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Physcomitrella patens has lipoxygenases for both eicosanoid and octadecanoid pathways

Aldwin Anterola^{a,*}, Cornelia Göbel^b, Ellen Hornung^b, George Sellhorn^c, Ivo Feussner^b, Howard Grimes^c

^a Southern Illinois University, Carbondale, IL 62901-6509, USA

^b University of Göttingen, D-37077 Göttingen, Germany

^c Washington State University, Pullman, WA 99164-4234, USA

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ABSTRACT

Mosses have substantial amounts of long chain C20 polyunsaturated fatty acids, such as arachidonic and eicosapentaenoic acid, in addition to the shorter chain C18 α -linolenic and linoleic acids, which are typical substrates of lipoxygenases in flowering plants. To identify the fatty acid substrates used by moss lipoxygenases, eight lipoxygenase genes from *Physcomitrella patens* were heterologously expressed in *Escherichia coli*, and then analyzed for lipoxygenase activity using linoleic, α -linolenic and arachidonic acids as substrates. Among the eight moss lipoxygenases, only seven were found to be enzymatically active *in vitro*, two of which selectively used arachidonic acid as the substrate, while the other five preferred α -linolenic acid. Based on enzyme assays using a Clark-type oxygen electrode, all of the active lipoxygenases had an optimum pH at 7.0, except for one with highest activity at pH 5.0. HPLC analyses indicated that the two arachidonic acid lipoxygenases form (12*S*)-hydroperoxy eicosatetraenoic acid as the main product, while the other five lipoxygenases produce mainly (13*S*)-hydroperoxy octadecatrienoic acid from α -linolenic acid. These results suggest that mosses may have both C20 and C18 based oxylipin pathways.

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1. Introduction

Lipoxygenases are non-heme iron containing dioxygenases involved in generation of lipid-derived signaling molecules in eukaryotes. They catalyze the addition of molecular oxygen to fatty acids containing a (1*Z*,4*Z*)-pentadiene functionality (e.g., arachidonic **1** and α -linolenic **2** acids), generating (1*S*,2*E*,4*Z*)-hydroperoxy fatty acids, which in turn serve as precursors to a variety of biologically active metabolites. Initially described as a lipoxidase in soybean (Balls et al., 1943), this class of enzyme decades later gained considerable attention in the biomedical community because of its involvement in the inflammatory response (Borgeat et al., 1976). Indeed, several inflammatory signaling molecules such as hydroxy eicosatetraenoic acids (HETEs), leukotrienes, lipoxins and hepoxilins (Brenner and Krakauer, 2003) are formed from arachidonic acid (**1**) via the action of different types (isoforms) of lipoxygenases in mammals. Formation of these eicosanoids is intriguingly similar to the formation of plant oxylipins from α -linolenic (**2**) and linoleic (**3**) acids, suggesting a possible generalized role of fatty acid oxygenation in defense signaling (see Schemes 1 and 2 for chemical structures).

In flowering plants, linoleic (3) and α -linolenic (2) acids are the most abundant fatty acids in lipid membranes and presumably serve as the major substrates of lipoxygenases, which insert oxygen either at the 9- or 13-positions of linole(n)ic acid, forming 9- and 13-hydroperoxy linole(n)ic acids, respectively. The 9-hydroperoxides formed, namely 9-hydroperoxy octadecadienoic acid (9-HPODE, 4) and 9hydroperoxy octadecatrienoic acid (9-HPOTE, 5), are precursors of a number of oxylipins, including: C9 aldehydes and oxoacids (found in fruit flavors and scents) formed via the action of hydroperoxide lyase (Mita et al., 2007); ketols and 10-oxo-11,15-phytodienoic acid derived from 9,10-epoxy octadecatrienoic acid produced by a 9-specific allene oxide synthase (Hamberg, 2000; Itoh et al., 2002; Stumpe et al., 2006); and colnele(n)ic acid generated by divinyl ether synthase (Itoh and Howe, 2001; Fammartino et al., 2007; Stumpe et al., 2001). 13-Hydroperoxy octadecatrienoic acid (13-HPOTE, 6) formed from α -linolenic acid (2) is similarly converted to another set of oxylipins. The latter includes C6 aldehyde hexenal and traumatin formed by hydroperoxide lyase (Matsui, 2006), etherolenic acid formed by divinyl ether synthase (Stumpe et al., 2008), and jasmonic





Abbreviations: H(P)ETE, hydro(pero)xy eicosaetraenoic acid; 13-H(P)ODE, (13 S,9Z,11E)-13-hydro(pero)xy-9,11-octadecadienoic acid; 9-H(P)ODE, (9S,10E,12Z)-9hydro(pero)xy-10,12-octadecadienoic acid; 13-H(P)OTE, (13S,9Z,11E,15Z)-13hydro(pero)xy-9,11,15-octadecatrienoic acid; 9-H(P)OTE, (9S,10E,12Z,15Z)-9hydro(pero)xy-10,12,15-octadecatrienoic acid; KODE, keto octadecadienoic acid; KOTE, keto octadecatrienoic acid; KETE, keto eicosatetraenoic acid (a.k.a. keto arachidonic acid).

^{*} Corresponding author. Tel.: +1 618 453 3222; fax: +1 618 453 3441. *E-mail address*: anterola@siu.edu (A. Anterola).





Scheme 2. Chemical structures of selected oxylipins.

acid formed via a series of steps initiated by allene oxide synthase (Wasternack, 2007). 9- and 13-Hydroperoxides are also converted by peroxygenase (Blee, 1998) and epoxy alcohol synthase (Hamberg, 1999) to yield different epoxy alcohols.

Oxylipins have been implicated in defense and signaling (Blee, 2002; Rosahl and Feussner, 2005), although lipoxygenases themselves have also been proposed to be involved in seed germination (via lipid mobilization with lipoxygenases acting on triglycerides) (Feussner et al., 2001) and in nitrogen storage (with lipoxygenases serving as vegetative storage proteins) (Fuller et al., 2001). Interestingly, although flowering plants do not contain polyunsaturated fatty acids such as arachidonic (1) and eicosapentaenoic acids, lipoxygenases (e.g., from soybean, barley, and potato) have been found to use arachidonic acid (1) as substrate (Vörös et al., 1998; Shimizu et al., 1984; Cook and Lands, 1975; Bild et al., 1977, 1978). It has been hypothesized that arachidonic (1) and eicosapentaenoic acid from oomycete (such as *Phytophtora infestans*) are utilized by plant lipoxygenases during pathogen attack to form defense-related compounds (Schewe and Nigam, 1997).

