Molecular and biochemical studies on genes responsible for green leaf volatile biosynthesis in plants; lipoxygenase in *Marchantia polymorpha* and hydroperoxide lyase in *Camellia sinensis*

A dissertation submitted in partial fulfilment of the requirements for the degree of Doctor of Philosophy at Yamaguchi University

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Moataz M. Tawfik

Graduated student

Graduate School of Medicine

Special dedication

To the lovely memory of my late father

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GENERAL INTRODUCTION

Green leaf volatiles (GLVs) are plant secondary metabolites found in most green plants. Their amounts are usually low when plant tissues are intact, but once the plant is under biotic or abiotic stress, like mechanical wounding, herbivore attack, or pathogen attack (bacterial, fungal), plants start to produce GLVs in a few seconds or minutes to protect themselves from damage (Shiojiri et al. 2006). GLVs also function in the plant-plant interaction as the plant recognizes the surrounding plants by sensing these volatiles (Rodriguez-Saona et al. 2013). GLVs also have a role in the plant sexual reproduction; for example, GLVs can act as good fragrance to attract pollinators (Lucas-Barbosa et al. 2011; Rusch et al. 2016).

GLVs follow a biosynthetic pathway that starts with lipids through a lipase to form polyunsaturated fatty acids (PUFAs). The reaction of lipoxygenase (LOX) is to add an oxygen to PUFA to yield fatty acid hydroperoxide (HPO). The metabolites that derive from oxidation of PUFAs via a LOX-catalyzed step as well as metabolites that derive from alternative oxidation reactions and subsequent reactions are collectively named oxylipins (Andreou & Feussner 2009). The subsequent rearrangement reaction cleaves the HPO of the fatty acid by hydroperoxide lyase (HPL) to form C6 aldehydes. Moreover, allene oxide synthase (AOS) also acts on HPOs to produce allene oxide, which is further metabolized to form 12-oxo phytodienoic acid (OPDA) and subsequently converted into jasmonic acid and its derivatives, called jasmonates (JAs) (Mosblech et al. 2009). In some cases, the LOX can carry out the HPL-like step by exerting cleavage reaction to form aldehydes like I will show in this study (Figure I)

LOX was first discovered in plants as an enzyme to carry out several functions in plants, mammals, and microorganisms (Andreou et al. 2009b; Oliw 2002). LOXs are known as non-heme, non-sulfur iron containing dioxygenases (Andreou & Feussner 2009) that can act on lipids containing (1*Z*,4*Z*)-pentadiene moiety, for example, PUFAs like arachidonic acid, eicosapentaenoic acid, α-linolenic acid, and linoleic acid to give 1-hydroperoxy-(2*E*,4*Z*)-pentadiene moeity. The HPO further reacts with LOX or other enzymes like HPL and AOS and gives volatile aldehydes, alcohols, and corresponding oxo fatty acids. Liverworts and mosses are members of bryophytes that locate in the basal diverging lineage of embryophytes. Liverworts evolved as one of the first land plants. Thus, study on liverworts may improve our understanding of the genetic mechanisms, which allowed plants to evolve from their aquatic algal ancestors and

to adapt to a terrestrial environment. *M. polymorpha* is a widely distributed dioecious liverwort that can be found in the moisture areas (Sharma et al. 2014). Bryophytes (liverworts and mosses) as well as algae form PUFAs like linoleic acid (18:2), α-linolenic acid (18:3), arachidonic acid (20:4), and eicosapentaenoic acid (20:5) in significant amounts. On the other hand, the fatty acid composition in vascular plants was mainly represented by linoleic acid (18:2) and α-linolenic acid (18:3) (Liavonchanka & Feussner 2006).

Sixteen LOX genes in *M. polymorpha* genome had been identified through RNA-sequencing analysis, and only three of them have been cloned, and their properties were studied and reported (Kanamoto et al. 2012). Liverworts contain huge amounts of terpenoids, and they also contain the biosynthetic pathway to form OPDA (Koeduka et al. 2015). Nonetheless, *M. polymorpha* does not have *HPL* gene that is responsible for the formation of GLVs (Koeduka et al. 2015). It has been reported that LOX has a side reaction to form oxylipin by-products through forming alkoxy radicals (Gardner 1989). Meanwhile, the activity to form C6-aldehydes from 13-HPO of C18 fatty acids had been detected in cultured cells of liverworts (Matsui et al. 1996) but the details are unknown. Furthermore, the activity of LOX enzyme to form C8 oxylipins had been reported with a moss by Senger et al. (2004), as well as the activity to form volatile oxylipins in a red algae had been reported by Shen et al. (2014). The liverwort *M. polymorpha* is a suitable model plant because efficient gene-transfer techniques including genome editing with the CRISPR/Cas9 system has been established (Sugano et al. 2014). Its genome sequence has also been reported (Ishizaki et al. 2008, 2016).

The tea made from *Camellia sinensis* L. is one of the most widely consumed beverages in the world (Krafczyk & Glomb 2008). There are a number of reports concerning human health benefits and the bioactive compounds in tea, such as anti-bacterial, anti-viral, anti-histaminic and protective effects against many chronic diseases (Chen et al. 2014). Upon herbivore attack, tea releases large amounts of volatile organic compounds (VOCs) as a part of defense mechanism. The GLVs released by tea plant attract both herbivores and their natural enemies (Wang et al. 2012; Sun et al. 2012). HPL has been found in tea leaves (Matsui et al. 1991). However, limited information on the mechanism of tea HPL is available. C6 aldehydes play a role in forming part of the aroma profile of green tea. Moreover, HPL-catalyzed production of C6 aldehydes may be a key step of a built-in resistance mechanism of plants against some sucking insect pests

(Vancanneyt et al. 2001), and it is also discussed that C6 aldehydes are parts of the volatile mixture that attract predators upon herbivore attack (Arimura et al. 2005).

In this study, I attempted to elucidate biochemical basis of GLV biosynthesis in *M. polymorpha* and *C. sinensis*. Previous study revealed two *CYP74* genes in the genome of *M. polymorpha* encoding AOSs, and showed no *HPL* to form C6 aldehydes from 13-HPO of C18 fatty acid. An ability to form C6 aldehydes was reported with one of the LOXs in a red alga. Therefore, I examined the ability to form C6 volatiles with LOX enzyme in *M. polymorpha*.

I also show that a cytochrome P450 of *C. sinensis*, *CYP74B24,* encodes functional 13HPL. Cloning and heterologous expression in *Escherichia coli* was done, and substrate specificity was examined. Furthermore, I quantified the amount of GLVs in tea leaves.

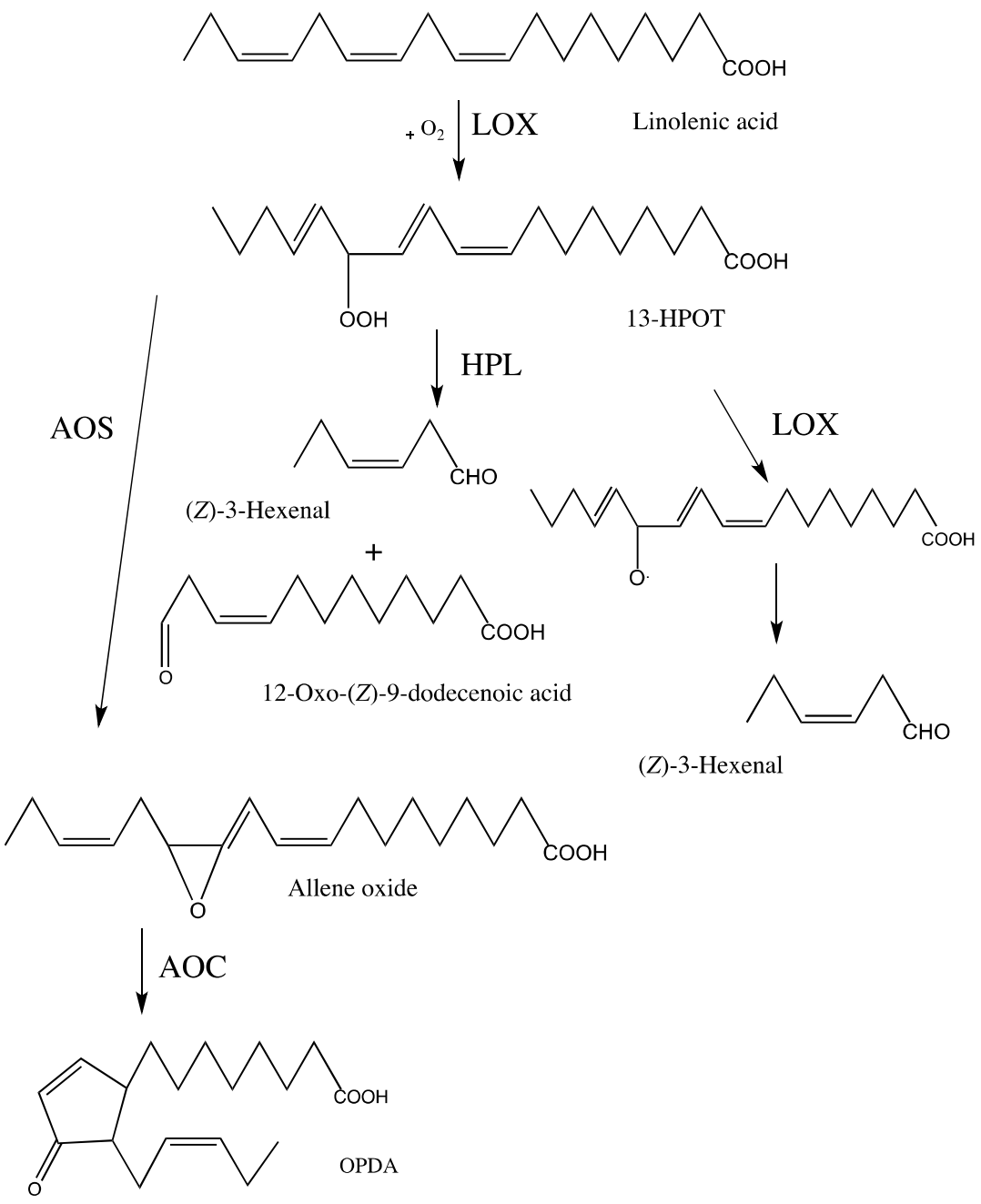


Figure I. Biosynthetic pathway to form green leaf volatiles and oxylipins.

References

Andreou A, Brodhun F, Feussner I. Biosynthesis of oxylipins in non-mammals. Prog. Lipid Res. 2009b; 48: 148-170.

Andreou A, Feussner I. Lipoxygenase – Structure and reaction mechanism. Phytochemistry. 2009; 70:1504–1510.

Arimura G, Kost C, Boland W. Herbivore-induced, indirect plant defences. Biochim. Biophys. Acta. 2005; 1734: 91–111.

Chen Z, Mei X, Jin Y, Kim E H, Yangb Z, Tua Y. Optimisation of supercritical carbon dioxide extraction of essential oil of flowers of tea (*Camellia sinensis* L.) plants and its antioxidative activity. J. Sci. Food Agric. 2014; 94: 316–321.

Dani Lucas-Barbosa D, Loon J A V, Dicke M. The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. Phytochemistry. 2011; 72: 1647-1654.

Gardner W H. Oxygen radical chemistry of polyunsaturated fatty acids. Free Radic. Biol. Med. 1989; 7: 65-86.

Ishizaki K, Chiyoda S, Yamato K T, Kohchi T. Agrobacterium-mediated transformation of the haploid liverwort *Marchantia polymorpha* L., an emerging model for plant biology. Plant Cell Physiol. 2008; 49: 1084- 1091.

Ishizaki K, Nishihama R, Yamato K T, Kohchi T. Molecular genetic tools and techniques for *Marchantia polymorpha* research. Plant Cell Physiol. 2016; 57: 262- 270.

Kanamoto H, Takemura M, Ohyama K. Cloning and expression of three lipoxygenase genes from liverwort, *Marchantia polymorpha L*., in *Escherichia coli*. Phytochemistry. 2012; 77:70–78.

Koeduka T, Ishizaki K, Mwenda CM, Hori K, Sasaki-Sekimoto Y, Ohta H, Kohchi T, Matsui K. Biochemical characterization of allene oxide synthases from the liverwort *Marchantia polymorpha* and green microalgae *Klebsormidium flaccidum* provides insight into the evolutionary divergence of the plant CYP74 family. Planta. 2015; 242:1175–1186.

Krafczyk N, Glomb M A. Characterization of phenolic compounds in rooibos tea. J. Agric. Food Chem. 2008; 56: 3368–3376.

Liavonchanka A, Feussner I. Lipoxygenases: Occurrence, functions and catalysis. J. Plant Physiology. 2006; 163: 348-357

Matsui K, Kaji Y, Kajiwara T, Hatanaka A. Developmental changes of lipoxygenase and fatty acid

hydroperoxide lyase activities in cultured cells of *Marchantia polymorpha*. Phytochemistry. 1996; 41:177–182.

Mosblech A, Feussner I, Heilmann I. Oxylipins: structurally diverse metabolites from fatty acid oxidation. Plant Physiol. Biochem. 2009; 47:511–517.

Oliw E H. Plant and fungal lipoxygenases. Prostag. Oth. Lipid Mediators. 2002; 68– 69, 313–323.

Rodriguez-Saona C R, Mescher M C, De Moraes C M. F. Baluska (ed.), Long-Distance Systemic Signaling and Communication in Plants, Signaling and Communication in Plants. 2013; pp. 393–412.

Rusch C, Broadhead G T, Raguso R A, Riffell J A. Olfaction in context — sources of nuance in plant–pollinator communication. Curr. Opin. Insect Sci. 2016; 15: 53–60.

Senger T, Wichard T, Kunze S, Goebel C, Lerchl J, Pohnert G, Feussner I. A multifunctional lipoxygenase with fatty acid hydroperoxide cleaving activity from the moss *Physcomitrella patens*. J. Biol. Chem. 2005; 208:7588–7596.

Sharma N, Jung C H, Bhalla P L, Singh M B. RNA Sequencing Analysis of the Gametophyte Transcriptome from the Liverwort. *Marchantia polymorpha*. PLoS One. 2014; 9: e97497.

Shen J, Tieman D, Jones J B, Taylor M G, Schmelz E, Huffaker A, Bies D, Chen K, Klee H J. A 13-lipoxygenase, TomloxC, is essential for synthesis of C5 flavour volatiles in tomato. J. Exp. Bot. 2014; 65:419–428.

Shiojiri K, Kishimoto K, Ozawa R, Kugimiya S, Urashimo S, Arimura G, Horiuchi J, Nishioka T, Matsui K, Takabayashi J. Changing green leaf volatile biosynthesis in plants: An approach for improving plant resistance against both herbivores and pathogens. Proc. Natl. Acad. Sci. U. S. A. 2006; 103: 16672–16676.

Sugano S S, Shirakawa M, Takagi J, Matsuda Y, Shimada T, Hara-Nishimura I, Kouchi T. CRISPR/Cas9-mediated targeted mutagenesis in the liverwort *Marchantia polymorpha* L. Plant Cell Physiol. 2014; 55: 475–481.

Sun X, Wang G, Gao Y, Chen Z. Screening and field evaluation of synthetic volatile blends attractive to adults of the tea weevil, *Myllocerinus aurolineatus*. Chemoecology. 2012; 22: 229–237.

Vancanneyt G, Sanz C, Farmaki T, Paneque M, Felix Ortego, Castanera P, Sa´nchez-Serrano J J. Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. Proc Natl. Acad. Sci. U.S.A. 2001; 98: 8139–8144.

Wang G, Sun X, Cai X, Chen Z. Effects of Tea volatiles on foraging behavior of *Evarcha albaria*. J. Ecol. Rural Environ. 2012; 28: 394–398.

CHAPTER ONE

n-Hexanal and (*Z*)-3-hexenal are generated from arachidonic acid and linolenic acid by a lipoxygenase in *Marchantia polymorpha* L.

Abstract

Most terrestrial plants form green leaf volatiles (GLVs), which are mainly composed of six-carbon (C6) compounds. In my effort to study the distribution of the ability of lipoxygenase (LOX) to form GLVs, I found that a liverwort, *Marchantia polymorpha*, formed *n*-hexanal and (*Z*)-3-hexenal. Some LOXs execute a secondary reaction to form short chain volatiles. One of the LOXs from *M. polymorpha* (MpLOX7) oxygenized arachidonic and α-linolenic acids at almost equivalent efficiency and formed C6-aldehydes during its catalysis; these are likely formed from hydroperoxides of arachidonic and α-linolenic acids, with a cleavage of the bond between carbon at the base of the hydroperoxy group and carbon of double bond, which is energetically unfavorable. These lines of evidence suggest that one of the LOXs in liverwort employs an unprecedented reaction to form C6 aldehydes as by-products of its reaction with fatty acid substrates.

