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Cloning and expression of three lipoxygenase genes from liverwort, *Marchantia polymorpha* L., in *Escherichia coli*

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ABSTRACT

Three genes homologous to plant lipoxygenase genes were identified from the EST libraries of *Marchantia polymorpha*, in order to clarify the function of LOXs in bryophytes. Full-length genes were isolated using 5′- and 3′-RACE methods and named *MpLOX1*, *MpLOX2*, and *MpLOX3*, respectively. To investigate the enzymatic activities of liverwort LOXs, recombinant MpLOX1, MpLOX2, and MpLOX3 proteins were prepared from *Escherichia coli* cells expressing the corresponding gene. LC–MS/MS analyses and chiral column chromatography of their reaction products showed that *MpLOX1* codes for 11*S*/15*S*-lipoxygenase against eicosapentaenoic acid and for 15*S*-lipoxygenase against arachidonic acid, and that *MpLOX2* and *MpLOX3* code for 15*S*-lipoxygenase against eicosapentaenoic and arachidonic acids. Phylogenetic analysis showed that the liverwort lipoxygenase genes separated from the ancestor of higher plants in the early stages of plant evolution. Quantification analyses suggested that arachidonic acid and eicosapentaenoic acid were preferred substrates. Furthermore, each liverwort lipoxygenase exhibited highest activity at pH 7.0 and dependency on Ca^{2+} ion in the oxygenation reaction.

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1. Introduction

Lipoxygenases (LOXs) are nonheme, nonsulfur iron dioxygenases found in many plants and animals that act on lipid substrates (Andreou and Feussner, 2009). They catalyze the positional and stereospecific insertion of molecular oxygen into polyunsaturated fatty acids (PUFAs) containing a (1*Z*,4*Z*)-pentadiene system, such as linoleic acid (LA, **1**), α-linolenic acid (ALA, **2**), arachidonic acid (AA, **3**), and eicosapentaenoic acid (EPA, **4**), thereby resulting in the formation of a (2*E*,4*Z*)-hydroperoxydiene (Schneider et al., 2007) (Scheme 1).

In plants, C18 PUFAs such as LA (**1**) and ALA (**2**) are the most common substrates for LOXs (Liavonchanka and Feussner, 2006). LOX activities are classified with respect to their positional specificity of oxygenation in PUFA (Gaffney, 1996). In the case of ALA (**2**), LOX catalyzes insertion of oxygen at either 9- or 13-positions of ALA (**2**) to form 9-hydroperoxyoctadecatrienoic acid (9-HPOTrE, **5**) or 13-HPOTrE (**6**), referred to as 9-LOX and 13-LOX activity, respectively (Feussner and Wasternack, 2002). In mammals, several kinds of LOX activity, such as 5-, 8-, 12-, and 15-LOX against EPA (**4**) and AA (**3**), have been identified. 5-LOX activity is mainly involved in the arachidonic cascade for leukotriene biosynthesis, whereas both 12-LOX and 15-LOX are involved in lipoxin biosynthesis. Lipoxins are implicated in the pathogenesis of various inflammatory conditions, such as arthritis, psoriasis, and bronchial asthma (Kantarci and Van Dyke, 2003, 2005). It has been reported that *Marchantia polymorpha* contains 5-LOX and 13-LOX activity against AA (**3**) and LA (**1**), respectively (Kanamoto et al., 2009; Matsui et al., 1996), thereby suggesting that liverwort includes both C18 and C20 fatty acid metabolic pathways. Octadecanoid metabolic pathways are involved in signaling for wounding and pathogen attack, and cell death in plants (Porta and Rocha-Sosa, 2002). Although liverworts contain C20 fatty acids such as AA (**3**) and EPA (**4**) (Shinmen et al., 1991), the physiological function of eicosanoids derived from C20 fatty acid metabolic pathways has not been clarified in plants. In the present study, attempts to clone three LOX genes from the liverwort *M. polymorpha* were carried out, with the resulting proteins characterized in terms of enzymatic activities to clarify their functions.

Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; CaMV, cauliflower mosaic virus; EPA, eicosapentaenoic acid; EST, expressed sequence tag; HEPE, hydroxy eicosapentaenoic acid; HETE, hydroxy eicosatetraenoic acid; HODE, hydroxyoctadecaenoic acid; HOTrE, hydroxyoctadecatrienoic acid; HPETE, hydroperoxy eicosatetraenoic acid; HPEPE, hydroperoxy eicosapentaenoic acid; HPOTrE, hydroperoxy octadecatrienoic acid; LA, linoleic acid; LOX, lipoxygenase; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; RACE, rapid amplification of cDNA ends.