Unlike flowering plants, algae and mosses may have substantial levels of arachidonic (1) and/or eicosapentaenoic acids, which can serve as substrates for their endogenous lipoxygenases. For example, the red algae *Rhodymenia pertussa* (Jiang et al., 2000), *Polyneura latissima* (Jiang and Gerwick, 1997), and *Gracilariopsis lemaneifor*- mis (Gerwick et al., 1991) have been reported to have oxylipins and/or enzyme activities that suggest the existence of arachidonate/eicosapentaenoate 5-, 9- and 12-lipoxygenases, whereas Chondrus crispus (another red alga) uses both arachidonic (1) and linole(n)ic (2, 3) acids to produce predominantly 12-hydroperoxy eicosatetraenoic acid (12-HPETE, 7) and 13-HPOTE (6)/13-HPODE (8), respectively (Bouarab et al., 2004). Brown algae from the Laminaria genus also use both C20 and C18 fatty acids, but apparently only those belonging to the ω 6-group such as arachidonic (1) and linoleic (3) acids, to form 15-HPETE (9) and 13-HPODE (8), respectively (Gerwick, 1994). In the green algae Chlorella pyrenoidosa (Nunez et al., 2002), a lipoxygenase active against linoleic acid (3) has been described, which produces roughly equal amounts of 9- and 13-HPODE (4 and 8). Hence both the eicosanoid and octadecanoid pathways have been apparently found in diverse forms of algae. Cvanobacteria have also been shown to harbor 13-lipoxygenases in the case of *Nostoc* sp. (Lang and Feussner, 2007; Lang et al., 2008), and 9/13-lipoxygenases in the case of Oscillatoria sp. (Beneytout et al., 1989).

In mosses, only a single lipoxygenase, PpLOX1 from P. patens, has so far been identified and characterized (Senger et al., 2005). It is mainly an arachidonate 12-lipoxygenase (producing 12-HPETE, 7) with 13-lipoxygenase activity against linole(n)ic acid (2, 3). Although the observed activity of this single enzyme accounted for oxylipins previously detected in P. patens (Wichard et al., 2004), this does not preclude the existence of other lipoxygenase isoforms in this moss. Indeed, in most plant species, lipoxygenases typically exist as members of multi-gene families (reviewed in Feussner and Wasternack, 2002), whose encoded proteins may have different biochemical properties. Soybean, for example, has eight isoforms known, of which three are expressed in seeds (Axelrod et al., 1981) and five are in vegetative tissues (e.g., leaves) (Fuller et al., 2001). These isoforms differ in terms of their substrate specificities, pH optima and the relative proportion of products formed (Fuller et al., 2001). Added to the fact that lipoxygenases have been found in various subcellular locations including the mitochondria (Braidot et al., 2004), cytosol, vacuole, chloroplast and the plasma membrane (reviewed in Feussner and Wasternack, 2002), such differences may reflect the variety of physiological functions lipoxygenases perform in plants, most of which are not yet fully understood.

Mosses are especially interesting to study because they are phylogenetically distinct from seed plants, and are hypothesized to have evolved separately from algal predecessors (Graham et al., 2000). While extant marine algae are known to have polyunsaturated fatty acids and even produce mammalian-like eicosanoids (Gerwick and Bernart, 1993), flowering plants appear to have lost this metabolic capacity in the course of evolution. Mosses, along with other non-flowering plants, have retained the ability to form polyunsaturated fatty acids, but it is still not clear what types of lipid signaling pathways these earlier land plants have, e.g., what fatty acids are used to form lipoxygenase-derived oxylipins. In this contribution, we report the substrate preferences, product and pH profiles of seven heterologously expressed lipoxygenases in *P. patens*, which suggest that both C20 and C18 fatty acid based signaling pathways may be present in this moss.

2. Results

2.1. Identification and sequence analysis of **Physcomitrella** lipoxygenase genes

Twelve Physcomitrella putative transcripts (i.e., expressed sequence tags or ESTs) in Physcobase (http://moss.nibb.ac.jp/), a DNA database maintained by the National Institute of Basic Biology (NIBB) in Okazaki, Japan (Nishiyama et al., 2003), were found to be highly similar (>40%) to sovbean lipoxygenases via a BLAST search using soybean lipoxygenase-1 as a query sequence. EST clones that had the longest 5' region were identified from the database, and then sequenced to obtain full-length nucleotide information, which indicated that only eight of them (pphn43n22, pphb33n05, pphb15j12, pphb13L22, pphb14h18, pphn9o24, pphn50o06, and pph20m14) encoded ~100 kDa lipoxygenases. The other four cDNAs are most likely "processed pseudogenes" which though transcribed into mRNAs are not translated into functional proteins (Vanin, 1985). Indeed, these pseudogenes contain only part of the lipoxygenase sequence which would have predicted translational products of only 30-65 kDa, whereas all known naturally occurring lipoxygenases from flowering plants are ~100 kDa.

Among the remaining eight putative LOX genes, one of them (pph20m14) did not yield an active LOX protein under the expression and assay conditions used. Hence, only seven had been functionally proven to be active LOXs in vitro (Table 1), with one of them (pphb33n05) being essentially identical (99.6% at the amino acid level) to the previously reported *Physcomitrella* lipoxygenase PpLOX1 (Senger et al., 2005). Their 5' and 3' untranslated regions were nearly identical, except for the longer cDNA sequence of pphb33no05. The latter EST also had an additional codon (GAG) in position 1378 of the coding region of the cDNA, which added a glutamate in the translated protein. The other two differences in the coding region involved adenine to guanine base changes in positions 658 and 2496 in the pphb33n05 sequence, which changed a glutamate to a glycine in the N-terminal region and a lysine to a glutamate in the C-terminal region of the protein, respectively. Hence, this EST represents either a more complete sequence of the same gene than originally reported, a splice variant, or a distinct gene altogether. To distinguish this EST from the original sequence, while at the same recognizing that they may be the same gene, this EST is thus referred to in this paper as PpLOX1b.

Following the earlier nomenclature, the six other active LOXs were named PpLOX2 (pphn43n22), PpLOX3 (pphb15j12), PpLOX4 (pphb13L22), PpLOX5 (pphb14h18), PpLOX6 (pphn9o24) and PpLOX7 (pphn50o06). The seven PpLOXs encode 105, 105, 103, 104, 108, 106 and 109 kDa proteins, respectively. The last EST

Table 1

Structural and functional characteristics	of lipoxygenase genes	in Physcomitrella	patens
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Name	Accession no.	5'-UTR (bases)	3'-UTR (bases)	Coding region (bases)	Predicted protein size (kDa)	Preferred fatty acid substrate	Activity on preferred substrate
PpLOX1b	ABF66647	479	259	2817	105	Arachidonate	(12S)-LOX
PpLOX2	ABF66648	174	229	2817	105	Arachidonate	(12S)-LOX
PpLOX3	ABF66649	245	359	2763	103	α-Linolenate	(13S)-LOX
PpLOX4	ABF66650	397	218	2763	104	α-Linolenate	(13S)-LOX
PpLOX5	ABF66651	216	252	2856	108	α-Linolenate	(13S)-LOX
PpLOX6	ABF66652	217	294	2871	106	α-Linolenate	(13S)-LOX
PpLOX7	ABF66653	199	138	2901	109	α-Linolenate	(13S)-LOX

(pph20m14) had a calculated protein molecular weight of 94 kDa. The putative 94 kDa protein had only 828 aa compared to at least 920 in other PpLOXs, with less amino acids in the N-terminal. While the C-terminal appears to be the same length as the other PpLOXs, the C-terminal isoleucine residue found conserved in other plant LOXs is missing (Fig. 1).