1.1 Introduction

Lipoxygenases (LOXs) are non-heme iron dioxygenases that can catalyze fatty acids containing (1*Z*,4*Z*)-pentadiene moiety, such as arachidonic, eicosapentaenoic, α-linolenic, and linoleic acids to yield the corresponding fatty acid hydroperoxides with 1-hydroperoxy-(2*E*,4*Z*)-pentadiene moiety (Andreou and Feussner 2009). Thus, the fatty acid hydroperoxides formed are further metabolized to form an array of bioactive lipid-mediators, which include leukotrienes and lipoxins in mammalian cells (Kuhn et al. 2015). In seed plants, green leaf volatiles (GLVs) consisting of six-carbon (C6) aliphatic aldehydes, alcohols, acetates, and jasmonates, among others, are extensively studied metabolites derived from LOX-produced fatty acid hydroperoxides (Mosblech et al. 2009). In seed plants, linoleic acid and α-linolenic acid are oxygenated by LOX to form the corresponding 13-hydroperoxides, which are further metabolized with hydroperoxide lyase (HPL) to yield C6 aldehydes, such as *n*-hexanal and (*Z*)-3-hexenal, respectively. A portion of the C6 aldehydes is further reduced to form the corresponding alcohols and further converted into their acetates (Matsui 2006). Allene oxide synthase (AOS) also acts on the 13-hydroperoxides. The product formed by AOS from α-linolenic acid 13-hydroperoxide is further metabolized to form 12-oxo phytodienoic acid, and subsequently to form a phytohormone (jasmonic acid) and its derivatives (jasmonates) (Mosblech et al. 2009). C6 volatiles and jasmonates, cumulatively called plant oxylipins, are involved in defense against pathogens and herbivores (Mosblech et al. 2009). Since a portion of C6 volatiles is emitted from the plant source to the atmosphere, they have been proposed to be involved in indirect defense in attracting carnivores of herbivores and in plant-plant communication (Matsui 2006; Scala et al. 2013). HPL and AOS share sequence homology and belong to a subfamily of cytochrome P450, CYP74 (Mosblech et al. 2009).

One of the questions to be resolved is how and when the ability to form plant oxylipins was acquired. The ability to form C6 aldehydes was found in a wide variety of plants ranging from liverworts to angiosperms (Hatanaka 1993; Matsui et al. 1996). A brown alga, *Laminaria angustata*, and a red alga, *Pyropia haitanensis*, are also reported to have the ability to form C6 aldehydes (Boonprab et al. 2003; Chen et al. 2015). Recent analysis on the genome sequence of a moss, *Physcomitrella patens*, indicated that the genome has two AOSs involved in oxophytodienoic acid synthesis and one HPL involved in C9 aldehyde synthesis. Two CYP74 genes found in the genome of *Marchantia polymorpha* encoded AOSs and showed no HPL activities to form C6 aldehydes from 13-HPO of C18 fatty acid (Koeduka et al. 2015).

Unexpectedly, in the course of examining the occurrence of the ability to form C6 volatiles with several non-seed plants, I found that *M. polymorpha* has this ability. It was assumed that an enzyme or enzymes other than HPL are accountable for the ability. Some LOXs are known to form volatile compounds during their catalysis of reactions with fatty acid substrates as by-products (Gardner 1991; Shen et al. 2014; Mochizuki et al. 2016). Recently, an ability to form C6 aldehyde was reported with one of the LOXs in a red alga (Chen et al. 2015). I therefore examined whether one of the LOXs in *M. polymorpha* has the ability to form C6 aldehydes.

1.2 Material and methods

1.2.1 *Plant materials*.

To clone the *LOX* gene, an archegoniophore of *M. polymorpha* (a female strain; Yam3) (Tanaka et al. 2016), was collected from the Yoshida campus of Yamaguchi University, Yamaguchi, Japan in May 2014. For volatile analysis and determination of transcript levels of genes, the thalli and archegoniophore of the female strain (Yam3) and antheridiophore of the corresponding male strain (Yam4) were collected from the same location on October 2, 2016.

1.2.2 *RT-PCR cloning and heterologous expression of MpLOX7*.

Total RNA was isolated from archegoniophore (100 mg Fresh Weight) using RNeasy kit (Qiagen, Hilden, Germany). After confirming the intactness of RNA with denatured agarose gel electrophoresis, genomic DNA was removed with DNAase I, and then, cDNA was synthesized using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's protocol. The forward primer 5′-CGCGGATCCATGGAGGGTTCTCTAGAGCGCAG-3′ (underline: *Bam*HI site) and the reverse primer 5′-CGCGGTACCTTAGATGGAGGTACTGTATGGAACC-3′ (underline: *Kpn*I site) were used to amplify the coding region of MpLOX7 with KOD Plus polymerase (Toyobo, Osaka, Japan). After adding the A-overhang using ExTaq polymerase (Takara Bio Inc., Otsu, Japan), the amplified fragment was cloned into the pGEM-T Easy Vector (Promega, Madison, WI, USA). After confirming the sequence, the open reading frame was sub-cloned into the pQE-30 expression vector and transformed into *Escherichia coli* (SG13009) (Qiagen). The cells were incubated overnight at 37°C with shaking in 3 mL Luria Bertani (LB) medium containing 100 $\mu g \cdot mL^{-1}$ of ampicillin, then transferred to 50 mL LB with ampicillin, and incubated at 37°C for another 2 h

with shaking until an OD600 of the culture reached 0.6. Isopropyl thio-β-D-galactoside was added to a final concentration of 0.5 mM, and bacteria were incubated for an additional 36 h at 16°C with shaking. Bacteria were centrifuged, and the pellet was suspended in 10 mL lysis buffer containing 50 mM sodium phosphate, pH 7.4, 300 mM NaCl, 10 mM imidazole, and 100 μg mL^{-1} lysozyme (Sigma-Aldrich, St. Louis, MO, USA). The samples were incubated on ice for 30 min with occasional stirring, and the cells were disrupted with a tip-type sonicator (10 sec × 3 times; Ultrasonic disruptor UD-201, TOMY, Tokyo, Japan). The lysate was centrifuged at 15,000 ×*g* for 30 min at 4°C, and the supernatants were applied to a 1.5 mL Ni-NTA column (Qiagen). The tagged enzyme was eluted with 3 mL of 50 mM sodium phosphate buffer at pH 7.4, 300 mM NaCl, and 250 mM imidazole. Imidazole was removed with gel filtration on PD-10 column (GE Healthcare Life Sciences, Chicago, IL, USA) equilibrated with 50 mM potassium phosphate buffer (pH 7.4). Protein was determined using Pierce BCA (Thermo Fisher Scientific) with bovine serum albumin as a standard.

1.2.3 *Phylogenetic analysis*.

Phylogenetic tree was conducted using the neighbor-joining method in MEGA6 software (Tamura et al., 2013). All the genes showing similarity with Arabidopsis LOX2 based on a BLASTP analysis in the genome database of *Chlamydomonas reinhardtii*, *Klebsormidium flaccidum*, *M. polymorpha*, *P. patens*, and *Selaginella moellendorffii*, concomitant with several representative seed plants (*Arabidopsis thaliana*, *Zea mays*, *Populus trichocarpa*, and *Picea abies*) (JGI Genome Portal, http://genome.jgi.doe.gov/) were chosen and aligned by using the L-INS-i method of MAFFT version 7 (http://mafft.cbrc.jp/alignment/software/). Gblocks (Talavera and Castresana 2007) was used to remove any poorly conserved regions.

1.2.4 *LOX activity*.

LOX activity was determined following an increase in absorption at 234 nm with a photometer (UV-160A, Shimadzu, Kyoto, Japan) with 50 mM potassium phosphate buffer (pH 7.4) at 25°C. In most cases, the fatty acid substrate was suspended at 50 mM with detergent (0.2% Tween 20, w/v) solution by using a tip-type sonicator under N_2 or Ar atmosphere in order to avoid autooxidation, and 5 μL of the fatty acid suspension was used in 1 mL of the reaction (final concentrations of fatty acid and Tween 20 were 0.25 mM and 0.001%, respectively). The reaction

was initiated by adding the enzyme solution. In most cases, a lag phase was observed, and the reaction velocity gradually increased after the onset of the reaction. Therefore, the maximum velocity of the reaction that appeared under the condition (typically after 1 to 3 min of the onset of reaction) was used for calculation.

1.2.5 *Kinetic parameter of LOX enzyme*.

The enzyme activity were determined in a different series of substrate concentration (from 5 to 100 μM) in triplicates. The experiment was done at room temperature in 1 mL enzyme mixture with potassium phosphate buffer (pH 7.4), and the obtained kinetic parameters were used to draw S/V plot and applied on a Lineweaver-Burk plot to get the kinetic parameters (k_m, Vmax, k_{cat} and k_m / k_{cat}) by using Hyper32 software (developed by the University of Liverpool).

1.2.6 *pH Selectivity.*

Following the same procedures used in kinetic parameter analysis. The activity were examined with Tris-HCl, MES-KOH and potassium phosphate, of 50 mM at pH from 5 to 9. Activity was calculated according to increase in the absorbance at 234 nm with time and by using molecular coefficient ε = 25,000 M^{-1} cm^{-1}, and the activity was shown in nKat.mL^{-1} unit.

1.2.7 *Analysis of the product*.

For product analysis, purified protein (6 μg) was incubated with 10 μL of 8 mM linoleic acid (suspended with 0.2% Tween 20) in 50 mM potassium phosphate buffer (pH 7.4) for 10 min at 25°C. The reaction was terminated by adjusting the pH to 4.0 with 1N HCl, and the products were extracted with 20 mL diethylether. Triphenylphosphine was added into the extract to reduce the unstable hydroperoxides to the corresponding hydroxyl derivatives. After drying over anhydrous Na_2SO_4, the solvent was removed under vacuum, and the resultant residues were dissolved in a small volume of ether. Fatty acid hydroperoxides were separated with a straight phase HPLC with a ZORBAX SIL column (4.6 × 250 mm, 5 μm) (Agilent Technologies, Santa Clara, CA, USA) with a solvent consisting of hexane:2-propanol:acetic acid (98.5:1.5:0.05, v/v/v) at 0.8 mL min^{-1} at 40°C. A photodiode detector, SPD-M20A (Shimadzu), was used, and in most cases, absorption at 234 nm was monitored. The positional and geometrical isomers of hydroperoxides of linoleic, linolenic, and arachidonic acids were identified with the corresponding

authentic compounds prepared with partially purified soybean LOX1 [13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (13-HPOD), 13-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid (13-HPOT), and 15-hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid (15-HPETE) were the main products] (Boonprab et al. 2003, Yanagi et al. 2011), concomitant with a mixture of HPOD prepared with autooxidation (overnight at 40°C), and (*S*)-HETE HPLC mixture purchased from Cayman Chemical (Ann Arbor, MI, USA).

1.2.8 *Volatile analysis*.

In order to examine volatile formation during MpLOX7 catalysis on fatty acids, the reaction was carried out in a 12 mL glass vial containing 1 mL enzyme mixture consisting of purified MpLOX7 (1.8 μg), 8 μL of 2.5 mM substrate (suspended with 0.2% Tween 20 and final concentration at 20 μM), and 972 μL of 50 mM potassium phosphate buffer (pH 7.4). After reacting for 7 min at 25°C, the reaction was stopped by adding 1 mL saturated $CaCl_2$ solution. The volatiles were collected by exposing an SPME fiber (50/30 μm DVB/Carboxen/PDMS, Supelco, Bellefonte, PA, USA) to the headspace of the vial at 40°C for 30 min. Immediately after collection, the volatiles were desorbed in the insertion port of a GC-MS (QP-5050, Shimadzu) set at 200°C. Injection of the volatiles was carried out in the splitless mode with 1 min sampling time. A DB-WAX column (30 m length × 0.25 mm diameter × 0.25 μm film thickness; Agilent Technologies) was used to separate volatiles at 40°C (1 min) to 180°C (1 min) at 15°C min^{-1}. The carrier gas (He) was delivered at 86.1 kPa. The fiber was held in the injection port for 10 min to fully remove any compounds from the matrix. The mass detector was operated in the electron impact mode with ionization energy at 70 eV. To identify each compound, retention index and MS profile of the corresponding authentic specimen were used, and some of the compounds were quantified with calibration curves prepared using the standard compound under the same volatile collection conditions.

Volatiles from different organs (100-150 mg fresh weight) of *M. polymorpha* were collected with the SPME fiber in the glass vial as described above. For mechanical wounding, each organ was pinched seven times with forceps and quickly placed into the vial, which was then closed tightly. SPME collection was carried out at 25°C for 30 min, and then, the adsorbed volatiles were analyzed with GC-MS as described above.

1.2.9 MpLOX7 *transcriptional level*.

Total RNA was extracted with FavoPrep plant total RNA mini kit (Favorgen Biotech Corporation, Ping-Tung, Taiwan) from the organs harvested at the same time with those used for volatile analysis. Mechanically wounded organs were harvested 10 min after wounding. After confirming the intactness of the RNA through denatured agarose gel electrophoresis, genomic DNA was digested with DNase I, and cDNA was synthesized with oligo d(T) primer using ReverTra Ace qPCR RT kit (Toyobo). The primers used were: 5′-TCGAGTGAAACCGGAAAGAAG-3′ (sense) and 5′-TTGAAGGCGTCGTGGTAATC-3′ (antisense). KOD FX Neo polymerase (Toyobo) was used to amplify, and *MpEF1α* gene was used as a control gene (Kanazawa et al., 2016).

1.3 Results

1.3.1 *Formation of carbon six aldehydes.*

M. polymorpha has been reported to contain terpenoids, especially sesquiterpenoids, as well as C8 volatiles (Suire et al. 2000; Asakawa et al. 2013; Kihara et al. 2014; Tanaka et al. 2016), but so far no report on the formation of C6 volatiles in the organs of *M. polymorpha* is available. The amounts of *n*-hexanal and (*Z*)-3-hexenal formed from intact organs of *M. polymorpha* were low, and trace amount of *n*-hexanal was detected only with intact archegoniophore and antheridiophore (Fig. 1-1A). Mechanical wounding enhanced their formation in thalli, archegoniophore, and antheridiophore (Fig. 1-1B). Reproductive organs, i.e., archegoniophore and antheridiophore, showed higher abilities to form *n*-hexanal than thalli. The amount of (*Z*)-3-hexenal formed from wounded archegoniophore was higher than that from wounded thalli or wounded antheridiophore. Archegoniophore also higher amount of C5 and C8 than other organs (Figures 1-2A, B)

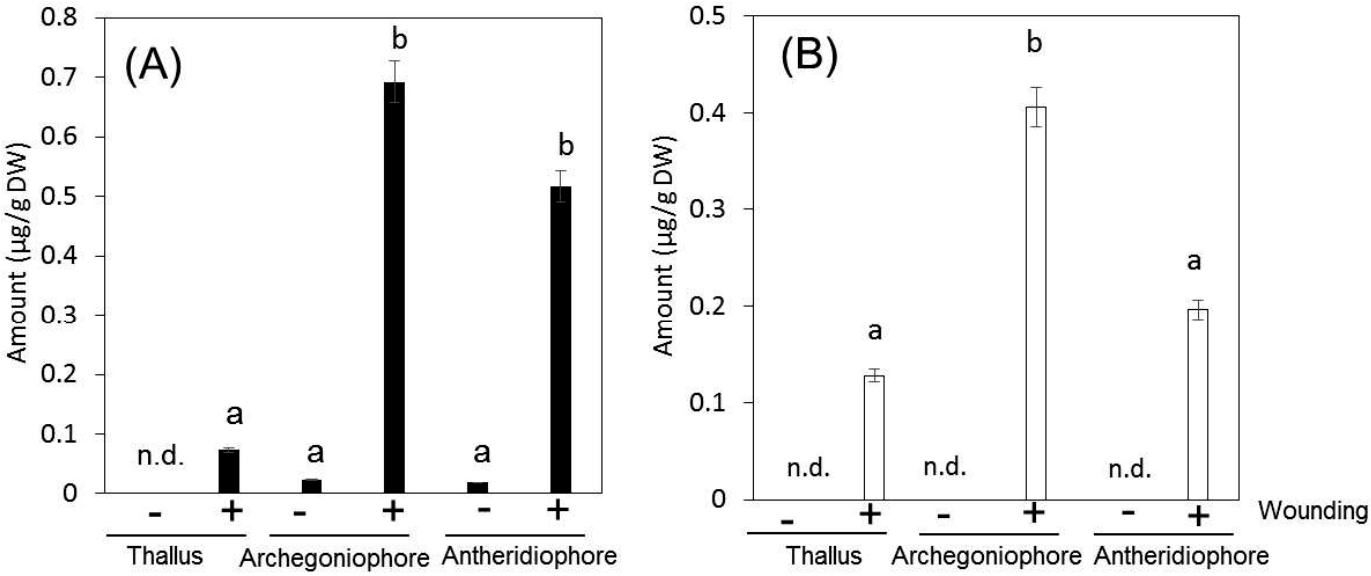
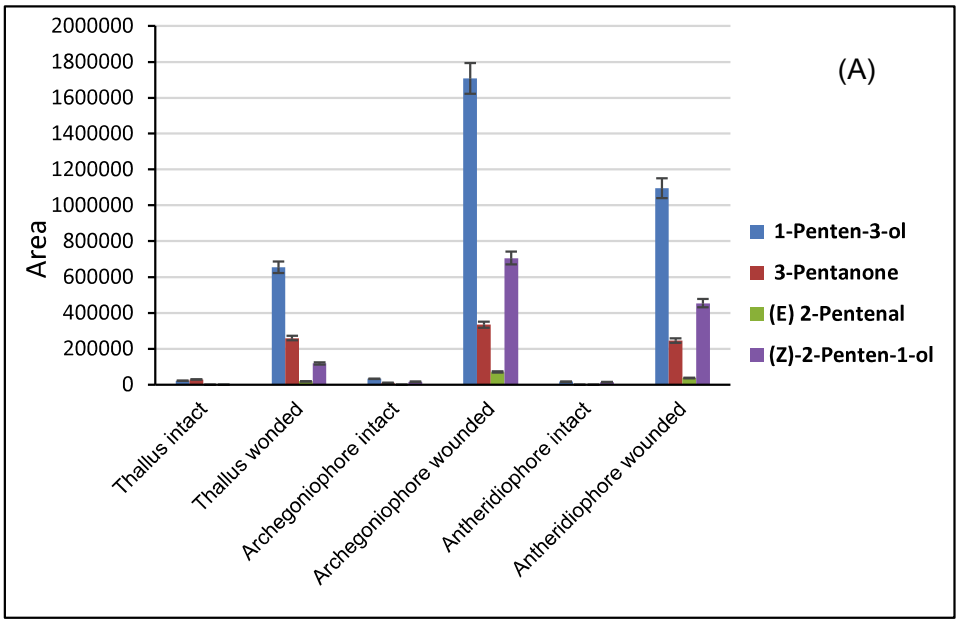


Figure 1-1. The amounts of *n*-hexanal (A) and (*Z*)-3-hexenal (B) formed from intact or mechanically wounded thalli, antheridiophores, and archegoniophores. Mean ± standard error is shown ($n = 3$). Different letters indicate significant differences between means ($P < 0.01$, two-way ANOVA with Bonferroni test for *n*-hexanal, and one-way ANOVA with Bonferroni test for (*Z*)-3-hexenal).