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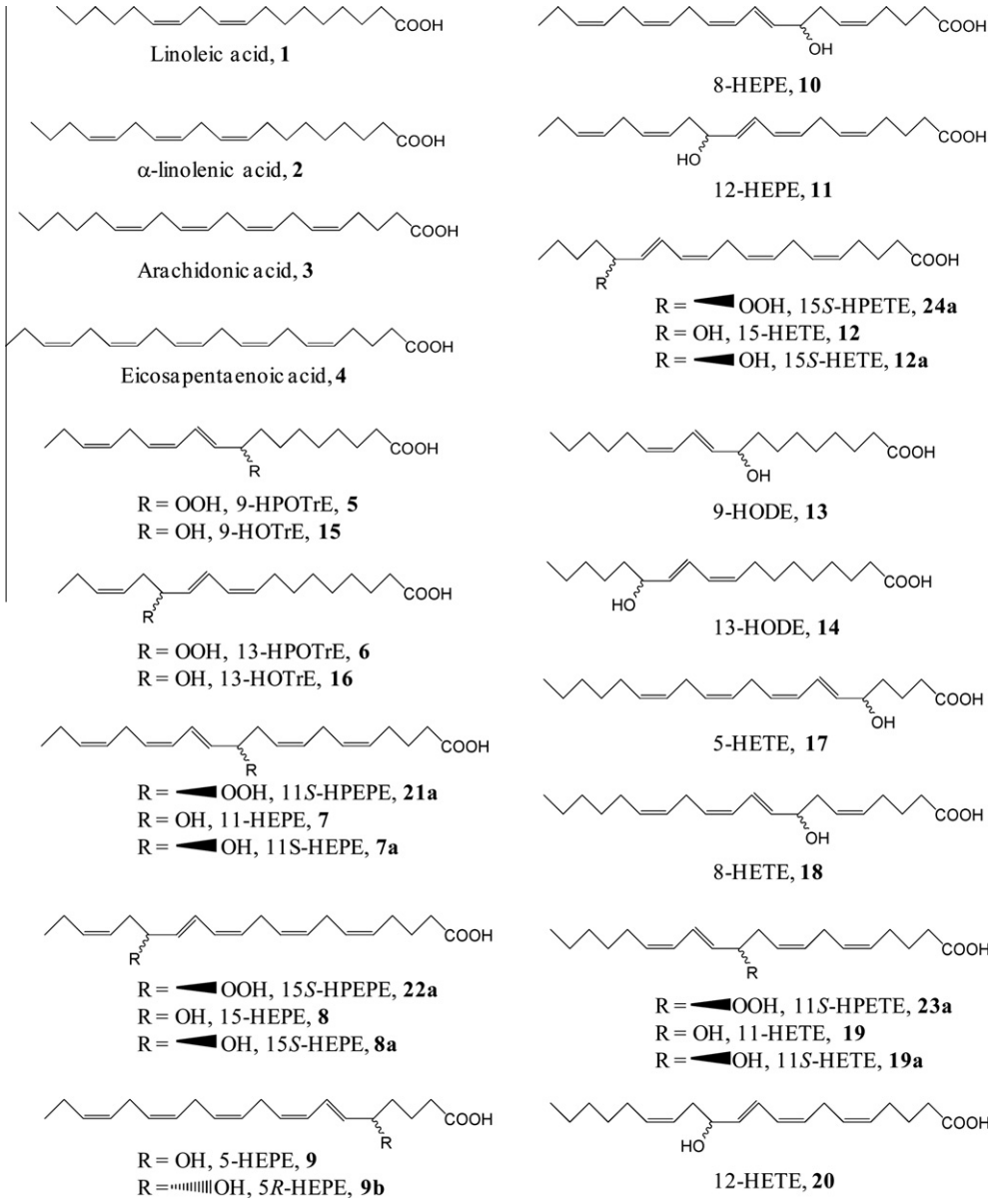

Scheme 1. Chemical structures of selected fatty acids and oxylipins. Arrow heads indicate enantiomers of oxylipins (◀, *S*-enantiomer; ▷, *R*-enantiomer).

2. Results

2.1. Identification of lipoxygenase genes in M. polymorpha

Three LOX-like sequences from the *M. polymorpha* EST database were identified by homologous sequence search comparing previously known plant and animal lipoxygenase genes. The complete cDNA clones for each LOX gene were acquired by 5′- and 3′-RACE based on information from the partial sequences of the EST clones. The sequences of three LOX cDNAs, named *MpLOX1*, *MpLOX2*, and *MpLOX3*, were found to be 3384, 3699, and 3224 nucleotides long, respectively. The first ATG was identified as a translational start site for the LOX mRNA since a stop codon was followed by the first ATG site in the same reading frame. The deduced amino acid sequences of MpLOX1, MpLOX2, and MpLOX3 showed that they consisted of 955, 985, and 955 amino acid residues, respectively, with estimated molecular weights greater than 106 kDa. Since all known lipoxygenases from flowering plants are approximately 100 kDa, the molecular weights of these putative MpLOXs were slightly larger than the typical molecular weight of plant lipoxygenases. The amino acid sequences in 5′ terminals of *MpLOX1*, *MpLOX2*, and *MpLOX3* genes are longer than those of mammalian *LOXs* (data not shown). Because the Wolf PSORT program predicted the subcellular localization of MpLOXs as chloroplast localization, signal peptide sequences might be contained in their 5′ terminals. The results of a BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) indicated that these MpLOXs had high amino acid sequence identities with *Physcomitrella patens* LOX6. An amino acid sequence alignment of MpLOX1, MpLOX2, MpLOX3, and PpLOX6 is shown in Fig. 1. All MpLOXs were found to be closely related to PpLOX6, with 38.4–40.6% identity at the amino acid level. The topography of the MpLOX proteins was investigated using Conserved Domain Architecture Retrieval Tool (CDART) software (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi). The results of this study showed that the MpLOX proteins consisted of two domains; namely, a PLAT (Polycystin-1, Lipoxygenase, Alpha-Toxin) domain in the amino-terminal region and a LOX domain in the downstream carboxyl-terminal region. The generally accepted function

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