Among the active PpLOX genes, PpLOX1b and 2 are most closely related to each other, being 96.1% identical at the amino acid level.

PpLOX1b PpLOX2 PpLOX3 PpLOX4 PpLOX5 PpLOX6 PpLOX7 pph20m14	1 1 1 1 1 1 1	MMLNRLETGSLASAFRGSREEFVVSRGRSPGRSQ
PpLOX1b PpLOX2 PpLOX3 PpLOX4 PpLOX5 PpLOX6 PpLOX7 pph20m14	68 68 77 81 102 90 81 62	KTATSTSTRGPSGDNVQYTGVATTMKKLKVLDLIDVADICIDTSIVGGKRWTVCLVSKDVDPKTGESMKSEVIFPNAGLEGPAASLIDIVLEF KTATTSTGGSSGDNVQVTATATIMKLKLLDIDIRVADICIDTSIVGGKRWTVCLVSKDVDPKTGESMKSSEVIFPNAGLEGPAASNIDIVLEF LFGGKQGSKDPN-LMCIKTSVTLVRSNFRGLDVGNVFSDIILGOSIIG-NOVTLCLVSNDVDPATGSGKLSGKVTFEHATKPRNLLRSTPFEINMF LFDTRPANSH-DVRLQISVVLMRSTFAGLDVGNLLGDIRLGOSIIK-NOVTLCLVSNDVDPATGSGRVSKSVTFEHATKPRNLLIFKLFOINLOC LQSPFVLYEVESSLVDLKATIVLCKKEFFESSVAAEDGKVMILEDISG-KRWYLCLVSNDVDSSTGKAMKTSEMMIEMATESSTSSHILSTYPTKVVNF LFHSVLPTASSNGDFVTLQAVVTVQKKLLVLDLIDLIADSG DVASILG-QHSIMLVSNKLDAKTKVLVSEDVPIKDAMTADMLYPTNFROMEF VRNTNSVRPSFTGLSTVGAVITVKK-KRLSRDQIEEAVUVFSDIG-SNGFCLVSEDAGAG-GAGKRCKEFTIKLWTKKAVQADKVVVTFF KKVQGPSKEG-VGETVTFKGTAVIVKLKLLFDVMDRVADIQCDASSIRG-KKWTFCLVSEDSSTGELVTSEEVTLQGLKIFDSITAEKLSSNVF
PpLOX1b PpLOX2 PpLOX3 PpLOX4 PpLOX5 PpLOX6 PpLOX7 pph20ml4	165 165 172 173 201 186 175 159	TV PK-SEGFEGALL KNAHPNEFL VSFE E HOKSKAH VTNSW YNTGK-TGAR IFECNTAM LEDETEASH KALREQEI IN REDETEE TV PK-SEGVEGALL KNAHPNEFL VSFE E HOKSKAH VTNSW YNTGK-TGAR IFECNTAM LEDETEASH KALREQEI IN REDETEE TV PK-NEGTEGALI FWEGNNLDVPLISTITTEFK TDAH VV POKNVIGE FCNSW FASDMAKDEGLEFAN LVTD TASETAGL KKLANEL BELGEDETEE FV SR-NEGTEGALI HNGHKNLVLPQLN-ITTEFK TKAQ TV PDRVVT LCDSW YASEKDKGGRIFFANKVYTEACTEPGURKLRKDEI KEI GEDETEE RVK-EFGEPGALIFVKFNRNEFL VSFSL FENNVIGE FCNSW YASEKDKGGRIFFANKVYTEACTEPGURKLRKDEI KEI GEDETEE RVK-EFGEPGALIFVKFNR
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PpLOX1b PpLOX2 PpLOX3 PpLOX4 PpLOX5 PpLOX6 PpLOX7 pph20m14	737 737 722 722 750 755 764 722	AA LCBSELMTWOT WEEK HED KKDSH@PELNSKEAU VDI TT IN DS SCHAAVVPEQYD AG MEHHET TR LL EEHGNEK KADFNKNPEEYYTTS AA LCBSELMTWOT WEEK HED KKDSH@PELNSKEAU VDI TT IN DS SCHAAVVPEQYD AG MEHHET TR LL EEHGNEK KADFNKNPEKYYTTS KA KADEBLONOUT ARTK HAD IT - ECOILAD KKDLVOI ITT IN ASCH HAAVVPEQYL AG MEHHET TR LL EEHGNEK KADFNKNPEKYYTTS RA QADEBLONOUT IN KHAD IN - ECOILAD KKDLVOI ITT IN ASCH HAAVVPEQYL AG MENHES TR LL EEGGTP- WDALQLNPEKYNDS GE EERRELOSMUN I BYVH GOKKDAECOPTINGKASH HI ITT VUN ASCH HAAVVPEQYL AG MENHEN TR LL EEGGTP- WEAMQQNEKYLISM SA 2010 IN TO INF KHAD IN - ECOILAD KKDLVOI ITT IN ASCH HAAVVPEQYL AG MENHEN TR LL EEGGTP- WEAMQQNEKYLISM GE EERRELOSMUN I BYVH GOKKDAECOPTINGKASH HI ITT VUN ASCH HAAVVPEQYL AG MENHEN TR LL EEGGTP- WEAMQQNEKYLISM SA 2011 I DANTT I BYVH GOKKDAECOPTING SEL YTI ITT IN ASCH HAAVVPEQYLA GAMENFEST TR FEBERT - WEALKNER YLDS CIL LDAELQOMUT I BYVH GOKKDAECOPTING SEL YTI ITT IN ASCH HAAVVPEQYLA GAMENFEST FEBERT - WEALKDER YLDS SV QCAELONGYN Y BYVH HAKRAAPGN I PMD KKNLVEI I TT IN ASCH HAAVVPEQYD AG FENCECIAR FY BEVTGP - VELLKDPHK MUQT SV QCAELONGYN Y BYVH HAKRAAPGN I PMD KKNLVEI I TT OW PIAN HAR FINGED I AG MEHES TR I EEGGT - WEALAD VYK YL ST
PpLOX1b PpLOX2 PpLOX3 PpLOX4 PpLOX5 PpLOX6 PpLOX7 pph20m14	839 839 822 822 851 857 857 866 821	V SNIDSTTTA, SVYBVISALCPIBE, I GERGNA NNEKVLAA KGEKES NEADAU, RARNA PKL NRGEPVK FYQLLPHSKPCJTSG, DNS I SNIDSTTA, SVYBVISALSPIBE, I GERGNA DNAEALAA KREKER, NEADAU, RARNA PKL NRGEPVK, FYQLLPHSKPCJTSG, DNS I ANAVQSKLN TTIBIJST SNBE I GERGOMA DDERVKAA KKSTR EEISA KSRNK PAN NRLEAVK, FYDLYPKGEG TNKC PNS I ANAVMTELN TTIBIJST SNBE I GERGOMA DDERVKGV KRSKR DEICN GERNA FKN NNN PVK FYDLYPKGEG TNKC PNS I SSETGATIJTTTG SSBE I GORSTP-NN SDEKISAV GROER GEIEL KARNO KNL NNK PVK FYDLYPKGEG TOKC PNS I SSTASIS STIBIATA IDER I GORSTP-NN NDERVLNA AKTOK VNVEN HELHA SSL NRA PVC FYDLYPKGEG TOKC PNS I SSTQASIS STIBILATA IDER I GORSTP-NN NDERVLNA AKTOK VNVEN HELHA SSL NRA PVC FYDLYPKGEG TOKC PNS I SSTQASIS STIBILATA NDER I GORSTP-NN NDERVLNA AKTOK VNVEN HELHA SSL NRA PVC FYDLYPKGEG TOKC PNS I SSTQASIS STIBILATA ANDER I SORSTP-NN NDERVLNA AKTOK VNVEN HELHA SSL NRA PVC FYDLYPKGEG TOKC PNS I SSTDATTTA AVEV AAAAAFKE VER-TP-TM ONEGKAAA GRIDK REVK INN HELAR SSL NRA PVC FYDLYPKGEG TOKC PNS I SSTDTTTA AVEV AAAAFKE VER-TP-TM ONEGAKAAA GRIDK REVK INN HELAR SSL NRA PVC FYDLYPK FYG FYGR PNS I SSTDTTTA AVEV AAAAFKE VER-TP-TM ONEGAKAA GRIDK REVK INN HELAR SFL PRE SSL PAC FYGR PNS I SSTDTTTA AVEV AAAFKE VER-TP-TM ONEGAKAA GRIDK WITN INN HELAR SFL PRE SSL PAC FYGR PNS I SSTDTTTA AVEV AAAFKE VER-TP-TM ONEGAKAA GRIDK VNVEN HELHA SSL PRE FYFE TGE FIGRE PNS I