C5 volatiles

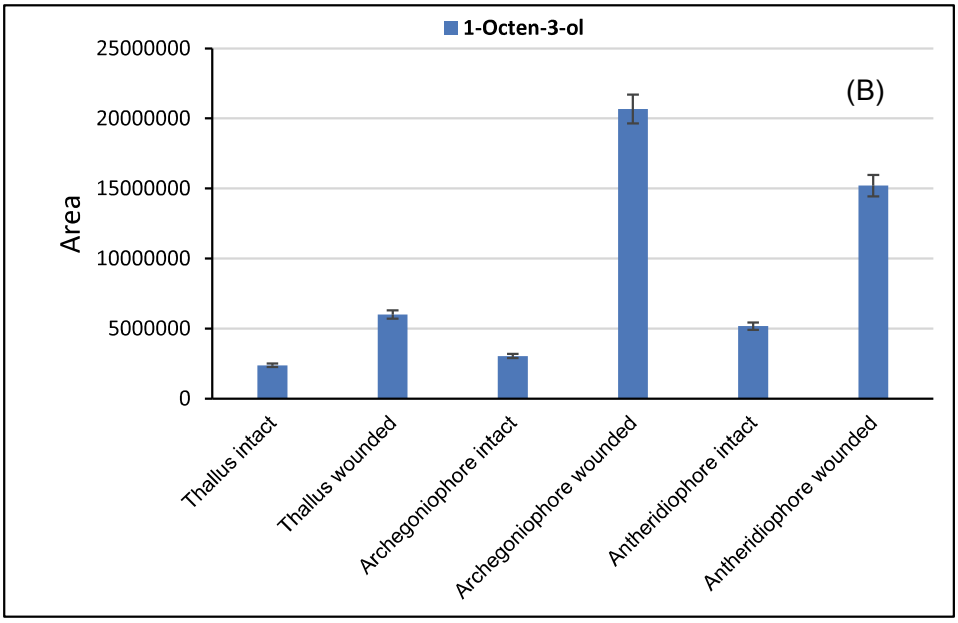


Figure 1-2. The C5 (A) and C8 (B) volatiles emitted out from intact, wounded thalli, antheridiophores, and archegoniophores. Volatiles were collected with SPME, and subsequently analyzed with GC-MS.

1.3.2 *Lipoxygenases in* M. polymorpha.

When the genome database of *M. polymorpha* (Marchantia Genome, marchantila.info/genome) was blast-searched with the amino acid sequence of Arabidopsis lipoxygenase 2 (At3g45140) as a query, 16 genes (including one pseudogene) showing significant similarities to the query sequence were found (Figure 1-3). Based on the results of ChloroP analyses (www.cbs.dtu.dk/services/ChloroP/), they were classified into chloroplast-localized (11 genes) and others (4 genes) (Figure 1-3). The phylogenetic analysis with LOXs found in reported reference genomes of non-seed plants, *C. reinhardtii*, *K. flaccidum*, *M. polymorpha*, *P. patens*, and *S. moellendorffii*, PpLOX1, a multifunctional arachidonic acid 12-LOX with fatty acid hydroperoxide cleaving activity (Senger et al. 2005), and PpLOX2, the other *P. patens* 12-LOX (Anterola et al. 2009), were located in a separated clade together. LOXs from green algae, *C. reinhardtii* and *K. flaccidum*, are also located with 12-LOXs from *P. patens*.

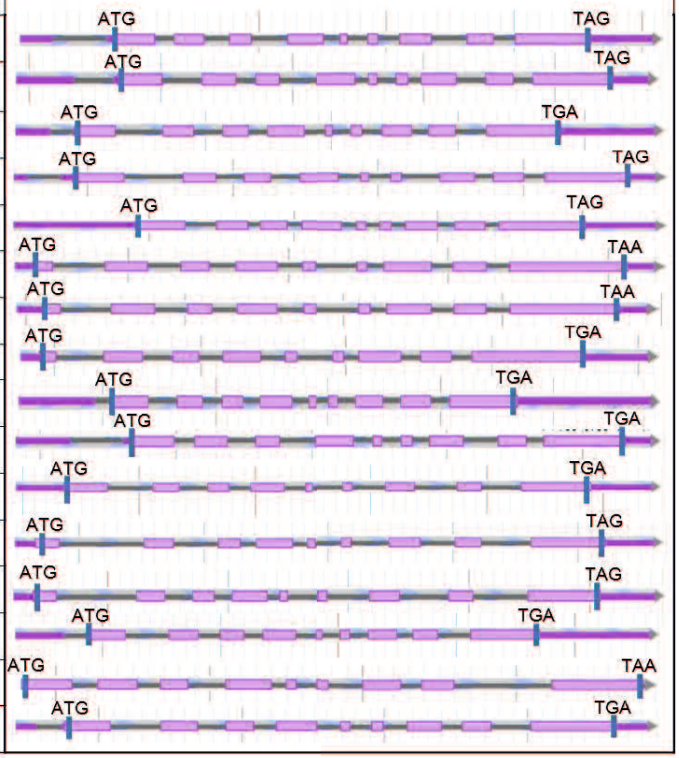
Gene short name	MpLOX order	ChloroP (judgement, potential length of transit peptide)	Genes with exons, introns, 3' UTR and 5' UTR representation
Mapoly0028s0085.1	MpLOX1	Y 63	
Mapoly0191s0014.1	MpLOX2	Y 65	
Mapoly0006s0130.1	MpLOX3	Y 32	
Mapoly0106s0031.1	MpLOX4	Y 72	
Mapoly0001s0529.1	MpLOX5	Y 35	
Mapoly0023s0182.1	MpLOX6	- 46	
Mapoly0026s0145.1	MpLOX7	- 72	
Mapoly0026s0148.1	MpLOX8	- 74	
Mapoly0055s0019.1	MpLOX9	Y 41	
Mapoly0063s0014.1	MpLOX10	Y 63	
Mapoly0063s0050.1	MpLOX11	Y 42	
Mapoly0064s0034.1	MpLOX12	- 19	
Mapoly0079s0056.1	MpLOX13	- 78	
Mapoly0083s0028.1	MpLOX14	Y 69	
Mapoly0191s0015.1	MpLOX15	Y 26	
Mapoly0420s0001.1	MpLOX16	Y 40	

Figure 1-3. Detailed information on *Marchantia polymorpha* lipoxygenases genes.

Among MpLOXs in the non-seed plant LOX clade, MpLOX1-3 with transit peptide sequences for chloroplast localization on their N-termini has been already studied (Kanamoto et al. 2012). Since a *P. haitanensis* gametophyte LOX (PhLOX2) that shows multifunctional enzyme activity to form C6 aldehydes is a cytosolic protein (Chen et al. 2015), I focused on one of the cytosolic LOXs in the specific clade of non-seed LOXs, MpLOX7, for further analyses (Figure 1-4).

The open reading frame of MpLOX7 encoded 917 amino acid polypeptides with an estimated molecular weight of 104.6 kDa (Figure1-5). The sequence contains PLAT (polycystin-1, lipoxygenase, alpha-toxin) domain that is generally found in most LOXs ranging from plants to mammals as the domain to interact with lipids or membrane through forming β-barrel structure (Eek et al. 2012, Newcomer and Brash 2015). It also has three histidine residues, one asparagine residue, and one carboxy-terminal isoleucine residue as the ligands for the non-heme iron atom in positions among amino acid sequences conserved among most plant LOXs (Andreou & Feussner 2009). The region spanning from L298 to F308 seemed to be a region showing substantial homology only among the LOXs in the non-seed plant LOX clade (Figure 1-6).

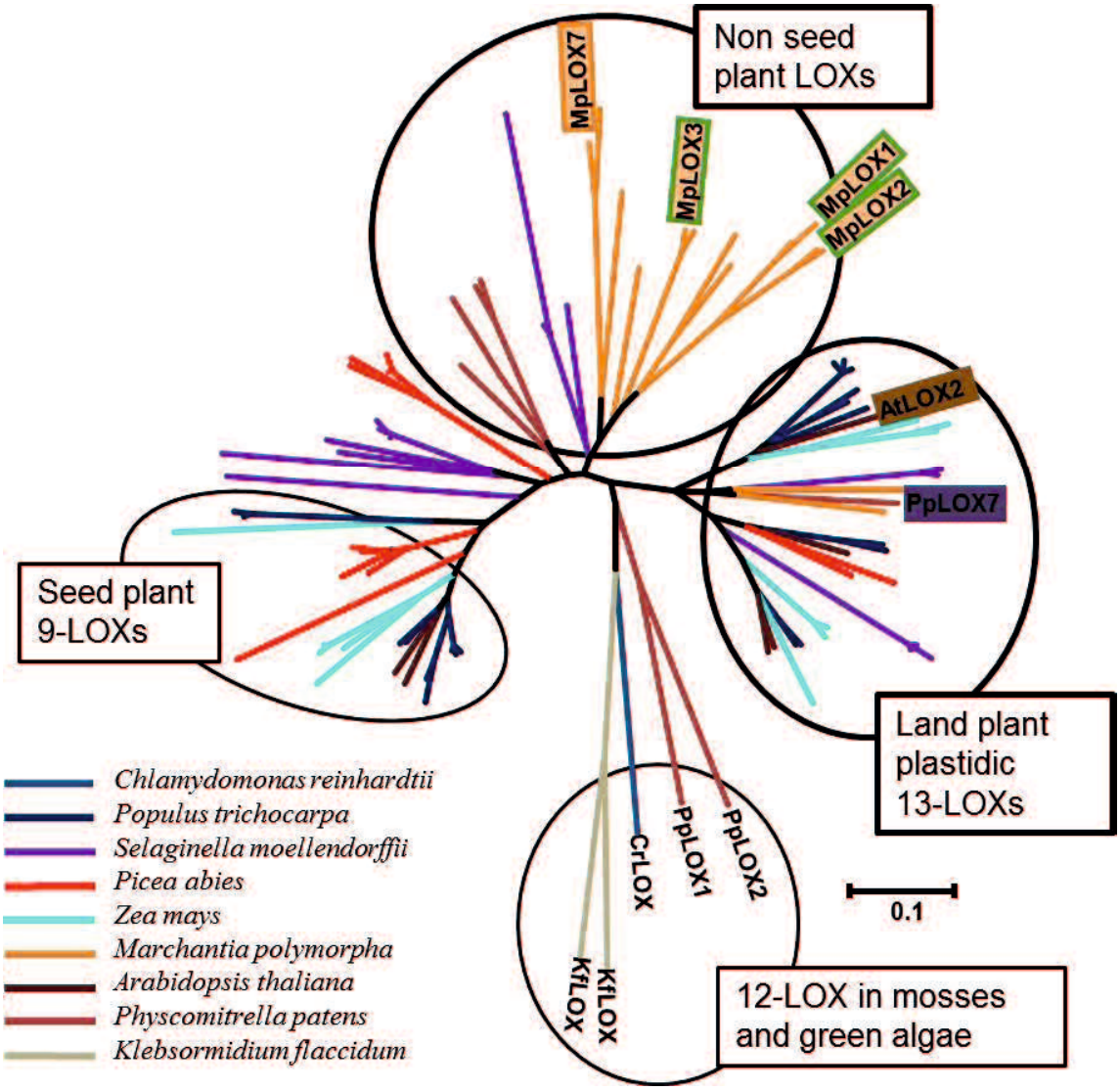


Figure 1-4. Phylogenetic analysis of MpLOXs with the LOXs from *Chlamydomonas reinhardtii*, *Klebsormidium flaccidum*, *Marchantia polymorpha*, *Physcomitrella patens*, *Selaginella moellendorffii*, *Arabidopsis thaliana*, *Zea mays*, *Populus trichocarpa*, and *Picea abies*. All the genes that showed similarity with Arabidopsis lipoxygenase 2 are aligned, and the phylogenetic analysis is performed using the neighbor-joining method of MEGA6 software. The list of LOXs used for the phylogenetic analysis is in supplemental table S1. The names of LOXs mentioned in the text are shown.

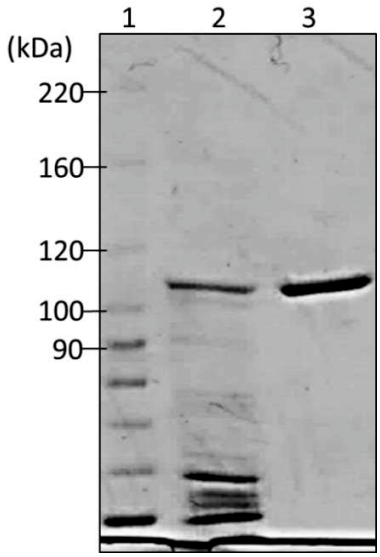


Figure 1-5. The SDS-PAGE gel image of expressed recombinant MpLOX7. Lane 1: molecular weight marker, lane 2: crude lysate, lane 3: protein after purification with Ni-affinity column.

1.3.3 *Properties of recombinant MpLOX7.*

The open reading frame of MpLOX7 was RT-PCR-cloned with cDNA prepared from archegoniophore, and subsequently expressed in *E. coli* as an N-terminal His-tagged recombinant protein. The properties of recombinant MpLOX7 were determined after purification to a homogenous state with affinity chromatography (Figure 1-5).

pH Selectivity, This experiment showed that MpLOX7 prefer the medium pH of 7.4, more than acidic pH or basic pH (Figure 1-7). The best buffer was potassium phosphate buffer, after comparing activity with Tris-HCl and MES-KOH to form HPO by following absorption at 234 nm. While MpLOX1, 2, 3 prefer the same buffer and in almost similar pH (potassium phosphate, pH 7) (Kanamoto et al. 2012), and *P. patens* lipoxygenases prefers phosphate buffer (pH 7) (Anterola et al. 2009).

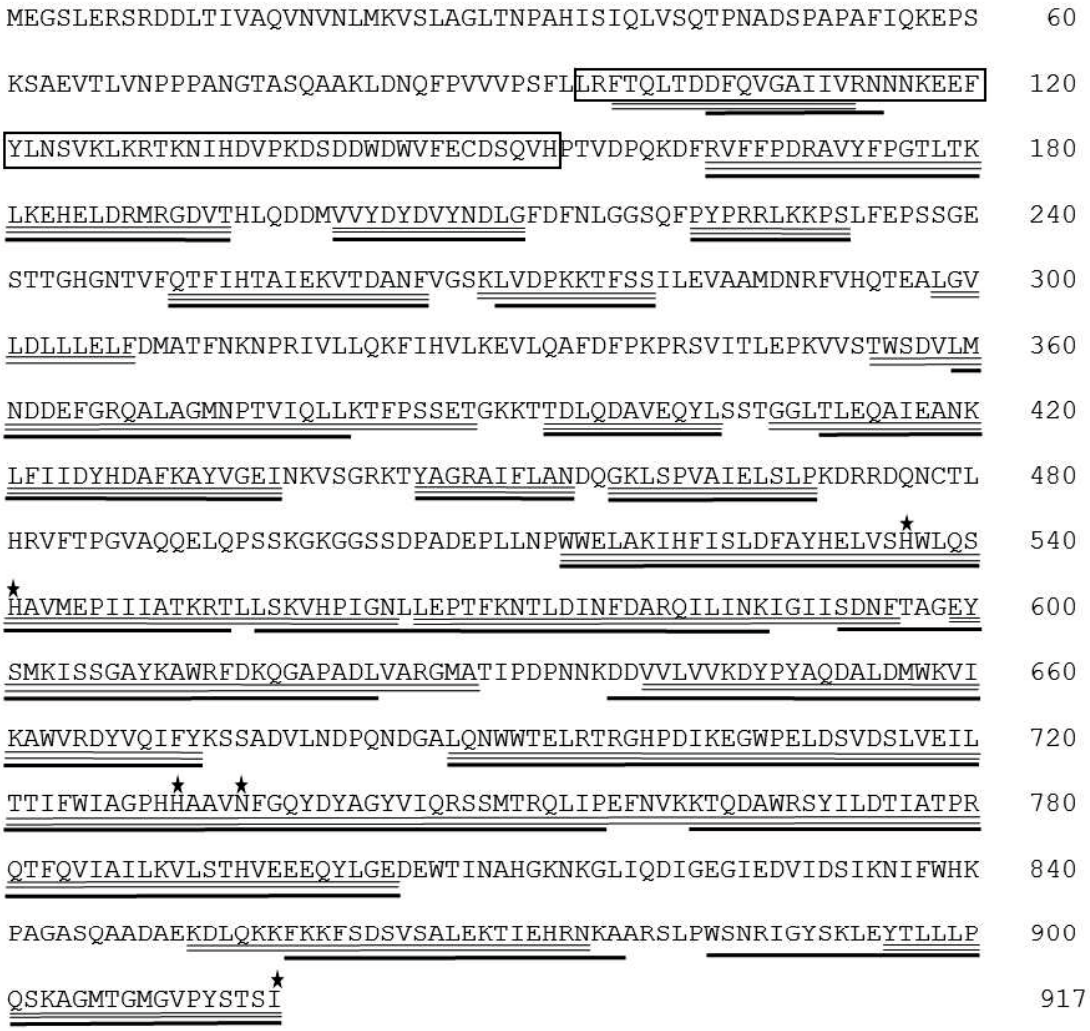


Figure 1-6. Amino acid sequence of MpLOX7. Histidines, asparagine, and isoleucine residues involved in binding iron atom are indicated with stars. PLAT domain is shown in the box. Domains showing similarity among all the LOX sequences used for making the phylogenetic tree in Fig. 2 are underlined, and those conserved among non-seed plant LOX clade are double underlined. Alignment of the sequences is performed using the L-INS-i method of MAFFT version 7, and those reasonably aligned positions are chosen with Gblocks.

