DAF, when cotyledons are not yet developed. The same gene is also weakly expressed in other almond tissues (roots and postgerminative cotyledons). Our results indicated that at least one other LOX gene, showing a different expression pattern, is present in the almond genome. Such multiplicity and differential regulation of LOX isoforms is common in other plant species and presumably reflects the various biological roles of these enzymes. Although genetic 'knockouts' of specific LOXs have indicated roles for the enzyme in wound-induced jasmonate synthesis [34] and resistance to pathogen invasion [35], there is no indication of the physiological role for the LOX isoforms identified in seeds. In soybean, seed LOXs seem to be superfluous in the sense that they can be genetically removed without adverse effects on crop performance [36].

In some cases, including the almond LOX described here, gene expression and/or enzyme activity seem to be associated with mitotic activity in young developing tissues. In pea seeds, a LOX translation product has been described at very early stages of cotyledon development [14], when growth is almost exclusively mitotic. Similarly, a particular LOX (L2) has been reported to be expressed in young maize embryos [37]. In nitrogen-fixing nodules, several different LOX genes are active, but one LOX in particular is associated with the region of meristematic activity [38]. Despite these clear associations, the role played by LOX in young dividing tissues is not obvious.

The almond LOX has no N-terminal transit peptide and a low level of identity to those LOXs known to be targeted to chloroplasts. Thus, according to the standard nomenclature proposed by the Commission on Plant Gene and Nomenclature [39] is a LOX1 and it should be named *Lox1:Pd:1*. It produces almost entirely 9-hydroperoxides when linoleic acid is used as substrate and does not seem to be able to produce any carbonyl compounds as secondary products of PUFA hydroperoxidation (data not shown). The determinants of LOX positional specificity (the position on the PUFA at which oxygen is inserted) have recently been demonstrated, at least in a cucumber lipid body LOX, to be a consequence of the nature of specific amino acids at the base of the PUFA binding pocket, in the positions equivalent to 580/581 in the almond seed LOX [27]. A large residue (H) at the position equivalent to 581 results in 13-LOX activity, but a small residue (V) allows access of the substrate in inverted orientation, thus producing 9-hydroperoxides. From the sequence of the almond LOX at these positions (TV), 9-LOX activity would be predicted, which is consistent with the observed enzyme activity in early seed development.

Although the majority of 9-LOX activity and gene expression was at early stages of seed development, before lipid body deposition begins, there also appeared to be a small amount of LOX activity associated with the lipid body fraction late in development. Further studies, using antibodies that are specific to the almond LOX, should clarify the exact subcellular location of the LOX in relation to lipid body deposition. Production of recombinant almond seed LOX *in vitro,* from a cDNA, can both act as a means of producing such an antibody and also afford the opportunity to examine the possible targeting of the enzyme to lipid bodies.

LOX activity in fruits, seeds and vegetables is significant because of the influence of the reaction products on food quality [7,8]. Carbonyl compounds (keto fatty acids) are associated with the production of free radicals and the destruction of antioxidants, including pigments. Not all LOXs carry out this reaction and we could see no evidence for the formation of keto fatty acids by almond seed LOX. Hydroperoxides resulting from LOX action can be further metabolized by hydroperoxide lyases into organoleptically active aldehydes with positive and negative attributes [7]. The products of 9-hydroperoxides have aromas that are characteristic of melons and cucumbers and such aromas are readily detected in developing almond seeds; it is highly likely that they are a consequence of LOX activity.

Modulation of LOX genes, either genetically or environmentally, could therefore have significant implications for almond seed quality.

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