Fig. 1. Amino acid sequences of active lipoxygenases of *P. patens* (PpLOX1b to 7) aligned using Clustal W. Conserved histidines, asparagines and isoleucines are italicized and marked with arrows.



Fig. 2. Phylogenetic tree of *P. patens* lipoxygenases (PpLOX1 to 7) together with selected lipoxygenases from angiosperms (sequences taken from Feussner and Wasternack, 2002). PpLOX8 (EDQ50470) and PpLOX9 (EDQ67347) were from EMBL. The evolutionary history was inferred using the Neighbor-Joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

In the ESTs we sequenced, PpLOX1b cDNA was 3594 nucleotides long, with 259 bases in the 3'-UTR (untranslated region) followed by a polyA tail, and 479 bases comprising the 5'-UTR, which is significantly longer than in the previously isolated PpLOX1 mRNA (only 82 bases). With this new sequence information, we were able to confirm the previous assumption of the ATG translational start site for the PpLOX1 mRNA, since the two upstream ATGs in the same reading frame were both followed by a stop codon. For PpLOX2, a 3256-nucleotide cDNA sequence has been determined from the EST clone pphn43n22, having a relatively shorter 5'-UTR (174 bases) which did not show any stop codons prior to the first putative ATG start site. However, at the time of writing, a new EST (pph18f10) has been deposited in Physcobase which showed stop codons in the same reading frame prior to this presumed start codon, thus increasing the likelihood that we have obtained the full-length coding region of this gene. Additionally, PpLOX2 encodes a similar-sized protein (105 kDa) with same number of amino acids (938) as the new PpLOX1b sequence, hence it is very likely that we have the complete sequence for the encoded protein. Interestingly, PpLOX2 also has the additional "GAG" in the coding region, which inserts a Glu in the translated protein. Thus both PpLOX2 and the new PpLOX1b sequence have 938 amino acids, while the previously reported PpLOX1 has only 937. Nonetheless, the presence of this additional residue did not seem to affect LOX activity. Using the TargetP v1.0 algorithm (Emanuelsson et al., 2000), putative transit peptides were found in each of these protein sequences which possibly direct them to the chloroplast. They are therefore classified as type 2 lipoxygenases (Feussner and Wasternack, 2002).

PpLOX3 and 4, which share 73.4% amino acid identity, are more closely related to each other than to the rest of the PpLOX family. PpLOX3 cDNA from the EST clone pphb15j12 is found to be 3383 nucleotides long, which includes 245 bases in the 5'-UTR, 2763 in the coding region, and 359 in the 3'-UTR followed by a polyA tail. PpLOX4 cDNA is 3409 nucleotides long, with 397, 2763 and 218

bases in the 5'-UTR, coding region and 3'-UTR, respectively. In each case, the putative start site is preceded by a stop codon in the same reading frame in the 5'-UTR, which supports a predicted translational product of 920 amino acids for both cDNAs, each of which is only 40% identical to either PpLOX1 or PpLOX2. Neither PpLOX3 nor PpLOX4 has a putative N-terminal signal peptide, according to TargetP v1.0 prediction.

PpLOX5, 6 and 7 have the longest ORFs among the active PpLOXs, encoding a stretch of 951, 956, and 966 amino acids, respectively. They are also most divergent from each other and from the other active PpLOXs, with only 42–47% identity at the amino acid level. PpLOX5 cDNA has 216 bases in the 5'-UTR, a 2856-nucleotide coding region and a 3'-UTR with 252



Fig. 3. The enzymatic activities at various pHs of *P. patens* lipoxygenases PpLOX1b (A), PpLOX2 (B), PpLOX3 (C), PpLOX4 (D), PpLOX5 (E), PpLOX5 (F) and PpLOX7 (G), using arachidonic (1) (\bigcirc, \bullet) , α -linolenic (2) (\blacktriangle, Δ) and linoleic (3) (\blacksquare, \Box) acids as substrates. Open symbols: phosphate buffer; shaded symbols: citrate buffer.

bases followed by a polyA tail, totaling 3340 bases. PpLOX6, on the other hand, has 3398 nucleotides comprising the 217, 2871, 294 and 16 bases in the 5'-UTR, ORF, 3'UTR and polyA tail, respectively. PpLOX7 has a relatively short 5'-UTR (199 bases), the longest ORF (2901 bases) among the PpLOXs, and the shortest 3'-UTR (138 bases which include 16 adenines in the polyA tail). Based on the prediction results of TargetP v1.0, PpLOX5 is possibly mitochondrial, whereas PpLOX6 and PpLOX7 are plastidic.