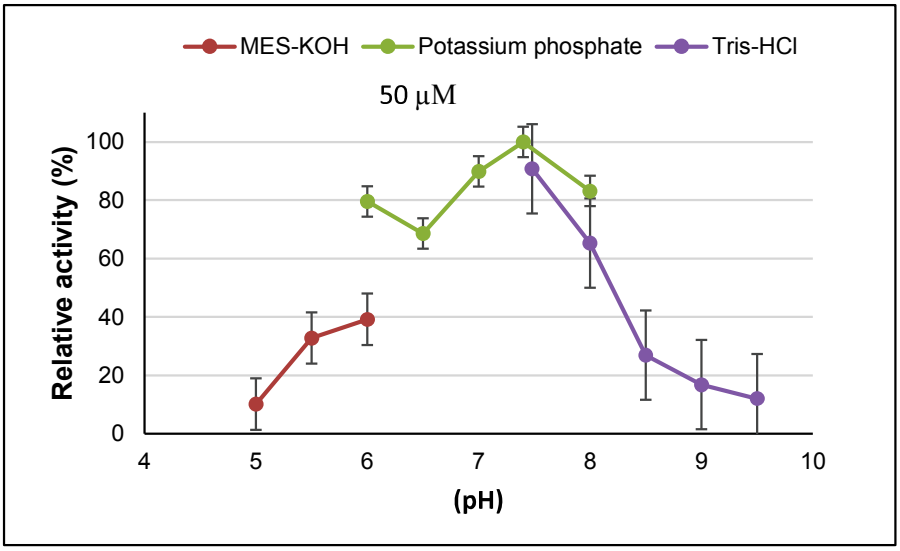


Figure 1-7. MpLOX7 enzyme activity against different buffers with different pH range from 5 to 9.5 using linoleic acid (18:2) as the substrate.

MpLOX7 was active with either C18 or C20 fatty acid with k_m and k_{cat} values equivalent to soybean LOX1 against linoleic acid (Sharma & Klinman 2015), and human 15-LOX-2 against arachidonic acid (Green et al. 2016) (Table 1). Among the four fatty acids examined in the present study, linoleic acid showed the lowest k_m value, whereas α-linolenic acid showed the highest k_{cat} value. Gamma-linolenic acid was a relatively poor substrate (Figure 1-8). Almost all bryophyte LOXs studied so far showed high affinity against arachidonic acid, eicosapentaenoic acid, and α-linolenic acid, but no or little activity was found with linoleic acid (Anterola et al. 2009, Kanamoto et al. 2012). PpLOX7, which showed high activity with linoleic acid but rather low activity with arachidonic acid and α-linolenic acid, was an exception. In this context, the wide substrate specificity of MpLOX7 should be noted. With C18 fatty acid, its 13-hydroperoxide was the main product, whereas with C20 fatty acid, its 15-hydroperoxide was the main product (Figures 1-9, 10, 11, 12) (Table 1).

Table 1. Kinetic parameters and main products of recombinant MpLOX7 with fatty acid substrates.

Substrate	k_m (μM)	*V*max (nkat·mg^{-1})	k_{cat} (sec^{-1})	k_{cat} / k_m (min^{-1}M^{-1}) ×106	Main product
Linoleic acid C18:2 (n-6)	**12.0**	**2270**	**237**	**1187**	13-Hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid
Arachidonic acid C20:4 (n-6)	**24.5**	**2300**	**241**	**589**	15-Hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid
α-Linolenic acid C18:3 (n-3)	**25.0**	**3000**	**314**	**753**	13-Hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid
Gamma-linolenic acid C18:3 (n-6)	**71.0**	**330**	**34.5**	**29**	13-Hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid

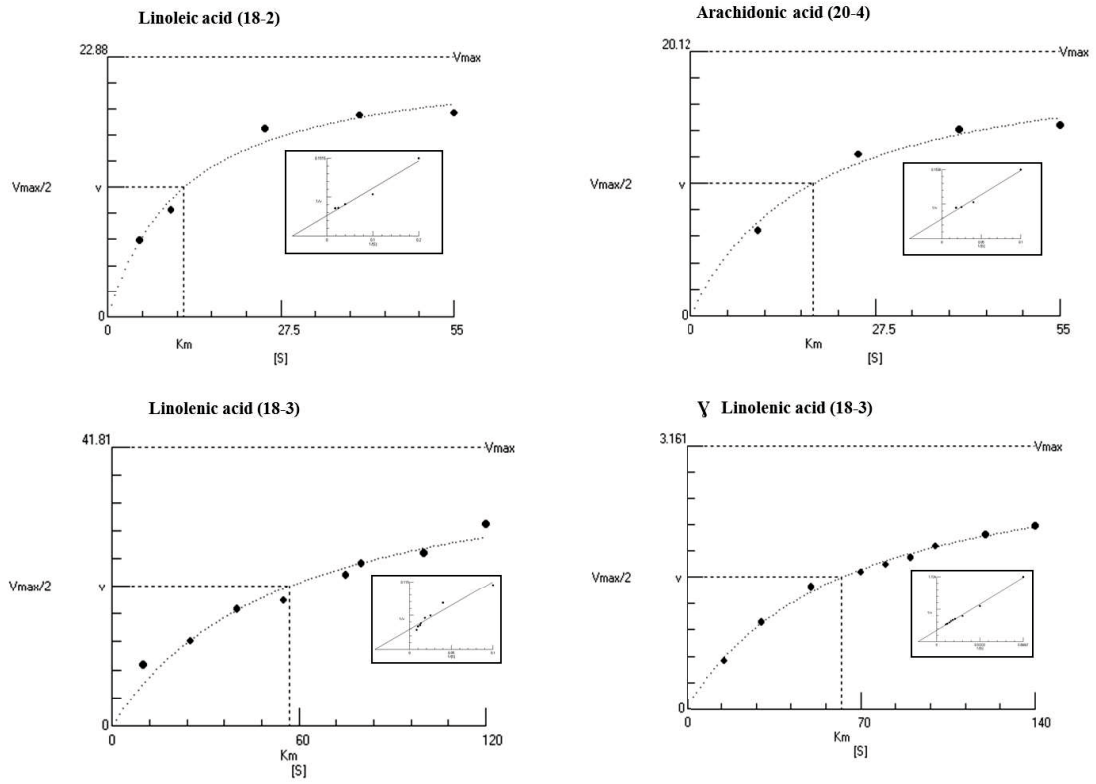


Figure 1-8. S/V plot and Lineweaver-Burk plot to show enzyme kinetics of MpLOX7.

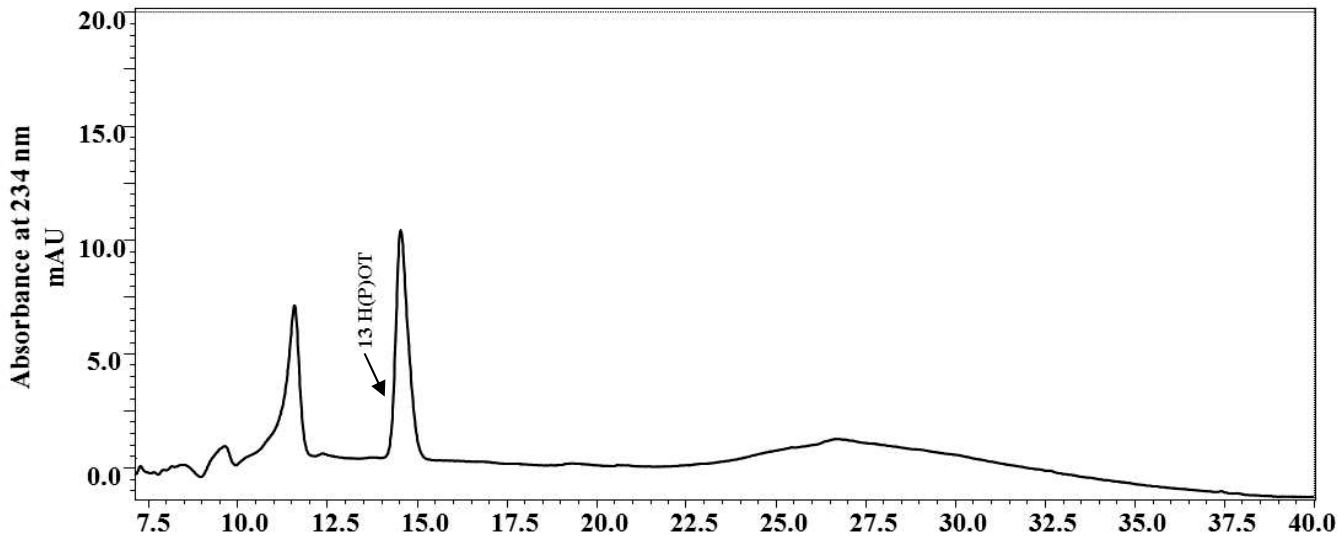


Figure 1-9. HPLC result showing the product of α-linolenic acid (18-3) with MpLOX7.

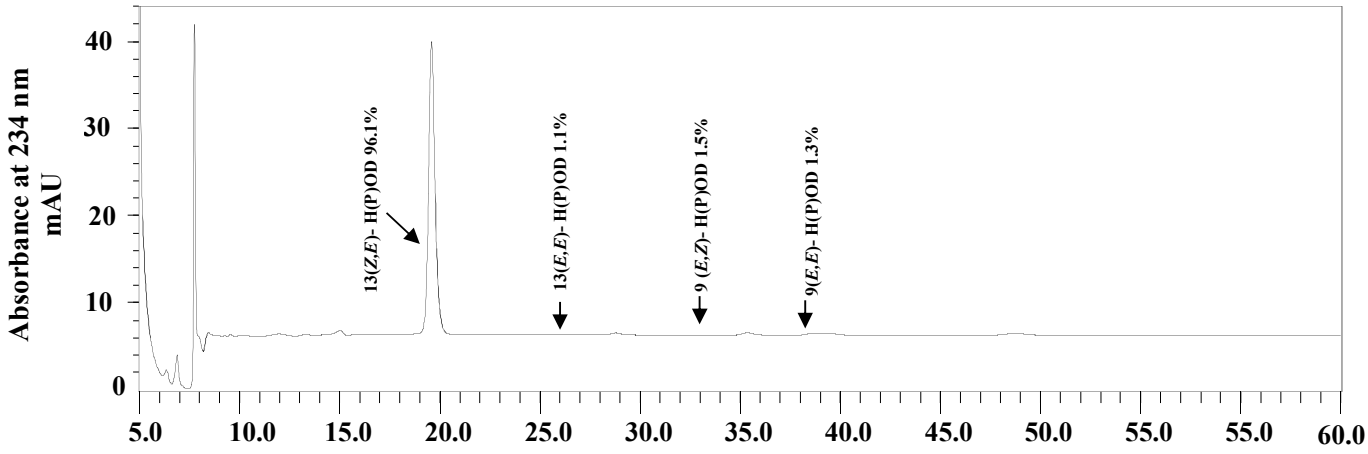


Figure 1-10. HPLC result showing the product of linoleic acid (18-2) with MpLOX7.

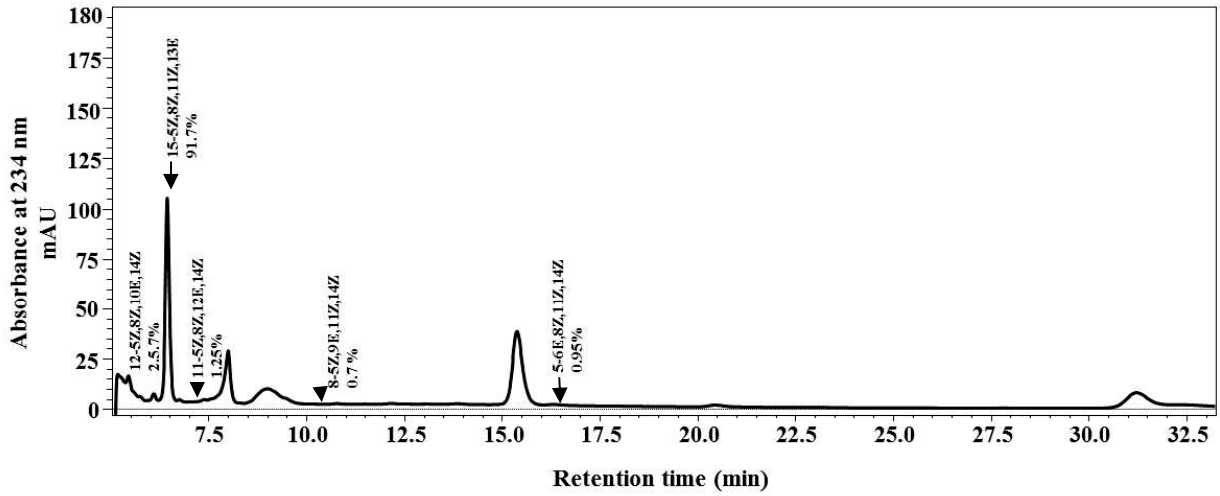


Figure 1-11. HPLC result showing the product of arachidonic acid (20-4) with MpLOX7.

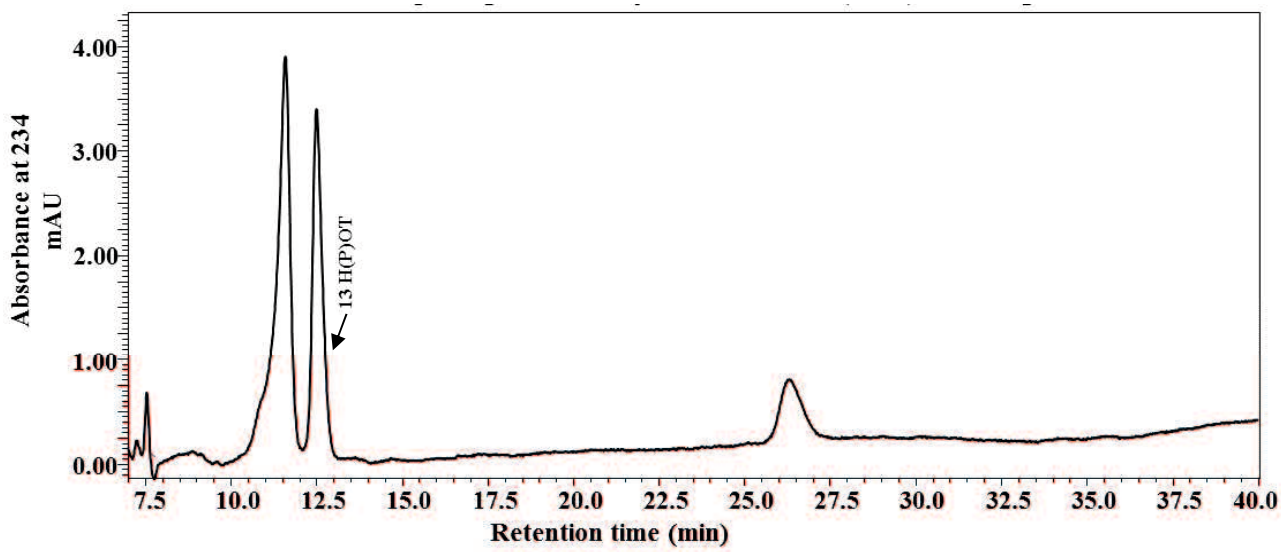


Figure 1-12. HPLC result showing the product of ɣ-linolenic acid (18-3) with MpLOX7.