All six lipoxygenase proteins (PpLOX2–PpLOX7) described above harbor the conserved histidine, asparagine and isoleucine residues involved in iron binding (Fig. 1). However, as described for PpLOX1 (Senger et al., 2005), none of them harbored the determinants involved in positional specificity described for lipoxygenases from flowering plants. Phylogenetic analysis with selected lipoxygenases from flowering plants showed that PpLOX7 group together with type 2 lipoxygenases from flowering plants, while the other PpLOXs form a distinct clade (Fig. 2).

2.2. Substrate specificity and pH profiles of PpLOXs

The open reading frames of PpLOX1b to 7 and EST clone pph20m14, were heterologously expressed in *Escherichia coli* using pET101 as vector and BL21DE3 as host strain. After induction with IPTG at 20 °C for 24 h, bacterial lysates were assayed for LOX activity at different pHs, using linoleic (**3**), α -linolenic (**2**) and arachidonic (**1**) acid as substrates, respectively. Using the Clark-type oxygen electrode, only PpLOX1b to 7, and not pph20m14, were found to be active, each with varying substrate preferences and pH optima.

Consistent with their high sequence similarity, PpLOX1b and 2 had the same pH profile and substrate preference (Fig. 3A and B). Both proteins have pH optima at pH 7.0 and a preference for arachidonic acid (1), which agrees with previous findings on PpLOX1 (Senger et al., 2005). Both PpLOX1b and PpLOX2 are most active against arachidonic acid at a relatively narrow pH range from pH 6.5 to 7.5, with a gradual decrease in LOX activity as the pH is de-



Fig. 4. HPLC analyses of LOX reaction products. PpLOX1b with arachidonic acid (1) (A), PpLOX7 with arachidonic acid (1) (B) and with α-linolenic acid (2) (C). The insets show the respective enantiomer separation of the products.



Fig. 5. Relative amounts of products generated by *P. patens* lipoxygenases as analyzed and quantified by normal phase HPLC, using linoleic (**3**), α-linolenic (**2**) and arachidonic (**2**) acids as substrates.

creased from pH 6.0 to 4.0. A sudden drop in the activity is seen at pH 8.0 and even decreases further as the pH is shifted to increased alkalinity. Under the current assay conditions used, no significant activity has been observed when either linoleic (**3**) or α -linolenic (**2**) acid is used as substrate. Although our data show that PpLOX2 is more active than PpLOX1b, this may simply be due to differences in expression levels. The latter, however, cannot be determined at this point, because the heterologously expressed proteins cannot be distinguished from endogenous bacterial proteins in SDS–PAGE gels (data not shown). The expression levels can therefore still be optimized prior to our next step of purifying these proteins.

PpLOX3 and 4, while similar in their primary structure, have distinct substrate preferences (Fig. 3C and D). PpLOX3 preferentially utilizes both α -linolenic (**2**) and arachidonic (**1**) acids as substrates, whereas PpLOX4 clearly prefers α -linolenic acid (**2**) only. Nevertheless, closer inspection of the pH profiles of PpLOX3 and PpLOX4 activities against α -linolenic acid (**2**) shows a very similar trend. In each case, gradual decrease in activity was observed as the pH is adjusted away from their respective pH optima (6.5 for PpLOX3 and 7.0 for PpLOX4), with a discernible shoulder in the alkaline region (pH 9.5 and pH 9.0, respectively) reflecting a slight increase in activity.

The activity of PpLOX3 against arachidonic acid (**1**) is highest at pH 6.5 to 8.0, being relatively constant at this pH range, which then gradually decreases as the pH is changed in either direction. With α -linolenic acid (**2**), PpLOX3 activity is highest at pH 7.0, showing a

gradual decline as the pH is increased to pH 10.0, until activity is no longer detected at pH 10.5. Following a drop in activity at pH 6.5 from pH 7.0, a gradual decrease in activity is observed from pH 6.5 to 4.0, until the enzyme is no longer active at pH 3.5.

PpLOX4 is slightly active against arachidonic acid (1) with highest activity observed at pH 7.0, though the activities at pH 7.0 and 8.0 are almost up to the same level. In fact, PpLOX4 activity against arachidonic acid (1) did not decrease significantly until the pH is either below pH 6.0 or at pH 8.5 and higher, with negligible activity detected at pH 3.5 and 9.5. This is somewhat similar to the pH profile of PpLOX4 activity with linoleic acid (3) as substrate, except that the enzyme remains active at pH 9.0–10.0, but not at pH 10.5.

PpLOX5 prefers α -linolenic acid (**2**) as substrate, although it also has significant, albeit lower, activity with arachidonic acid (1), and much less activity against linoleic acid (3) (Fig. 3E). With α -linolenic acid (2), the pH optimum appears to be at pH 7.0, although almost the same level of activities have been observed at pH 5.5 and 6.0, following a significant drop at pH 6.5. A steep decline in activity then ensues with further increase in pH from 7.0 or a decrease from pH 5.5. With arachidonic acid (1), the pH optimum of PpLOX5 is at pH 6.5, with gradual decrease in activity as the pH decreases to 4.0. A steeper decline is observed from pH 7.0-8.0, with the enzyme no longer active at pH 8.5. Much less activity was observed with linoleic acid (**3**) as substrate, though activity stayed constant at a broader pH range (5.5-7.0), with gradual decrease as the pH is decreased from 5.5 to 4.0 or when the pH is changed from 7.0 to 8.0. Linoleic acid (3)-dependent LOX activity of PpLOX5 may be considered negligible when compared to the activity against linolenic acid (2). PpLOX5 activities remained low at pH 8.5 and 9.0 using any of the three substrates.

PpLOX6 is most active under acidic conditions, with only minimal activity observed at pH 7.0 and higher (Fig. 3F). α -Linolenic acid (**2**) is the preferred substrate, being oxygenated 5-fold more than arachidonic acid (**1**), and 10-fold more than linoleic acid (**3**) at the optimal pH range (4.5–5.0). While the enzyme is still active against the preferred substrate even at pH 3.5, a steep decline in its activity against α -linolenic acid (**2**) is observed from pH 4.5 to 3.0, whereas the decrease in activity is more gradual when the pH is increased from 5.0 to 6.0. A sudden drop in activity is observed at pH 6.5 decreasing further at 7.0, where activity holds at a minimal level until pH 8.0. In the case of arachidonic (**1**) and linoleic (**3**) acids, the decrease in activity as the pH is adjusted from 4.5 to 5.0, respectively, is more gradual, as the activities with these substrates are already relatively low compared to that with α -linolenic acid.

PpLOX7 prefers α -linolenic (**2**) over either linoleic (**3**) or arachidonic (**1**) acids, with optimal activity at pH 7.0 (Fig. 3G). A steep decline in activity is observed as the pH is adjusted towards either acidic or alkaline conditions, although a considerable level of activity still persists around pH 8 before PpLOX becomes completely inactive at pH 9. It is interesting to note that none of the moss lipoxygenases exhibited optimal activity at alkaline pH.

2.3. LOX product analysis

The products formed by each PpLOX at different pHs (4.0–8.5) using the preferred substrates arachidonic (1) and α -linolenic (2) acids were initially analyzed by reversed phase HPLC. The elution conditions developed herein for the reversed phase HPLC analysis of expected LOX products from arachidonic acid (1) (see Section 5) allowed the separation of 15-HETE (10), 11-HETE (11), 12-HETE (12) and 5-HETE (12) and 11-HETE (14) and 9-HETE (15) coeluted with 12-HETE (12) and 11-HETE (11), respectively (data not shown). Under these separation conditions, PpLOX1b and PpLOX2 accumulated a major peak that coeluted with 8-HETE (14) and 12-HETE (12). Further HPLC analysis of the LOX products via a pro-

cedure which utilized reversed, normal and chiral phase HPLC (Senger et al., 2005), confirmed that both PpLOX1b and PpLOX2 were arachidonate (12S)-LOXs, similar to what was previously determined for PpLOX1 (Senger et al., 2005). We found no evidence of changes in the product specificity of either PpLOX1b or 2 that accompanied alteration in the pH of the reaction.

We achieved baseline separation of the LOX products of α -linolenic acid (**2**), i.e., 9-HOTE (**16**) and 13-HOTE (**17**), by reversed phase HPLC (data not shown), and thus were able to determine that PpLOX3, 4, 5, 6 and 7 are all 13-LOXs with respect to α -linolenic acid (**2**), and 15-LOXs with respect to arachidonic acid (**1**). This was confirmed by normal phase HPLC, which additionally was able to determine that 13-HODE (**18**) was formed as the major product from linoleic acid (instead of 9-HODE, **19**). Representative chromatograms of LOX products using normal phase and chiral HPLC are shown in Fig. 4.

Fig. 5 shows each of the PpLOX's product profiles, which were obtained by normal phase HPLC. PpLOXs are essentially indistinguishable from each other with respect to their activities against linoleic acid (**3**). PpLOX4 generated the highest proportion of 13-HODE (**18**) (~80%), followed by PpLOX5 (~67%) and PpLOX3 (~63%). PpLOX7 had the lowest proportion of 13-HODE (**18**) (~54%), whereas PpLOX6 had about the same proportion of 13-HODE (**18**) (~54%), whereas PpLOX6 had about the same proportion of 13-HODE (**18**) as that of PpLOX2 (58%). In each case, the remaining products were mostly 9-HODE (**19**) (17–38%), with minimal amounts of 13-KODE (**20**) (~0.7–5%) and 9-KODE (**21**) (~0.4–2%) detected. The similarity in their product profiles is not surprising given that none of them actually prefers linoleic acid (**3**) as substrate (see Schemes 1 and 2 for chemical structures).

With α -linolenic acid (**2**) as substrate, the order of PpLOXs in terms of the highest proportion of 13-HOTE (**17**) produced is different from that seen with using linoleic acid (**3**). Excluding PpLOX1b and PpLOX2 from the comparison, PpLOX4 and 5 had the lowest proportion of 13-HOTE (**17**) produced (~51 and 62%, respectively) whereas PpLOX6 had the highest (~93%). PpLOX1b and 2 can be excluded from this comparison since neither prefers α -linolenic acid (**2**) as substrate. Indeed, although PpLOX1b and 2 seemed to form 12-HOTE (**22**) as the major product, the absolute amounts of these hydroxy fatty acids were so low compared to those formed by PpLOX1b and 2 from arachidonic acid (**1**) (see below), that they could simply be derived from non-specific oxidation.

The product profiles of the PpLOXs with arachidonic acid (1) were most instructive for comparative purposes since all PpLOXs used this as substrate. Thus, PpLOX1b and 2 formed mostly12-HETE (12) (56% and 79%, respectively) with minor amounts of 15-HETE (10) (\sim 10–20%), 5-HETE (13) (\sim 4–7%), 8-HETE (14) (\sim 3–5%), and 11-HETE (11) (\sim 3–7%), whereas PpLOX3, 4, 5, 6 and 7 formed 15-HETE (10) as the major product (\sim 74%, 69%, 78%, 67% and 82%, respectively). These results show that PpLOX1b and 2 are arachidonate 12-LOXs, while the rest of the PpLOXs are arachidonate 15-LOXs, which is consistent with their respective activities with α -linolenic acid (2) as a 13-LOX.

Analysis of the enantiomers of LOX products formed by each PpLOX was carried out by chiral phase HPLC analysis, with the results shown graphically in Fig. 6. With linoleic acid (**3**) as substrate, PpLOX1b formed racemic mixtures of both 13-HODE (**18**) and 9-HODE (**19**), which is consistent with its lack of significant activity using this substrate. Hence, the LOX products formed from PpLOX1b incubation may simply be a result of non-specific oxidations. In the case of PpLOX3, 4, 5, 6 and 7, the *S*-enantiomer of 13-HODE (**18a**) is mostly formed (86%, 88%, 78%, 86%, 78%, respectively), whereas a racemic mixture of 9-HODE (**19**) was produced. Similar results were obtained with α -linolenic acid (**2**) as substrate. PpLOX1b generated a racemic mixture of either 13-HOTE (**17**) or 9-HOTE (**16**), whereas the other PpLOX3, 6 and 7



Fig. 6. Enantiospecificity of *P. patens* lipoxygenases with respect to the products generated from linoleic (**3**), α -linolenic (**2**) and arachidonic (**1**) acids. Proportions of the different isomers are given in Fig. 5.

generated almost exclusively the *S*-enantiomer of 13-HOTE (**23**) (96%, 98% and 94% e.e., respectively), whereas PpLOX4 and 5 had high proportions of (13*S*)-HOTE (**23**) as well (96% and 90%, respectively). This higher enantiomeric purity of the 13-HOTE (**17**) products (compared to 13-HODE, **18**) probably reflects their preference for linolenic acid (**3**) as substrate. Interestingly, PpLOX2 forms an excess of the *S*-enantiomers of 13-HODE (**18a**) and 13-HOTE (**17**) (36% and 66% e.e., respectively), instead of a racemic mixture.

In the case of arachidonic acid (1), PpLOX1b and 2 generated 95–99% of the (12*S*)-HETE (24) enantiomer, while the other products, 5-HETE (13), 11-HETE (11), 8-HETE (14) and 15-HETE (10), were racemic. The rest of the PpLOXs formed racemic products from arachidonic acid (1) except for 15-HETE (10), in which the *S*-enantiomer (25) is generated preferentially (\sim 79–94%), which is again consistent with their 13-LOX activity against α -linolenic acid (2). Hence, all of the PpLOXs cloned thus far stereospecifically generate LOX products of the *S*-configuration.