1.3.4 *By-product formation due to MpLOX7 activity.*

In order to investigate volatile by-product formation during catalysis of MpLOX7 with fatty acid substrates, α-linolenic acid or arachidonic acid was reacted with MpLOX7 for 7 min, and then, the volatiles formed were analyzed with headspace SPME-GC/MS. When arachidonic acid was used as the substrate, substantial amount of *n*-hexanal was detected (Figure 1-13A). The product of MpLOX7 from arachidonic acid, 15-HPETE, is unstable, especially when transition metals would be involved in the reaction mixture. Further, some LOXs examined so far have abilities to react on fatty acid hydroperoxides to form the corresponding alkoxyl radical (Gardner 1991; Salch et al. 1995; Shen et al. 2014). Therefore, the same concentration of 15-HPETE that was formed after the reaction of MpLOX7 with arachidonic acid for 7 min under the reaction condition employed here was incubated with active or heat-inactivated MpLOX7. The amount of *n*-hexanal formed from 15-HPETE with either active or heat-inactivated enzyme was significantly lower than that formed from active enzyme reacted with arachidonic acid (Figure 1-14A). Thus, at least a portion of *n*-hexanal detected in the present study was formed during catalysis of MpLOX7 on arachidonic acid. Under the experimental condition employed here, no formation of carbon five volatile compounds could be detected. When α-linolenic acid was used as the substrate, the formation of (*Z*)-3-hexenal was detected (Figure 1-14B). When 13-HPOT was reacted with active or heat-inactivated MpLOX7, no formation of (*Z*)-3-hexenal was detected (Figure 1-13B), which indicated that (*Z*)-3-hexenal was formed during catalysis of MpLOX7 on α-linolenic acid.

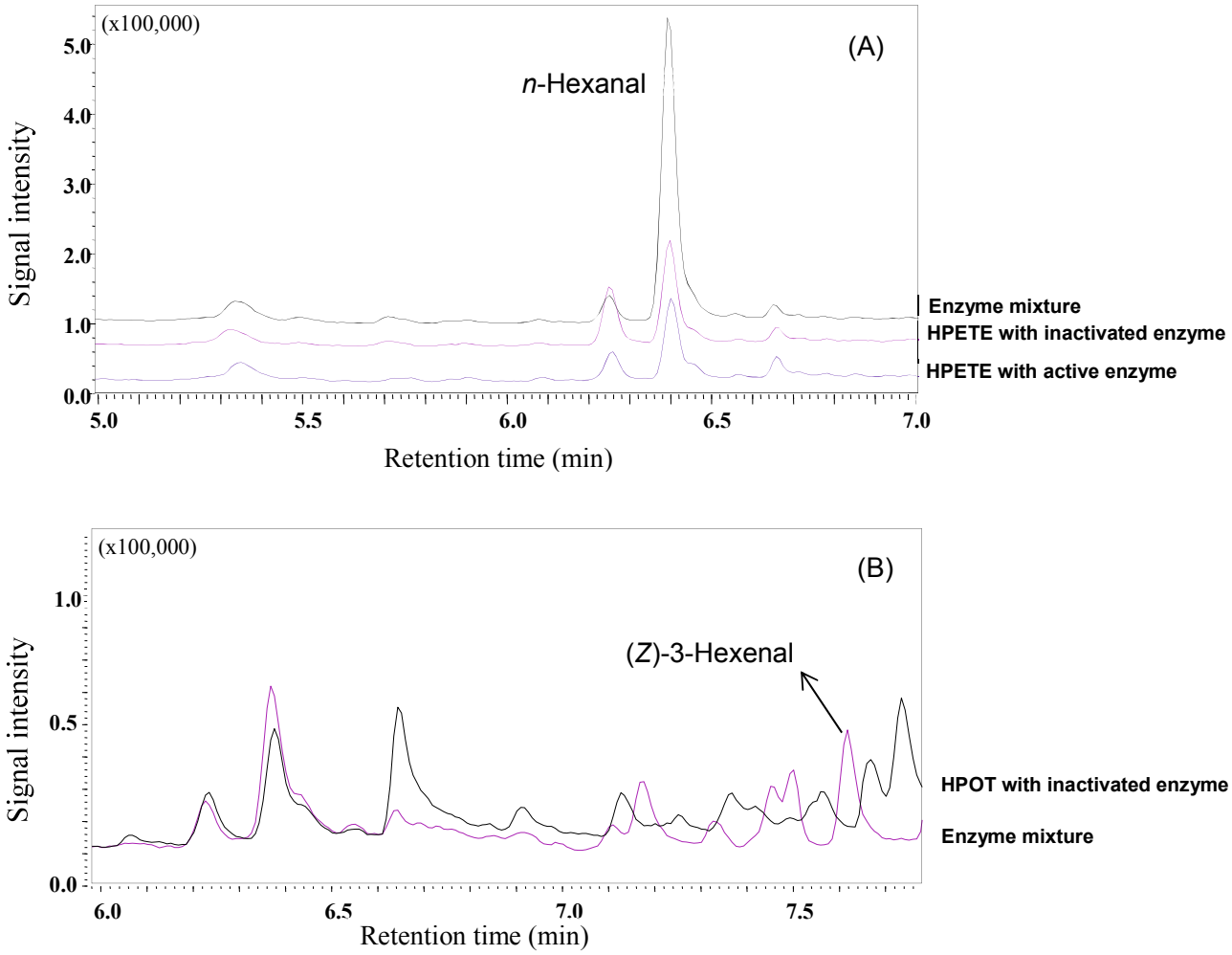


Figure 1-13. GC-MS result showing the enzymatic formation of C6 aldehydes from MpLOX7 reaction with active enzyme with arachidonic acid (A) and linolenic acid (B).

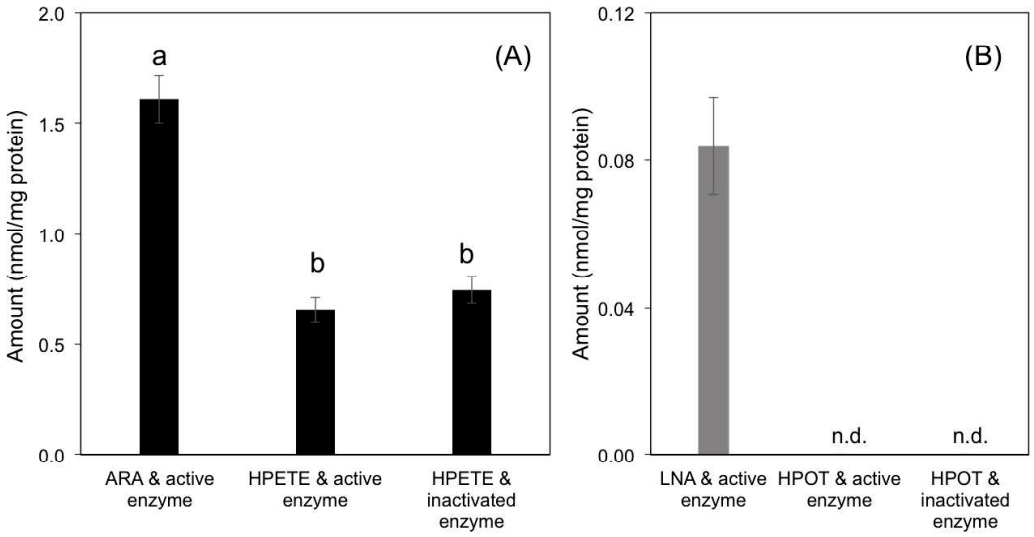


Figure 1-14. Formation of *n*-hexanal from arachidonic acid (ARA) (A) and (*Z*)-3-hexenal from α-linolenic acid (LNA) (B) during catalysis of recombinant MpLOX7. As a control experiment, hydroperoxide [arachidonic acid 15-hydroperoxide (HPETE) for (A) and α-linolenic acid 13-hydroperoxide (HPOT) for (B)] formed during catalysis is reacted with active or heat-inactivated MpLOX7, and *n*-hexanal and (*Z*)-3-hexenal formed are analyzed. Mean ± standard error is shown (n = 3). Different letters in (A) indicate significant differences between means (P <0.01, one-way ANOVA with Bonferroni test).

1.3.5 MpLOX7 *transcriptional level.*

The lines of evidence shown above suggests that MpLOX7 have an ability to form *n*-hexanal and (*Z*)-3-hexenal during its reaction on arachidonic and α-linolenic acids. In order to examine whether this reaction can occur *in vivo*, the transcript level of *MpLOX7* in thallus, archegoniophore, and antheridiophore was estimated with semi-quantitative RT-PCR with *elongation factor 1 α* (*MpEF1 α*) as a control gene (Figure 1-15). *MpLOX7* was expressed in all the three organs used here at an almost constant level. The transcript level was not changed after mechanical wounding.

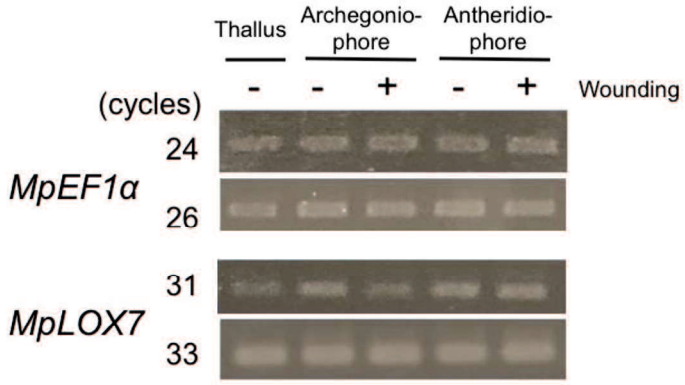


Figure 1-15. Expression level of *MpLOX7* in thalli, archegoniophores before mechanical wounding and after mechanical wounding, and antheridiophore before mechanical wounding and after mechanical wounding. *MpEF1a* is used as a control gene to standardize the amount of template used for PCR. Amplification with 31 or 33 cycles for *MpLOX7* (137 bp) and 24 or 26 cycles for *MpEF1α* (448 bp) is shown in order to compare their expression levels before saturation of amplification.

1.4 Discussion

In the present study, I found that *M. polymorpha* formed C6 aldehydes, such as *n*-hexanal and (*Z*)-3-hexenal (Figure 1-1), and that their formation was intensely induced after mechanical wounding. This reminded us of the rapid formation of GLVs including C6 aldehydes with leaves of angiosperms after mechanical wounding (Mochizuki et al. 2016; Christeller & Galis 2014). Six-carbon GLVs are formed from 13-hydroperoxides of linoleic and linolenic acids through the catalysis of a specialized cytochrome P450 belonging to CYP74 family, hydroperoxide lyase (HPL), in angiosperms. When the genome sequence of *M. polymorpha* was examined, two CYP74 genes were detected, but both encoded AOSs (Koeduka et al. 2015). There was no HPL activity with the two AOSs of *M. polymorpha* at least *in vitro* with the corresponding recombinant enzymes expressed in *E. coli*. Therefore, it was assumed that the other enzyme(s) was involved in C6 aldehyde formation in *M. polymorpha* (Figure 1-16).

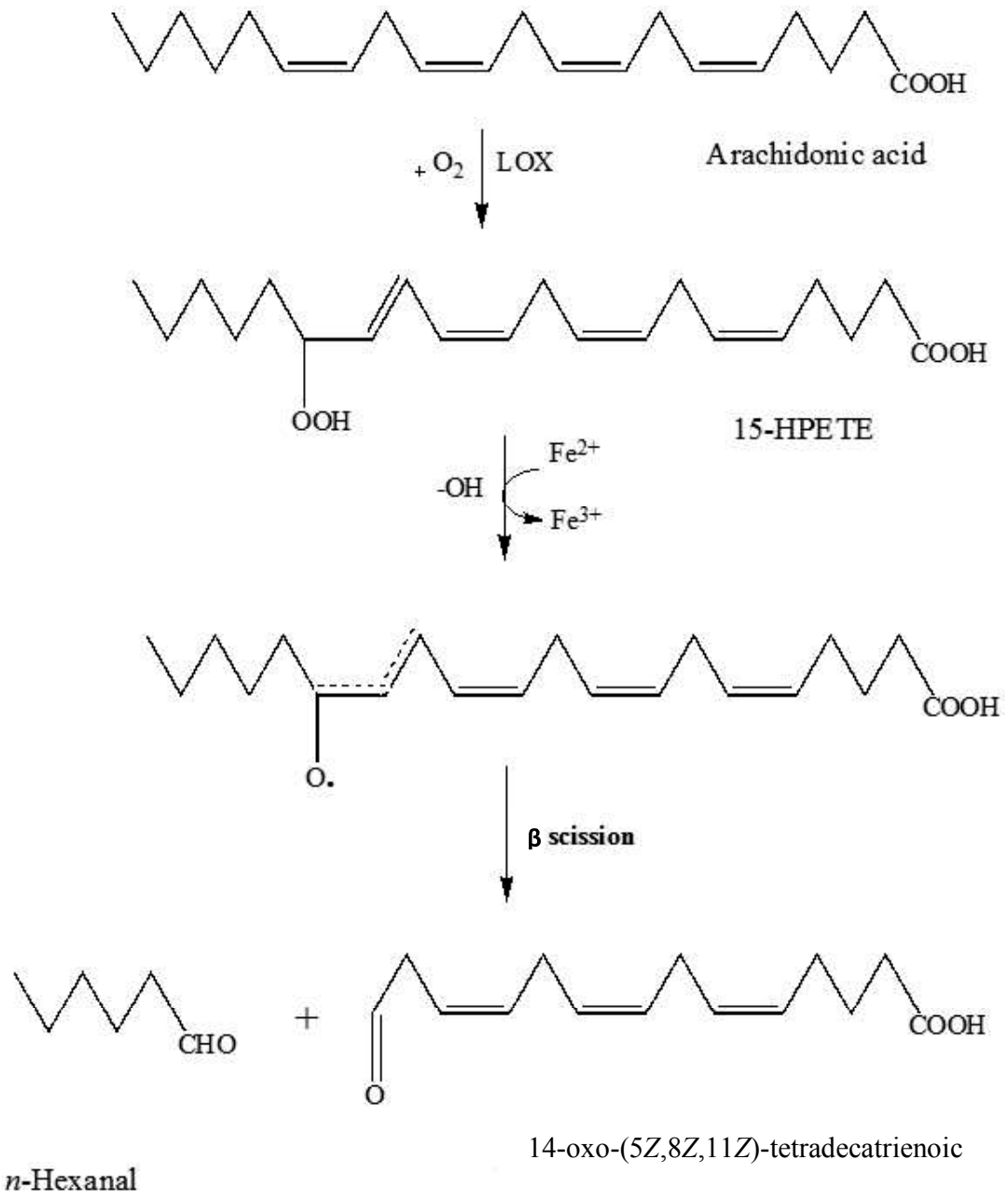


Figure 1-16. Biosynthetic pathway to form *n*-hexanal from arachidonic acid.

It has been reported that some LOXs, which insert molecular oxygen at the 13-position of C18 fatty acids, have an ability to form five-carbon volatile compounds (Gardner 1991; Salch et al. 1995; Shen et al. 2014; Mochizuki et al. 2016). Since the formation of C5 volatiles was enhanced when the LOX reaction was carried out under oxygen-deficient condition, it was

proposed that the alkoxy radical formed during LOX catalysis was directed to a spontaneous β-scission reaction to yield C5 volatile compounds (Gardner 1991) (route a in Figure 1-17). With β-scission on alkoxy radical, cleavage of the non-allylic C-C bonding is favorable, whereas cleavage of the allylic C-C bonding is unfavorable owing to its high bond dissociation energy (Gardner 1989) (route b in Figure 1-17). Therefore, spontaneous β-scission of alkoxy radical is not largely accountable to the formation of C6 aldehydes.

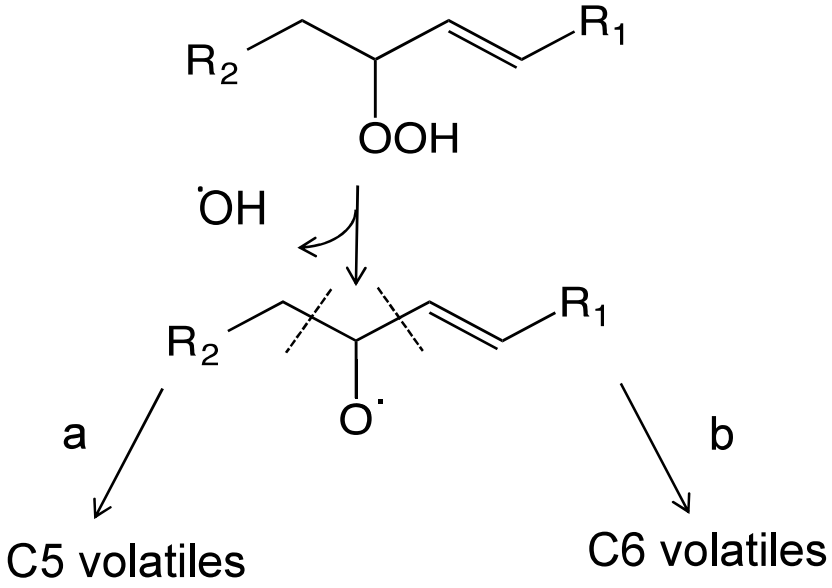


Figure 1-17. Proposed routes of β-scission of fatty acid hydroperoxides formed by lipoxygenases. In route a, the non-allylic C-C bonding would be cleaved to form C5 volatiles. In route b, the allylic C-C bonding (between the carbon at the base of alkoxyl radical and that of double bond) would be cleaved to form C6 volatiles. R1 = decenoic acid or dodecadienoic acid; R2 = butyl or butenyl.