3. Discussion

Full-length cDNAs from Physcobase facilitated the identification and functional characterization of lipoxygenase genes in *P. patens*. Since we no longer had to perform cDNA library screening and/or PCR-based cloning, functional characterization of these genes had been completed in less time and was also less prone to error. Furthermore, since Physcobase had already assembled the ESTs into putative unigenes, it is possible to verify the sequence and/or identify sequence variants by sequencing other ESTs belonging to one contig pair. On the other hand, these ESTs do not represent the complete genome of *P. patens*, and so there still is the possibility of more lipoxygenase isoforms present in the moss genome. Indeed, at least four more additional lipoxygenase genes may be present in P. patens, based on its recently sequenced genome (Rensing et al., 2008). However, none of these additional lipoxygenases reported in the moss genome are found in the EST databases. Given the limited number of *Physcomitrella* ESTs available at present, it is difficult to determine whether or not these additional lipoxygenase-like sequences are expressed and encode active lipoxygenases.

The functional characterization of lipoxygenases in the moss *P*. *patens*, following the report of a multifunctional lipoxygenase from the same species (Senger et al., 2005), sheds more light into the nature of oxylipin metabolism in non-flowering land plants. Mosses differ from higher plants in terms of their lipid composition, especially in having significant amounts of arachidonic acid (1) comparable to that of linoleic (3) and α -linolenic (2) acids (Dembitsky, 1993). P. patens is in fact reported to have more arachidonic (1) (16.1%) than either linoleic (3) (13.7%) or linolenic (2) (11.9%) acids (Grimsley et al., 1981). Hence, *P. patens* and other mosses could potentially have a C20-based lipoxygenase pathway similar to mammalian counterparts, which seemed the only plausible working hypothesis prior to our discovery that other lipoxygenases do exist in this moss that utilize α -linolenic (2) more so than arachidonic (1) acid. Linole(n)ic acid (2, 3) derived oxylipins such as 9-HPODE (4), 13-HPODE (8), 9-HPOTE (5) and 13-HPOTE (6) have previously been detected in P. patens (Stumpe et al., 2006), but this could have also been derived non-enzymatically or non-specifically via an arachidonate lipoxygenase. Now it is clear that both C18 and C20 fatty acids could be sources of oxvlipins in *P. patens* since this moss has a variety of lipoxygenases with distinct substrate preferences, including for C18 fatty acids. Since the existence of both C18- and C20-derived oxylipins has already been observed in algae (Moghaddam and Gerwick, 1990; Gerwick et al., 1999; Jiang et al., 2000; Bouarab et al., 2004; Tsai et al., 2008), it makes sense that mosses have the same types of oxylipins produced, as they represent a distinct lineage phylogenetically in between algae and higher plants.

There seems to be at least two *Physcomitrella* lipoxygenases acting on arachidonic acid (**1**), namely PpLOX1b and PpLOX2, which apparently have strong preference for this substrate, and produce (12*S*)-HPETE (**26**) as the major product. PpLOX3 also appear to utilize arachidonic acid (**1**) at apparently the same efficiency as α -linolenic acid (**2**), but the product formed is (15*S*)-HPETE (**27**). Furthermore, all three enzymes are predicted to be in the plastid. If this prediction based on phylogenetic tree analysis is correct, then (12*S*)-HPETE (**26**), as well as its lyase product 12-ODTE (**28**) (Senger et al., 2005), would be expected to be generated in the plastids. Whether or not LOX products generated in the plastids are substrates for other enzymes remains to be determined.

In higher plants, 13-HPOTE (**6**) is the substrate of AOS and HPL in the chloroplasts. 13-HPOTE (**6**) could preferentially be generated by PpLOX3 to 7, all of which may be localized in the plastid. PpLOX4, 5, 6 and 7 all appear to prefer α -linolenic acid (**2**) over ara-

chidonic (1) and linoleic (3) acids, so there is a strong possibility that they actually produce 13-HPOTE (6) *in vivo*. If this is the case, then mosses may also be producing octadecanoids, in addition to eicosanoids.

An optimal pH of 5.0 for PpLOX6 is compatible with the potential acidification of the thylakoid lumen (Lee and Kugrens, 1999), if this is where PpLOX6 is ultimately targeted, although all type 2 lipoxygenases have been localized to the stroma so far. A lipoxygenase from barley also had an acidic pH optimum at 6.0 (Lulai and Baker, 1976), but this has not been specifically localized to any organelle.

Interestingly, none of the Physcomitrella lipoxygenases are 9lipoxygenases with respect to α -linolenic acid (2) nor are there PpLOXs that prefer linoleic acid (3). Linoleic acid (3) is found mostly in seeds of flowering plants where lipoxygenases are thought to be important for germination. As early land plants do not have seeds, the substrate preference of PpLOXs is consistent with the possible evolution of linoleate-utilizing LOXs in seed plants. Furthermore, it seems that the distribution of 9-lipoxygenases in the plant kingdom may be restricted to flowering plants and the existence of 9/13-lipoxygenases in angiosperms suggest that the early forms of land plant lipoxygenases are 13-lipoxygenases, which later evolved to also produce 9-lipoxygenase products. It appears however that the ability to utilize arachidonic acid (1) is an ancient trait since this is also present in algae. Alternatively, the ability to use arachidonic acid (1) as a LOX substrate has evolved independently in several lineages of the plant kingdom.

Phylogenetic analysis of moss lipoxygenases together with other known plant lipoxygenases showed that all PpLOXs except for PpLOX7 clustered together into one clade (Fig. 2). This suggests that the ancestor of PpLOX7 gave rise to type 2 LOXs and perhaps more recently to type 1 LOXs in higher plants, while the other PpLOXs form a distinct clade that is probably bryophyte specific. It is also possible that PpLOXs and lipoxygenases from higher plants evolved from a common cyanobacterial ancestor that has been described recently (Lang and Feussner, 2007; Lang et al., 2008).

4. Concluding remarks

Physcomitrella patens has several lipoxygenases with different substrate and product specificities. Among the seven lipoxygenases es that have been functionally characterized, two are arachidonate (12*S*)-lipoxygenases and the remaining five are linolenate (13*S*)-lipoxygenases. These results are consistent with mosses having both eicosanoid and octadecanoid pathways, similar to those found in algae. This provisionally provides an evolutionary explanation for the arachidonate utilizing lipoxygenase activities observed in some angiosperms which do not produce arachidonic acid (1). The results also suggest that other arachidonic acid (1) producing plants (such as some gymnosperms and ferns), may have C20-derived oxylipins as well. Further investigation of lipoxygenases in these plants could thus result in more insights into the evolution and diversity of oxylipin pathways.

5. Experimental

5.1. Materials

All solvents used were HPLC grade (Fisher). Arachidonic (1), linolenic (2), linoleic (3) and hydroxyfatty acids (10–19) were purchased from Cayman Chemical (Ann Arbor, MI). All other reagents were analytical or molecular biology grade (from Sigma unless otherwise noted). *Physcomitrella* cDNA clones (pphn50006, pphb14h18, pphb33n05, pphb14L11, pph20m05, pphn43n22,

pphn35p15, pphn9o24, pphb15j12, pph17d11, pph20m14, pphb13L22) were obtained from RIKEN BioResource Center (Nishiyama et al., 2003).

5.2. cDNA sequencing and analysis

Lyophilized plasmid DNA (100 ng) harboring putative LOX genes (from RIKEN BRC) were reconstituted in nuclease-free H₂O (5 µL) and transformed into electrocompetent XL1-Blue cells (Stratagene) via electroporation. Transformants were grown in selective LB media containing ampicillin (100 µg/ml) at 37 °C. Plasmids were prepared from overnight-grown positive colonies after growing in 5 mL LB media containing ampicillin (100 g/mL) at 37 °C for 16 h, using a Promega Wizard[®] plus SV kit, which were then used as template for a DNA sequencing reaction containing BigDve reagent and T7 or T3 primer. PCR consisted of a 96 °C hot start for 3 min followed by 25 cycles of 96 °C/20 s denaturation. 50 °C/30 s annealing, and 60 °C/3 min elongation performed on an Amplitron II thermocycler. PCR products were purified by passing them through Performa[®] gel filtration cartridges (Edge BioSystems), and analyzed using an ABI sequencer. Subsequent DNA sequencing reactions were performed using internal primers designed from the initial sequences obtained to determine the fulllength sequences of the cDNAs, which were eventually assembled from the overlapping sequence data generated from these sequencing reactions. Sequence information was processed and analyzed using GCG software (University of Wisconsin, Madison, WI).

5.3. Heterologous expression

The coding region of each cDNA was amplified using plasmids (0.5 µL, 100 ng) prepared from XL1-Blue cells (see Section 5.2) as template. The 50 uL PCR mixture overlayed with mineral oil also contained 0.2 µM forward primer that contains a 5'-CACC upstream of the ATG initiation site, 0.2 µM reverse primer that spans the stop codon. 0.4 mM dNTPs, and 1 unit of Pfu hotstart Turbo DNA Polymerase (Stratagene). Thermal cycling (95 °C for 3 min. followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 3 min, ending in a 10 min incubation at 72 °C) was performed on a Robocycler II (Stratagene). PCR products were cloned into pET101 TOPO cloning vector (Invitrogen) and transformed into TOP10 cells (Invitrogen) according to the manufacturer's instructions. Positive colonies were grown and used as source of plasmids for heterologous expression. The plasmid inserts were sequenced to verify correct orientation of the cDNA along the vector's reading frame. These plasmids were then transformed into an expression host BL21DE3 (Invitrogen), following the manufacturer's protocol. Overnight cultures of BL21DE3 transformants were inoculated into 50 mL LB media containing ampicillin (50 μ g/mL) and grown until the absorbance of the culture at 600 nm reached 0.5. Protein expression was induced by addition of IPTG to a final concentration of 0.5 mM, and subsequent incubation at 20 °C for 24 h. Cells were harvested by centrifugation (4000g, 20 min, 4 $^\circ C)$ and pellets were stored at -20 °C until use. Protein concentration was determined using the BCA protein assay kit (Pierce). SDS-PAGE was performed according to Fuller et al. (2001).

5.4. Assay of LOX activity

Cell pellets were resuspended in 0.1 M phosphate buffer pH 7.0 (1 mL) and sonicated while on ice (3 \times 15 s, setting 4) using a Microson ultrasonic cell disruptor (Misonix). After centrifugation (23,000g, 30 min, 4 °C), the supernatant was collected for subsequent LOX assays. Lipoxygenase activity was determined polarographically at 25 °C using a Clark-type oxygen electrode

(Hansatech Instruments, Norfolk, UK) by measuring the decrease in oxygen levels in a 0.5 mL enzyme reaction mixture. The latter consisted of either 0.1 M phosphate or citrate buffer (485 μ L), 0.1–0.4 mg crude protein extract (i.e., 5–15 μ L bacterial lysate) and 5 μ M arachidonic (1), α -linolenic (2) or linoleic (3) acids.

5.5. HPLC analysis of LOX products

Bacterial lysates (prepared from cell pellets as in Section 5.4, 10 μ L) were incubated with 10 μ M arachidonic (**1**), α -linolenic (**2**) or linoleic (**3**) acids (10 μ L) in 0.1 M phosphate buffer pH 7.0 (380 μ L) for 30 min. After addition of HOAc–H₂O (0.5 mL, 3:97, v/ v), lipids were extracted using the Bligh and Dyer method (Bligh and Dyer, 1959) and the CHCl₃ extract was dried under reduced pressure using a centrifugal evaporator (Jouan RC10-22, Winchester, VA). Hydroperoxyfatty acids were reduced to the corresponding hydroxyfatty acids by addition of NaBH₄ (100 mM) in MeOH (50 μ L). After 30 min HOAc–H₂O (50 μ L, 0.5:99.5, v/v) was added prior to HPLC analysis of a 10 μ L aliquot.

To identify LOX products from arachidonic (1) and α -linolenic (2) acids, reversed phase HPLC was performed on a Waters[™] 626 LC system connected to a 996 Photodiode Array Detector and a 717 plus autosampler, using a Novapak C18 column $(3.9 \times 150 \text{ mm}, 4 \text{ }\mu\text{m} \text{ } \text{particle size})$. For analysis of arachidonic acid (1) derived products, samples were eluted with a linear gradient from MeCN-H₂O-HOAc (30:69.65:0.35) to MeCN-H₂O-HOAc (50:49.75:0.25) in 10 min, followed by isocratic elution with MeCN-H₂O (50:50) for 30 min. LOX products from α -linolenic acid (2) were separated via isocratic elution with MeCN-H₂O-HOAc (50:59.97:0.03). Hydroxyfatty acids derived from C18 and C20 fatty acids were detected at 234 and 237 nm, respectively. Lipoxygenase products were also analyzed independently by normal phase HPLC followed by chiral phase HPLC to determine their enantiomeric forms. LOX products were identified by comparison of their retention times and absorption spectra with authentic standards purchased from Cayman Chemical (Ann Arbor, MI) or generated in the laboratory, as described (Senger et al., 2005).

5.6. Phylogenetic analysis

Phylogenetic analysis was conducted using the Mega4 software (Tamura et al., 2007). Deduced amino acid sequences of selected LOXs were aligned by Thompson et al. (1994) with a gap opening penalty of 3 and a gap extension penalty of 1.8. Based on the resulting multiple alignment, a phylogenetic tree was drawn using the Neighborhood-Joining method (Felsenstein and Churchill, 1996) using default parameters in the Mega4 software.

Sequence data

The nucleotide sequences reported in this paper have been deposited in the GenBank with the following accession numbers: ABF66647, ABF66648, ABF66649, ABF66650, ABF66651, ABF66652, and ABF66653.

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