Recently, a LOX from red algae was reported to have the ability to form volatile aldehydes through cleavage of allylic C-C bond (Chen et al. 2015). It was also reported that an Arabidopsis ecotype (Col-0) that had no HPL activity still formed a substantial amount of *n*-hexanal (Duan et al. 2005). Even though the mechanism of the formation of these volatile aldehydes in either cases has not been fully elucidated yet, these inspired us to check the ability of *M. polymorpha* LOX to form C6 aldehydes as by-products. There are 16 genes encoding *LOX* in *M. polymorpha*. Among them, three showed relatively high sequence similarity with seed plant plastidic 13-LOXs, whereas

the other thirteen LOXs are located in a clade consisting of LOXs from lycophytes and bryophytes. One of the LOXs in the clade consisted of non-seed plant LOXs, namely, MpLOX7, which showed low but substantial ability to form *n*-hexanal and (*Z*)-3-hexenal from arachidonic acid and α-linolenic acid, respectively, *in vitro*. The formation of C5 volatiles was hardly observed with MpLOX7 under the reaction condition employed here. Since the main products of MpLOX7 reaction on arachidonic and α-linolenic acids were the corresponding 15-hydroperoxide and 13-hydroperoxide, the formation of C6 aldehydes should be produced through cleavage of the allylic C-C bonding of the hydroperoxides but not through cleavage of the non-allylic C-C bonding. MpLOX7 and PhLOX likely share the mechanism causing cleavage of allylic C-C bonding, but MpLOX7 was more specific to the allylic C-C bonding cleavage because the cleavage on the allylic or non-allylic C-C bonding proceeded at an almost similar degree with PhLOX (Chen et al. 2015). For cleavage on the allylic C-C bonding, the formation of epoxy allylic carbon radical from alkoxyl radical, followed by a rearrangement and subsequent hydroxylation to yield a hemiacetal ester is proposed by examining the reaction mechanisms of an angiosperm HPL (Grechkin & Hamberg 2004), and a catalase-related hemoprotein in coral (Teder et al. 2015). According to the proposed mechanism of cleavage on the allylic C-C bonding, the fate of alkoxyl radical must be controlled to avoid spontaneous reaction mostly leading to non-allylic C-C bonding, but instead be controlled to form epoxy allylic carbon radical. Presently, I do not know whether MpLOX7 employs the same mechanism to cleave the non-allylic C-C bonding, and further detailed study must be done.

In summary, I found that *M. polymorpha* formed C6 aldehydes, and that one of the LOXs that was relatively specific among the basal plant lineage might at least partly account for the formation of C6 aldehydes. Since there are twelve other LOXs in *M. polymorpha* that show high homology to MpLOX7, further study must be conducted to determine how much each MpLOX in the clade would account for the formation of C6 aldehydes *in vivo*. C6 aldehydes are formed as defense compounds in various angiosperms (Matsui 2006). During the evolution of plants, acquisition of HPL activity, probably through mutation of AOS, seemed to occur after emergence of moss (Koeduka et al. 2015). It is also important to recognize whether the C6 aldehyde formation in liverworts is involved in defense against biotic stresses.

References

Andreou A, Feussner I. Lipoxygenase – Structure and reaction mechanism. Phytochemistry. 2009; 70:1504–1510.

Anterola A, Göbel C, Hornung E, Sellhorn G, Feussner I, Grimes H. *Physcomitrella patens* has lipoxygenases for both eicosanoid and octadecanoid pathways. Phytochemistry. 2009; 70:40–52.

Asakawa Y, Ludwiczuk A, Nagashima F. Phytochemical and biological studies of bryophytes. Phytochemistry. 2013; 91:52–80.

Boonprab K, Matsui K, Akakabe Y, Yotsukura N, Kajiwara T. Hydroperoxy-arachidonic acid mediated *n*-hexanal and (*Z*)-3- and (*E*)-2-nonenal formation in *Laminaria angustata*. Phytochemistry. 2003; 63:669–678.

Chen H M, Zhu Z J, Chen J J, Yang R, Luo Q J, Xu J L, Shan H, Yan J X. A multifunctional lipoxygenase from *Pyropia haitanensis*- The cloned and functioned complex eukaryotic algae oxylipin pathway enzyme. Algal Res. 2015; 12:316–327.

Duan H, Huang M Y, Palacio K, Schuler M A. Variations in CYP74B2 (hydroperoxide lyase) gene expression differentially affect hexenal signaling in the Columbia and Landsberg erecta ecotypes of Arabidopsis. Plant Physiol. 2005; 139:1529–1544.

Eek P, Jarving R, Jarving I, Gilbert N C, Newcomer M E, Samel N. Structure of a calcium-dependent 11*R*-lipoxygenase suggests a mechanism for Ca^{2+} regulation. J. Biol. Chem. 2012; 287:22377–22386.

Gardner W H. Recent investigations into the lipoxygenase pathway of plants. Biochim. Biophys. Acta. 1991; 1084:221–239.

Gardner W H. Oxygen radical chemistry of polyunsaturated fatty acids. Free Radic. Biol. Med. 1989; 7: 65-86.

Grechkin A N, Hamberg M. The “heterolytic hydroperoxide lyase” is an isomerase producing a short-lived fatty acid hemiacetal. Biochim. Biophys. Acta. 2004; 1636:47–58.

Green A R, Barbour S, Horn T, Carlos J, Raskatov JA, Holman TR. Strict regiospecificity of human epithelial 15-Lipoxygenase-2 delineates its transcellular synthesis potential. Biochemistry. 2016; 55:2832–2840.

Hatanaka A. The biogeneration of green odor by green leaves. Phytochemistry. 1993; 34:1201–1218.

Kanamoto H, Takemura M, Ohyama K. Cloning and expression of three lipoxygenase genes from liverwort, *Marchantia polymorpha L*., in *Escherichia coli*. Phytochemistry. 2012; 77:70–78.

Kanazawa T, Era1 A, Minamino N, Shikano Y, Fujimoto M, Uemura T, Nishihama R, Yamato KT, Ishizaki K, Nishiyama T, Kohchi T, Nakano A, Ueda T. SNARE Molecules in *Marchantia polymorpha*: Unique and conserved features of the membrane fusion machinery. Plant Cell Physiol**.** 2015; 0:1– 18.

Kihara H, Tanaka M, Yamato K T, Horibata A, Yamada A, Kita S, Ishizaki K, Kajikawa M, Fukuzawa H, Kohchi T, Akakabe Y, Matsui K. Arachidonic acid-dependent carbon-eight volatile synthesis from wounded liverwort (*Marchantia polymorpha*). Phytochemistry. 2014; 107:42–49.

Koeduka T, Ishizaki K, Mwenda C M, Hori K, Sasaki-Sekimoto Y, Ohta H, Kohchi T, Matsui K. Biochemical characterization of allene oxide synthases from the liverwort *Marchantia polymorpha* and green microalgae *Klebsormidium flaccidum* provides insight into the evolutionary divergence of the plant CYP74 family. Planta. 2015; 242:1175–1186.

Kuhn H, Banthiya S, van Leyen K. Mammalian lipoxygenases and their biological relevance. Biochim. Biophys. Acta. 2015; 1851:308–330.

Matsui K, Kaji Y, Kajiwara T, Hatanaka A. Developmental changes of lipoxygenase and fatty acid hydroperoxide lyase activities in cultured cells of *Marchantia polymorpha*. Phytochemistry. 1996; 41:177–182.

Matsui K. Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr. Opin. Plant Biol. 2006; 9:274–280.

Mochizuki S, Sugimoto K, Koeduka T, Matsui K. Arabidopsis lipoxygenase 2 is essential for formation of green leaf volatiles and five-carbon volatiles. FEBS Lett. 2016; 590:1017–1027.

Mosblech A, Feussner I, Heilmann I. Oxylipins: structurally diverse metabolites from fatty acid oxidation. Plant Physiol. Biochem. 2009; 47:511–517.

Newcomer M E, Brash A R. The structural basis for specificity in lipoxygenase catalysis. Protein Sci. 2015; 24:298–309.

Salch Y P, Grove M J, Takamura H, Gardner H W. Characterization of a C-5,13-cleaving enzyme of 13(*S*)-hydroperoxide of linolenic acid by soybean seed. Plant Physiol. 1995; 108:1211–1218.

Scala A, Allmann S, Mirabella R, Haring MA, Schuurink RC. Green leaf volatiles: a plant's multifunctional weapon against herbivores and pathogens. Int. J. Mol. Sci. 2013; 14:17781–17811.

Senger T, Wichard T, Kunze S, Goebel C, Lerchl J, Pohnert G, Feussner I. A multifunctional lipoxygenase with fatty acid hydroperoxide cleaving activity from the moss *Physcomitrella patens*. J. Biol. Chem. 2005; 208:7588–7596.

Sharma SC, Klinman JP. Kinetic detection of orthogonal protein and chemical coordinates in enzyme catalysis: double mutants of soybean lipoxygenase. Biochemistrty. 2015; 54:5447–5456.

Shen J, Tieman D, Jones JB, Taylor MG, Schmelz E, Huffaker A, Bies D, Chen K, Klee H J. A 13-lipoxygenase, TomloxC, is essential for synthesis of C5 flavour volatiles in tomato. J. Exp. Bot. 2014; 65:419–428.

Suire C, Bouvier F, Backhaus R A, Begu D, Bonneu M, Camara B. Cellular localization of isoprenoid biosynthetic enzymes in *Marchantia polymorpha*. Uncovering a new role of oil bodies. Plant Physiol. 2000; 124:971–978.

Talavera G, Castresana J. Improvement of phylogeneis after removing divergent and ambiguously aligned blocks from protein sequence alignment. Syst. Biol. 2007; 56:564–577.

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 2013; 30:2725–2729.

Tanaka M, Esaki T, Kenmoku H, Koeduka T, Kiyoyama Y, Masujima T, Asakawa Y, Matsui K. Direct evidence of specific localization of sesquiterpenes and marchantin A in oil body cells of *Marchantia polymorpha* L. Phytochemistry. 2016; 130:77–84.

Teder T, Lõhelaid H, Boeglin W E, Calcutt W M, Brash A R, Samel N. A catalase-related hemoprotein in coral is specialized for synthesis of short-chain aldehydes. J. Biol. Chem. 2015; 290: 19823–19832.

Yanagi K, Sugimoto K, Matsui K. Oxylipin-specific cytochrome P450s (CYP74s) in *Lotus japonicas*: their implications in response to mechanical wounding and nodule formation. J. Plant Interact. 2011; 6:255–264.

Supplemental Table S1. List of genes used for the phylogenetic tree.

	gene genome name	gene short name	accession number
1	Mapoly0028s0085.1	MpLOX1	AB569089.1
2	Mapoly0191s0014.1	MpLOX2	AB569090.1
3	Mapoly0006s0130.1	MpLOX3	AB569091.1
4	Mapoly0106s0031.1	MpLOX4	OAE30885
5	Mapoly0001s0529.1	MpLOX5	
6	Mapoly0023s0182.1	MpLOX6	OAE29063
7	Mapoly0026s0145.1	MpLOX7	OAE28684
8	Mapoly0055s0019.1	MpLOX9	
9	Mapoly0063s0014.1	MpLOX10	OAE23731
10	Mapoly0063s0050.1	MpLOX11	OAE23761
11	Mapoly0064s0034.1	MpLOX12	OAE19683
12	Mapoly0079s0056.1	MpLOX13	OAE32277
13	Mapoly0083s0028.1	MpLOX14	BAJ46516
14	Mapoly0191s0015.1	MpLOX15	
15	Mapoly0420s0001.1	MpLOX16	
16	Phpat.015G051500.1.p	PpLOX1	DQ518920.1
17	Phpat.015G051800.1.p	PpLOX2	DQ518921.1
18	Phpat.017G059100.1.p	PpLOX3	ABF66649
19	Phpat.014G038800.1.p	PpLOX4	ABF66650
20	Phpat.011G019200.1.p	PpLOX5	DQ518924.1
21	Phpat.001G116900.1.p	PpLOX6	DQ518925.1
22	Phpat.012G053300.1.p	PpLOX7	DQ518926.1
23	AT3G45140	AtLOX2	NP_566875
24	AT1G67560	AtLOX6	NP_176923
25	AT1G17420	AtLOX3	NP_564021
26	AT1G72520	AtLOX4	NP_177396
27	AT1G55020	AtLOX1	NP_175900
28	AT3G22400	AtLOX5	NP_188879
29	MA_184054g0010	PaLOX	
30	MA_15951g0010	PaLOX	
31	MA_3501g0020	PaLOX	
32	MA_10427506g0020	PaLOX	
33	MA_10436100g0010	PaLOX	
34	MA_11043g0030	PaLOX	
35	MA_10429541g0010	PaLOX	
36	MA_153g0020	PaLOX	
37	MA_47100g0010	PaLOX	
38	MA_10427506g0010	PaLOX	
39	MA_161903g0010	PaLOX	

40	Smoe_441081	SmLOX	XP_002970196
41	Smoe_140773	SmLOX4	XP_002961703
42	Smoe_268169	SmLOX	XP_002978360
43	Smoe_439120	SmLOX	XP_002964909
44	Smoe_439121	SmLOX	XP_002964910
45	Smoe_446635	SmLOX	XP_002986491
46	Smoe_109226	SmLOX	XP_002978700
47	Smoe_170977	SmLOX	XP_002969954
48	Smoe_402722	SmLOX	XP_002961108
49	Smoe_402721	SmLOX	XP_002961107
50	Smoe_178158	SmLOX	XP_002980397
51	Smoe_112556	SmLOX	XP_002980228
52	Smoe_112468	SmLOX	XP_002980396
53	Smoe_437605	SmLOX	XP_002961193
54	Smoe_168576	SmLOX	XP_002966811
55	Potri014G018200	PtLOX	XP_002320037
56	Potri014G177200	PtLOX	XP_002320571
57	Potri010G089500	PtLOX	XP_002315780
58	Potri008G151500	PtLOX	XP_002311617
59	Potri013G022100	PtLOX	XP_002319015
60	Potri005G032800	PtLOX	XP_006382595
61	Potri005G032700	PtLOX	XP_006382594
62	Potri013G022000	PtLOX5	XP_011023610
63	Potri005G032600	PtLOX	
64	Potri005G032400	PtLOX	
65	Potri003G067600	PtLOX	XP_002304125
66	Potri001G167700	PtLOX	XP_006369132
67	Potri010G057100	PtLOX	XP_002314548
68	Potri008G178000	PtLOX	XP_002311724
69	Potri009G022400	PtLOX	XP_002314229
70	Potri001G015400	PtLOX	XP_002297796
71	Potri001G015300	PtLOX	XP_002299250
72	Potri001G015500	PtLOX	XP_006368564
73	Potri017G046200	PtLOX	
74	Potri001G015600	PtLOX	XP_006388115
75	GRMZM2G106748	ZmLOX9	NM_001112527
76	GRMZM2G040095	ZmLOX6	NM_001112506
77	GRMZM2G109056	ZmLOX4	NP_001105974
78	GRMZM2G076031	ZmLOX5	NP_001105975
79	GRMZM2G109130	ZmLOX	NP_001105515
80	GRMZM2G156861	ZmLOX	NP_001105003
81	GRMZM2G017616	ZmLOX	DAA43689
82	GRMZM2G104843	ZmLOX	XP_008666983
83	GRMZM2G070092	ZmLOX	AFW58386

84	GRMZM2G015419	ZmLOX	ABF01001
85	GRMZM2G009479	ZmLOX11	ABF01002
86	GRMZM5G822593	ZmLOX8	XP_008663576
87	kfl00028_0030	KfLOX1	GAQ79298
88	kfl00081_0200	KfLOX2	GAQ81478
89	Cre12.g512300	CrLOX	XP_001690882

LIST OF ABBREVIATIONS

PUFA: Polyunsaturated fatty acid

LOX: Lipoxygenase

AOS: Allene oxide synthase

HPL: Hydroperoxide lyase

k_m: Michaelis Menten constant

k_{cat}: Catalytic constant

V_{max}: Maximum velocity

HETE: Hydroxyl eicosatetraenoic acid

LNA: Linolenic acid

LA: Linoleic acid

ARA: Arachidonic acid

13-HPOD: 13-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid

13-HPOT: 13-hydroperoxy-(9*Z*,11*E*,15*Z*)-octadecatrienoic acid

15-HPETE: 15-hydroperoxy-(5*Z*,8*Z*,11*Z*,13*E*)-eicosatetraenoic acid

OPDA: 12-Oxo-phytodienoic acid

CHAPTER TWO

CYP74B24 is the 13-hydroxyperoxide lyase involved in biosynthesis of green leaf volatiles in tea (*Camellia sinensis*)

Abstract

Green leaf volatiles (GLVs) are C6-aliphatic aldehydes/alcohols/acetates, and biosynthesized from the central precursor fatty acid 13-hydroperoxides by 13-hydroperoxide lyases (HPLs) in various plant species. While GLVs have been implicated as defense compounds in plants, GLVs give characteristic grassy note to a bouquet of aroma in green tea, which is manufactured from young leaves of *Camellia sinensis*. Here we identify three *HPL*-related genes from *C. sinensis* via RNA-Sequencing (RNA-Seq) *in silico*, and functionally characterized a candidate gene, *CYP74B24*, as a gene encoding tea HPL. Recombinant CYP74B24 protein heterologously expressed in *Escherichia coli* specifically produced (*Z*)-3-hexenal from 13-HPOT with the optimal pH 6.0 *in vitro*. *CYP74B24* gene was expressed throughout the aerial organs in a rather constitutive manner and further induced by mechanical wounding. Constitutive expression of *CYP74B24* gene in intact tea leaves might account for low but substantial and constitutive formation of a subset of GLVs, some of which are stored as glycosides. Our results not only provide novel insights into the biological roles that GLVs play in tea plants, but also serve as basis for the improvement of aroma quality in tea manufacturing processes.

2.1 Introduction

Tea (*Camellia sinensis*) is one of the most consumed beverages in the world, and is classified into three categories, green tea, oolong tea and black tea based on degree of fermentation. Green tea, manufactured without fermentation process, has characteristic green note (recognized as glassy aroma) derived from six carbons (C6)-aliphatic aldehydes/alcohols/acetates, so called as green leaf volatiles (GLVs).

GLVs are a class of bioactive oxylipins derived from oxygenation of polyunsaturated fatty acids, and also generally observed volatile organic compounds (VOCs) in land plants (Matsui, 2006). Plants emit GLVs to counter biotic (e.g., mechanical wounding by insects and herbivores) and abiotic stresses (e.g., ozone exposure) (Arimura et al. 2009). For example, transgenic plants that can produce larger amount of (*Z*)-3-hexenal showed enhanced resistance to herbivores through the attraction of natural enemies and against fungi through the suppression of their growth compared to non-transformant (Shiojiri et al. 2006). Important to note, GLVs have been shown as transmitting molecules from a plant (tomato) damaged by herbivore attack to undamaged plants, in which (*Z*)-3-hexen-1-ol is taken up and stored as water-soluble diglycosides. Undamaged plants exposed to volatiles emitted by conspecifics infested with common cutworms (exposed plants) became more defensive against the larvae than those exposed to volatiles from uninfested conspecifics (control plants) partly because of accumulation of the glycosides in a laboratory condition (Sugimoto et al. 2014).

The first products of biosynthetic pathway to form GLVs, (*Z*)-3-hexenal and *n*-hexanal, is produced from 13-hydroperoxides (HPOs) of plastid-derived C18-fatty acids, such as linolenic and linoleic acids, respectively, by HPO lyases (HPL) (Mwenda & Matsui 2014). HPL are structurally similar to allene oxide synthase (AOS) and divinyl ether synthase (DES) that are involved in the biosynthesis of defense-related oxypilins, jasmonate and divinyl ether, respectively. HPL, AOS, and DES form distinct phylogenetic subclusters, but all belong to CYP74 family of the cytochrome P450 superfamily enzyme (P450) (CYP74B, CYP74A, and CYP74D, respectively) (Feussner & Wasternack 2002). Among other P450s, CYP74s are unique in that they lack N-terminal transmembrane domain that are characteristic to microsomal P450s and generate various products via rearrangement of their respective HPO substrates in the absence of molecular oxygen and reducing equivalent, which are generally essential to P450 oxido-reductases (Lee et

al. 2008). A portion of (*Z*)-3-hexenal is then reduced to (*Z*)-3-hexen-1-ol via an unknown reductase with the aid of NADPH (Matsui, et al. 2012), and further converted into various conjugates, such as acyl-ester (D'Auria et al. 2007) and glycoside form (Ohgami et al. 2015) in plants. In most land plants, the pathway to form GLVs *de novo* from endogenous fatty acids is rapidly activated when the plant tissues are disrupted by mechanical wounding, herbivore attack, necrotrophic pathogen attack, and so on (Figure 2-1) (Matsui 2006; D'Auria et al. 2007). Recent work on volatile metabolism of tea plant showed that (*Z*)-3-hexen-1-ol is glycosylated by two distinct UDP-sugar dependent glycosyltransferases (CsGT1/UGT85K11 and CsGT2/UGT94P1) and stored as water-soluble diglycoside form (primeveroside) in the leaves (Ohgami et al. 2015). The primeveroside is considered to be a storage form of (*Z*)-3-hexen-1-ol because it is released through specific hydrolysis by an extracellular β-primeverosidase (βPD) (Mizutani et al. 2002) (Figure 2-1). Release of bioactive compound via hydrolysis of stable glycoside form, is widely observed in plant specialized metabolites, e.g., 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA) and glucosinolates (β-thioglucoside-N-hydroxysulfates) (Frey et al. 2009). The metabolic system enables rapid production of bioactive volatiles even without *de novo* synthesis. Taken together, there are two metabolic pathways in tea leaves to form GLVs under stressed conditions, i.e., *de novo* synthesis and hydrolysis of the accumulated glycosides.

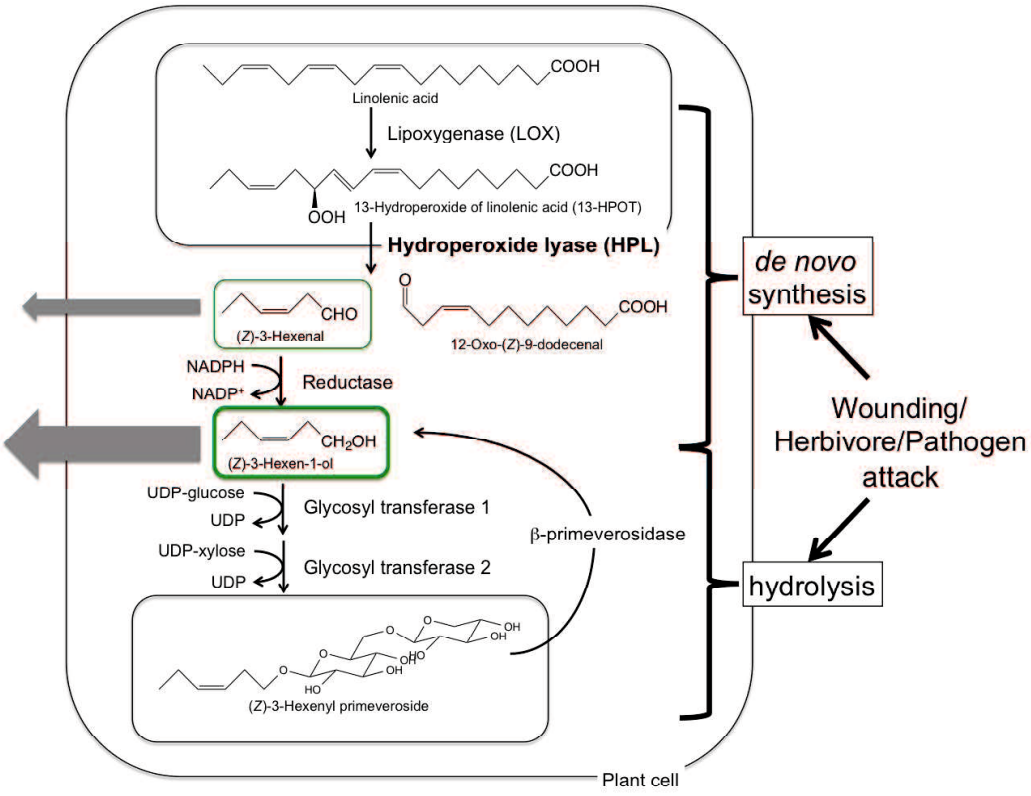


Figure 2-1. Illustration of HPL-mediated GLV metabolism in tea plant.

In tea plant, fatty acid HPO cleaving activity has been previously reported in the leaves (Matsui et al. 1991); however, a gene responsible for the HPL activity had remained unknown. More recently, a HPL homolog *CsiHPL1*, isolated form *C. sinensis* based on the sequence similarity to known HPLs, was shown to regulate defense response against *Prodenia litura* and *Alternaria alternata* by modulating GLVs release and jasmonic acid (JA)-mediated gene expression when heterologously expressed in tomato (Xin et al. 2014). However, biochemical property and transcriptional regulation of CsiHPL1 *in vitro* remain to be fully elucidated.

Here I attempt to elucidate biochemical basis of GLV biosynthesis in *C. sinensis*. I show that a cytochrome P450 of *C. sinensis*, *CYP74B24* encodes functional 13HPL. Substantial level of transcript of the gene was detected in various above-ground organs of tea plants, and its level was enhanced by 2 to 3 fold after mechanical wounding.

2.2 Materials and Methods

2.2.1. *RNA-Sequencing (RNA-Seq).*

RNA-Sequencing was basically performed by Dr. Ono as previously described in Natsume et al (2015). Total RNA was extracted from various aerial tissues (leaf, stem and leaf with mechanical wounding) of *C. sinenesis* cultivar Yabukita using RNeasy Plant Kit (Qiagen) in June 2014 when new leaves ready for harvest emerged. The RNA samples were treated with DNase Set (Qiagen) to remove contaminating genomic DNA. The quality of the RNA samples was evaluated by using BioAnalyzer (Agilent Technologies) with RNA6000 Nano Chip. A 1 mg aliquot of total RNA from each sample was used to construct cDNA libraries using TruSeq Stranded Total RNA with Ribo-Zero Plant (Illumina), according to the manufacturer's instructions. The resulting cDNA library was validated using BioAnalyzer with DNA1000 Chip and quantified using Cycleave PCR Quantification Kit (TAKARA BIO INC.). PE sequencing using 2 × 101 cycles was performed using HiSeq1500 (Illumina) in the high output mode.

Total reads were extracted with CASAVA v1.8.2 (Illumina). Then, PCR duplicates, adaptor sequences and low quality reads were removed from the extracted reads as follows. Briefly, if the first 10 bases of the two reads were identical and the entire reads showed >90% similarity, the reads were considered to be PCR duplicates. Base calling from the 50 to the 30 end was stopped when the frequency of accurately called bases dropped to 0.5. The remaining reads were assembled using Trinity (February 25, 2013 release) (Grabherr et al. 2011) using normalization with maximum coverage to 30. For each sample, the reads were aligned with a two-step method to obtain reliable fragment per kilobase of exon model per million mapped reads (FPKM) values. To eliminate the unreliable sequences, contigs with less than twenty mapped reads were removed. Contigs including a complete open reading frame of a putative HPL were identified based on a homology search of the Uniprot database by blastn. To evaluate whether there were enough reads to calculate reliable FPKM values, the total numbers of mapped reads were estimated using Bowtie (v2.1.0) (Langmead et al. 2009). The mapping of short reads by Bowtie was performed against the contigs generated by Trinity with the following parameters: single mutations were allowed, and the mismatch penalty for nucleotides with low quality value (<20) was ignored.

2.2.2. *qRT-PCR analysis.*

qRT-PCR was performed by Dr. Ono according to the procedure described by Noguchi et al. (2008). RNA was extracted from the following tissues: young leaves, mature leaves, stem, root, flower and young leaves at 30 min and 2 hours after mechanical wounding as described above. Real-time PCR was performed using a 7500 Real-Time PCR system (Life Technologies) and a Power SYBR Green PCR kit (Qiagen). Relative transcription levels were analyzed by the DD cycle threshold method (Life Technologies) after normalization to expression of an internal standard (18S rDNA). Gene amplification was performed using the primer sets: qRT-Cs FA-HPL-Fw (5'-ACC TCG TCC CGA TAC AAC AG-3') and qRT-Cs FA-HPL-RV (5' -GAT CTT GAC GGT GGG TAG GA-3') for CYP74B24 (LC063856) and Cs18SrRNA-FW: 5′- CAC GGG GAG GTA GTG ACA AT-3′ and Cs18SrRNA- RV: 5′-CCT CCA ATG GAT CCT CGT TA-3′ for 18S rRNA of *C. sinensis* (AB120309).

2.2.3. *Molecular cloning and functional expression of CYP74B24.*

Full-length cDNA fragment of CYP74B24 gene was amplified from cDNA of cv. Yabukita by reverse transcription-PCR using gene-specific oligonucleotides Cs FA-HPL-Art-FW (5' -GTG CCG CGC GGC AGC GGA TCC ATG TCG GCA GTG ATG GCG AAA-3') and Cs FA-HPL-Art-RV (5' -GGC TTT GTT AGC AGC CAA GCT TTC ACT TAG CTT TTT CAA CG-3'). For expression in *Escherichia coli*, the amplified fragment was ligated into the pENTR/D-TOPO vector (Life Technologies), and the sequence was verified. They were subcloned into the pET15b expression vector (Merck Millipore; www. merckmillipore.com) and transformed into *E. coli* BL21 (DE3; TOYOBO; www. toyobo-global.com).

The recombinant protein was expressed by Overnight Express Auto induction Systems (Novagen) according to the manufacture's instruction at 30°C for 24 hours. After the cells were disrupted with French Press (Thermo), membrane fraction was collected at 100,000 × g for 60 min. The recombinant enzyme was solubilized with treating the membrane suspension with 1% (w/v) Triton X-114 for 1 h, and cleaned with centrifugation at 100,000 × g for 1 h. The solubilized enzyme solution was fractionated through an anion-exchange column (0.79 i.d. × 7 cm) filled with DEAE-Toyopearl 650M （TOSOH, which was equilibrated with 40 mL of 10 mM Tris-HCl pH

8.0 including 1.0% Triton X-114. The active fraction eluted as proteins slightly interacted with the resin was collected and used for enzyme assay after examining the homogeneity with SDS-PAGE.

2.2.4. *Enzymatic assay.*

13-HPOs of linoleic and linolenic acid and their 9-isomers were prepared with partially purified soybean seed lipoxygenase-1 and with partially purified potato tuber lipoxygenase as described previously (Koeduka et al. 2014). The purity of each HPO was examined with the reversed phase HPLC (Mightysil PR18, 4.6 x 250 mm, with acetonitrile/water/acetic acid (60/40/0.01, v/v) with monitoring absorption at 234 nm). For HPL reaction, decrease of the absorption of substrate derived from the 1-hydroperoxy-2,4-pentadien moiety was continuously monitored with a reaction mixture (1 mL) consisting of 4 μL of the recombinant protein (82.8 ng protein) and 4 μL of substrate (10 mM of 9-HPOT, 9-HPOD, 13-HPOT or 13-HPOD; final concentration of 40 μM) in 992 μL of 50 mM MES-KOH buffer (pH 6.0). Optimal pH of the CYP74B24 was tested in a range of pH 3.0-8.0 using four buffers with different pH (Na-acetate, MES-KOH, Na-phosphate, and Tris-HCl from low pH to high pH).

2.2.5. *Volatile analysis.*

Tea shoots consisting of two leaves and one bud were cut out from tea plants (3 to 5 years old) grown in the Experimental Farm of Yamaguchi University in June 2015. The cut surface was immediately covered with water-impregnated cotton and further with aluminum foil, and incubated at 25°C for three hours under fluorescent light to settle down the wound-responses brought about during sampling. The leaves were either remained intact or mechanically wounded by an array of needles (25 G) 20 times (Figure 2-2A). The surroundings of holes turned brown within several minutes (Figure 2-2B). The shoot was carefully inserted into a glass tube (2.2 i.d. × 120 mm) with MonoTrap RCC18 (GL Science, Tokyo, Japan) to collect volatiles emitted into the headspace (Figure 2-2C). MonoTrap was exchanged at 10, 60, 120, and 180 min after the onset of volatile collection. The volatiles were eluted from MonoTrap matrix with 200 μL of CH_2Cl_2 containing 4.72 nmol mL^{-1} nonanyl acetate. GC-MS was performed with QP-5050 (Shimadzu,

Kyoto, Japan) equipped with a Stabilwax column (30 m × 0.25 mm i.d. × 0.25µm film thickness, Restek, Bellefonte, PA). The column temperature was programmed as follows: 40°C for 5 min, increasing by 7.5°C min^{-1} to 160°C for 1 min, subsequently, increasing by 15°C min^{-1} to 220°C for 10 min with He as the carrier gas (86.1 kPa). Splitless injection with a sampling time of 1 min was used. The temperatures of the injector and interface were 240 and 200°C, respectively. For quantification of (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, and linalool, a given concentration of each volatile compound was dissolved in 2 mL of water containing 0.5% (w/v) Tween 20, then, transferred into the glass tube holding 7 × 6 cm filter paper in its inside. The filter paper retained ca. 1 mL of aqueous solution, and gave almost the same surface area of tea leaves. The volatiles in headspace were collected for 10 or 50 min with MonoTrap and analyzed as shown above to construct calibration curve with the reference of internal standard (nonanyl acetate). For quantification of (*Z*)-3-hexen-1-yl acetate, molecular chromatogram at *m/z* of 82 was monitored in order to avoid interference of a compound overlapping with the peak for (*Z*)-3-hexen-1-yl acetate.

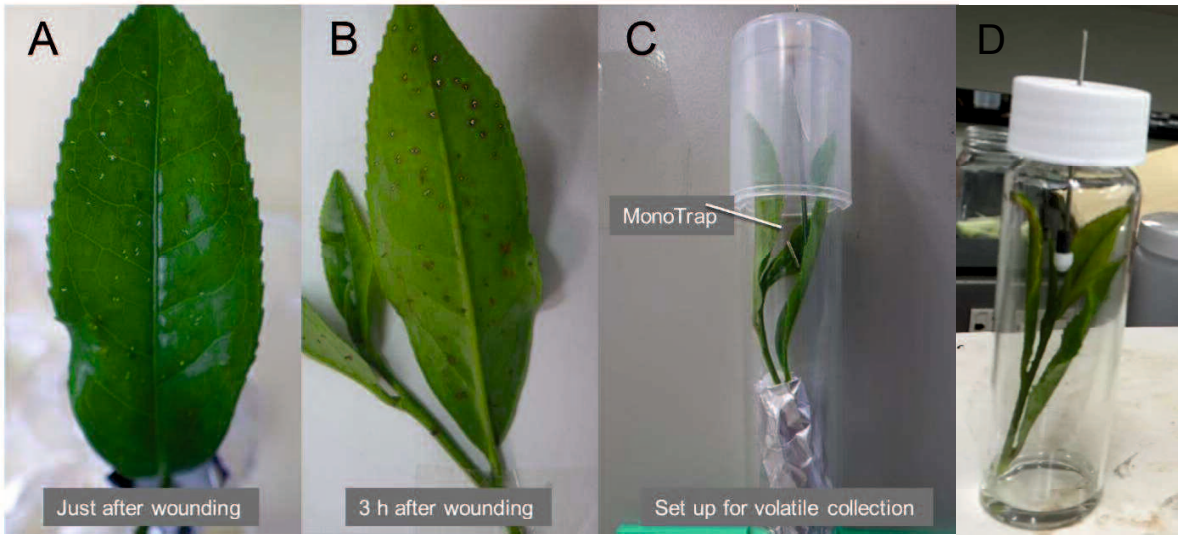


Figure 2-2. (A, B) The technique to perform mechanical wounding and, (C, D) collecting volatiles using MonoTrap.

2.3 Results

2.3.1. *Isolation of HPL-related genes of* C. sinensis.

To survey candidate genes responsible for HPL in *C. sinensis*, we performed deep RNA-Sequencing in three samples of young organs (stems, leaves, and mechanically wounded leaves) where primeverosides of GLVs accumulate (Ohgami et al. 2015). All the sequence reads were assembled together by TRINITY program, resulting in 47,652 contigs after filtering, from which candidate sequences were screened based on sequence similarity to plant *CYP74* genes identified so far by TBLASTX *in silico*. Subsequently, three candidate genes, each of which has an apparent full-length open reading frame for CYP74, were identified and designated as CYP74A51 (LC063857), CYP74A52 (LC063858), and CYP74B24 (LC063856) by the P450 nomenclature committee (Nelson, 2009) (Figure 2-3).

Figure 2-3. Partial amino acid alignment of Arabidopsis HPL, AOS and CYP74B24 of *C. sinensis*.

Phylogenetic analysis with previously characterized CYP74 genes demonstrated that CYP74B24 belongs to the HPL subcluster, whereas CYP74A51 and CYP74A52 are located in different subclusters for AOS (CYP74A) and 9/13HPL (CYP74C), respectively (Figure 2-4). CYP74B24 showed high structural similarity to previously isolated CsiHPL1 (Xin et al. 2014).

2.3.2. *Expression of HPL-related genes of* C. sinensis.

RNA-Sequencing analysis by Bowtie program showed that *CYP74B24* is highly expressed in all three tea organs compared to the other two *CYP74* genes, *CYP74A51* and *CYP74A52* (Figure 2-5). The expression of *CYP74B24* gene was higher in leaves with mechanical wounding than in intact young leaves, suggesting its possible role in wound-induced response in tea plant. Further gene expression analysis of *CYP74B24* in various tea organs (young leaf, old

leaf, stem, root, and flower) by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) showed that *CYP74B24* gene is highly expressed in flower, while its expression was detected from all the organs tested (Figure 2-5). Furthermore, the induction of *CYP74B24* expression within 2 hours after mechanical wounding (Figure 2-5) was basically consistent with the result of the RNA-Sequencing.

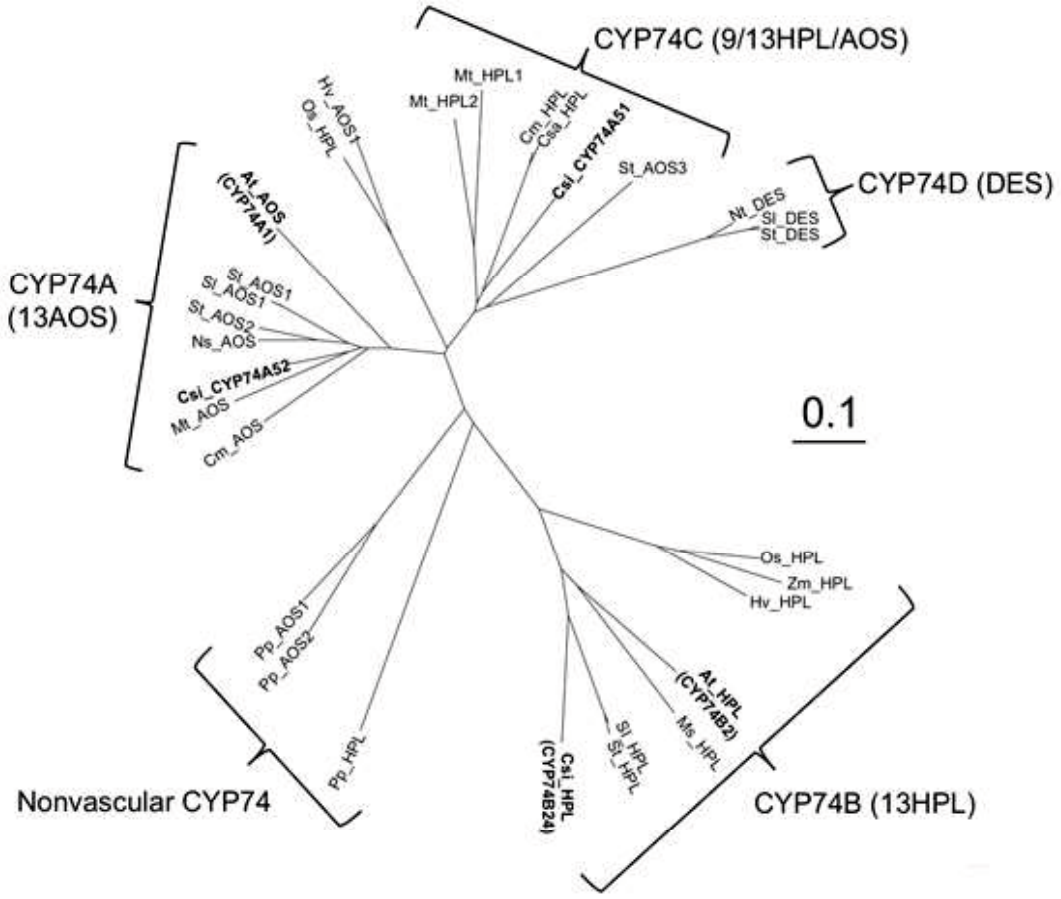


Figure 2-4. Phylogenetic tree of HPL-related P450s. The amino acid sequences of HPL-related P450s were aligned using Muscle bundled in SEAVIEW 4.5.4 (Edgar, 2004, Gouy et al. 2010). The unrooted phylogenetic tree was constructed by using the Bio Neighbor–Joining method (1,000 replicates). The alignment used for this analysis is available as the Supplementary Data. Bar size indicates 0.06 amino acid substitutions per site.

Csi: *Camellia sinensis*, At: *Arabidopsis thaliana*, St: *Solanum tuberosum*, Sl: *Solanum lycopersicum*, Nt: *Nicotiana tabacum*, Na: *Nicotiana attentuata*, Cs: *Cucumis sativum*, Cm: *Cucumis melo*, Mt: *Medicago truncatula*, Pp: *Physcomitrella patens*, Ms: *Medicago sativum*, Zm: *Zea mays*, Hv: *Hordeum vulgare*, Os: *Oryza sativa*

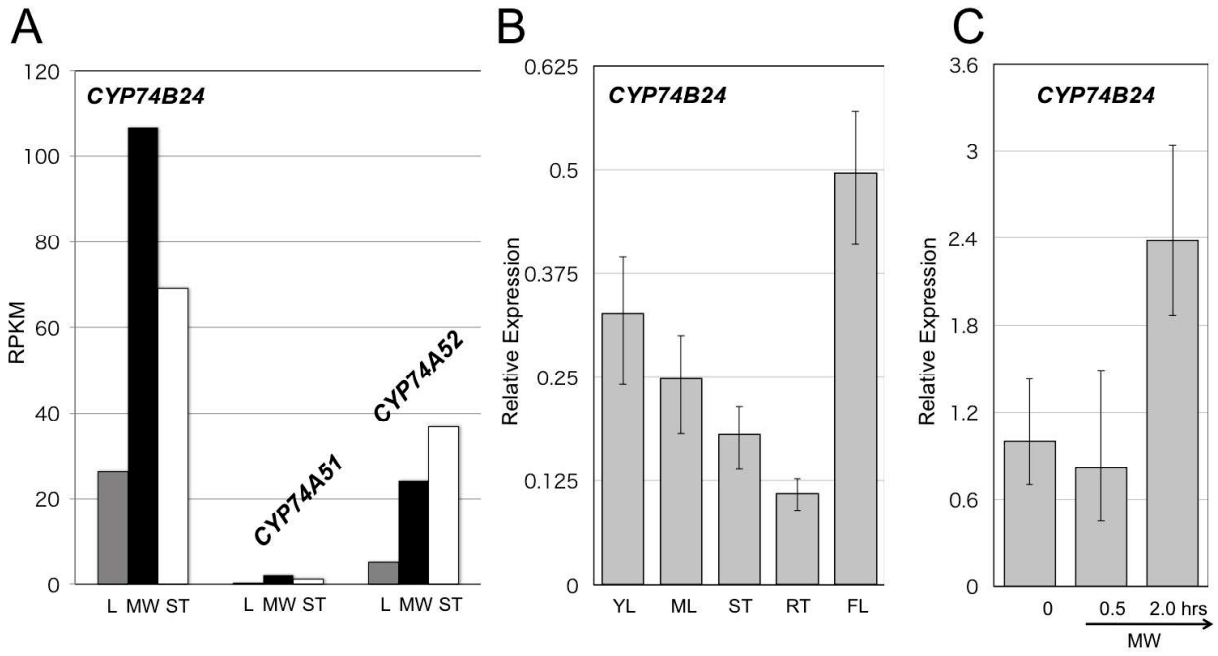


Figure 2-5. Gene expression of tea *HPL* homologs (A) RNA-Sequencing of three HPL-related genes from *C. sinensis* by Bowtei-mappin-g. (B) Tissue specific gene expression of *CYP74B24* by qRT-PCR. (C) Gene expression of *CYP74B24* in leaves upon mechanical wounding (MW).

2.3.3. *Biochemical characterization of recombinant CYP74B24.*

To address enzymatic activity of CYP74B24 *in vitro*, the recombinant protein was heterologously expressed in *E. coli* as a membrane binding protein. After solubilization with 1% (w/v) Triton X-114, the recombinant protein was purified to homogenous state using an anion exchange chromatography.

The recombinant protein was subjected to enzymatic assays with putative substrates (13-HPOT/D and 9-HPOT/D). Under a standard assay condition, recombinant CYP74B24 showed the highest activity towards 13-HPOT among the four substrates, followed by 13-HPOD, whereas it showed negligible activity to 9-HPOT and 9-HPOD (Figure 2-6). Kinetic parameters for 13-HPOT were determined to be k_m =11.4 μM and Vmax =6.03 μkat mg^{-1} (k_{cat} = 301 sec^{-1}), whereas that for 13-HPOD was to be k_m =18.2 μM and Vmax =1.70 μkat mg^{-1} (k_{cat} = 84.9 sec^{-1}). The optimal pH

was determined to be 6.0. Taken together, these biochemical data demonstrated that CYP74B24 is a *C. sinensis* 13HPL, which preferably produces (*Z*)-3-hexenal from 13-HPOT.

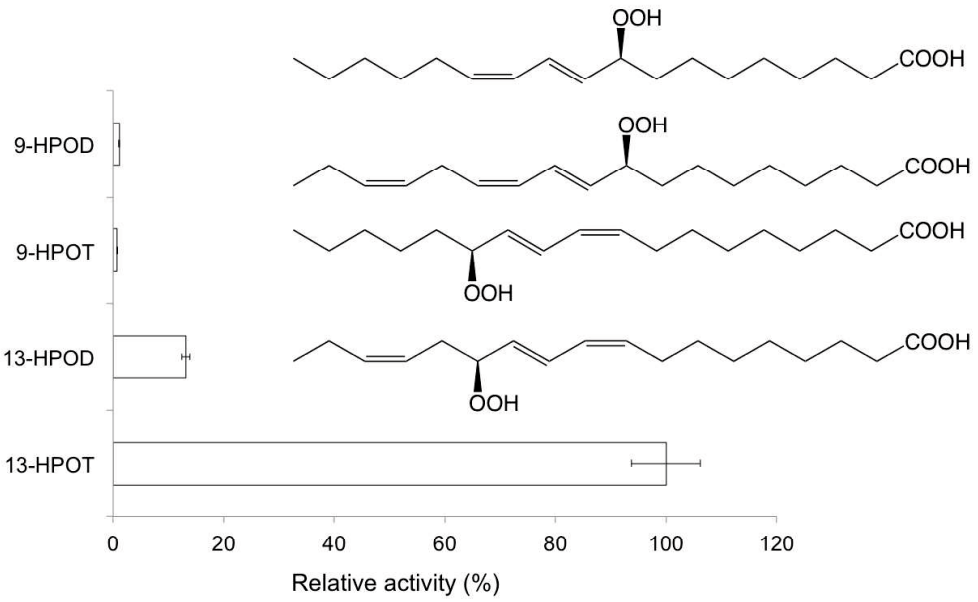


Figure 2-6. Substrate specificity of CYP74B24 for fatty acid hydroperoxides.

2.3.4. *Volatiles formed from fresh tea leaves*.

The results shown above indicated that an enzyme for GLV formation, i.e., 13HPL, rather constitutively expressed in various organs. Because glycosides of (*Z*)-3-hexen-1-ol occur in intact leaves and stems (Ohgami et al. 2015), it was assumed that GLVs were constantly formed in intact organs, albeit at low levels. I collected volatiles emitted from intact and mechanically wounded tea leaves to monitor profiles for forming GLVs in tea leaves (Figures 2-7 and 2-8).

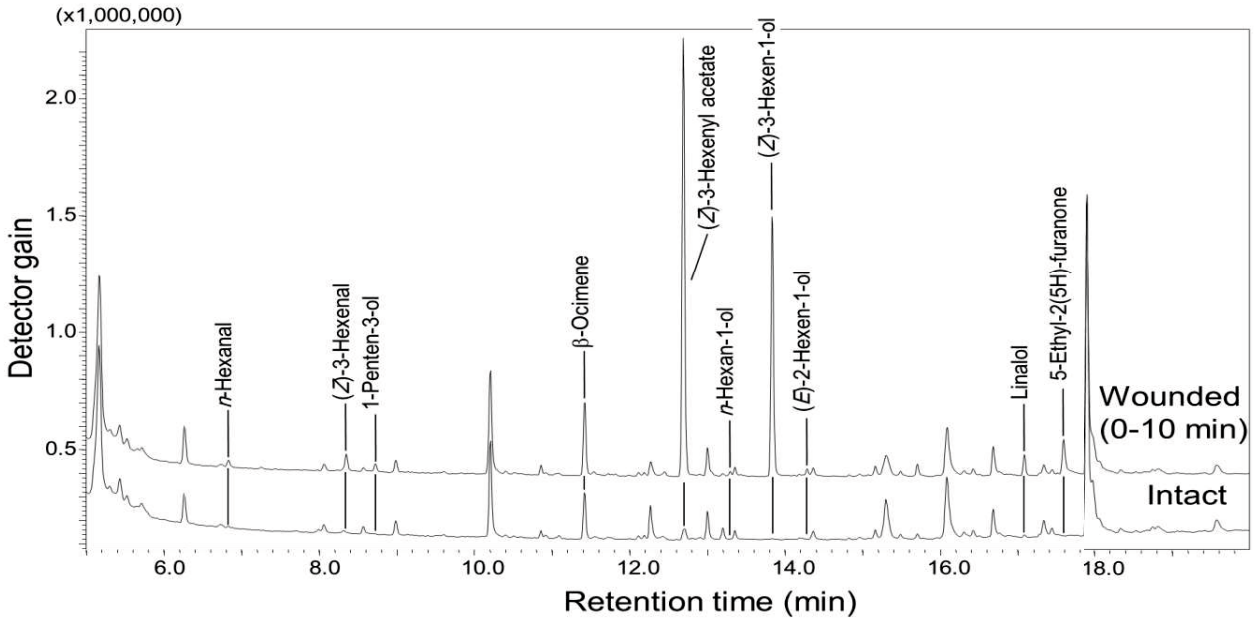


Figure 2-7. GC-MS result of volatiles formation from intact and mechanically wounded tea leaves.

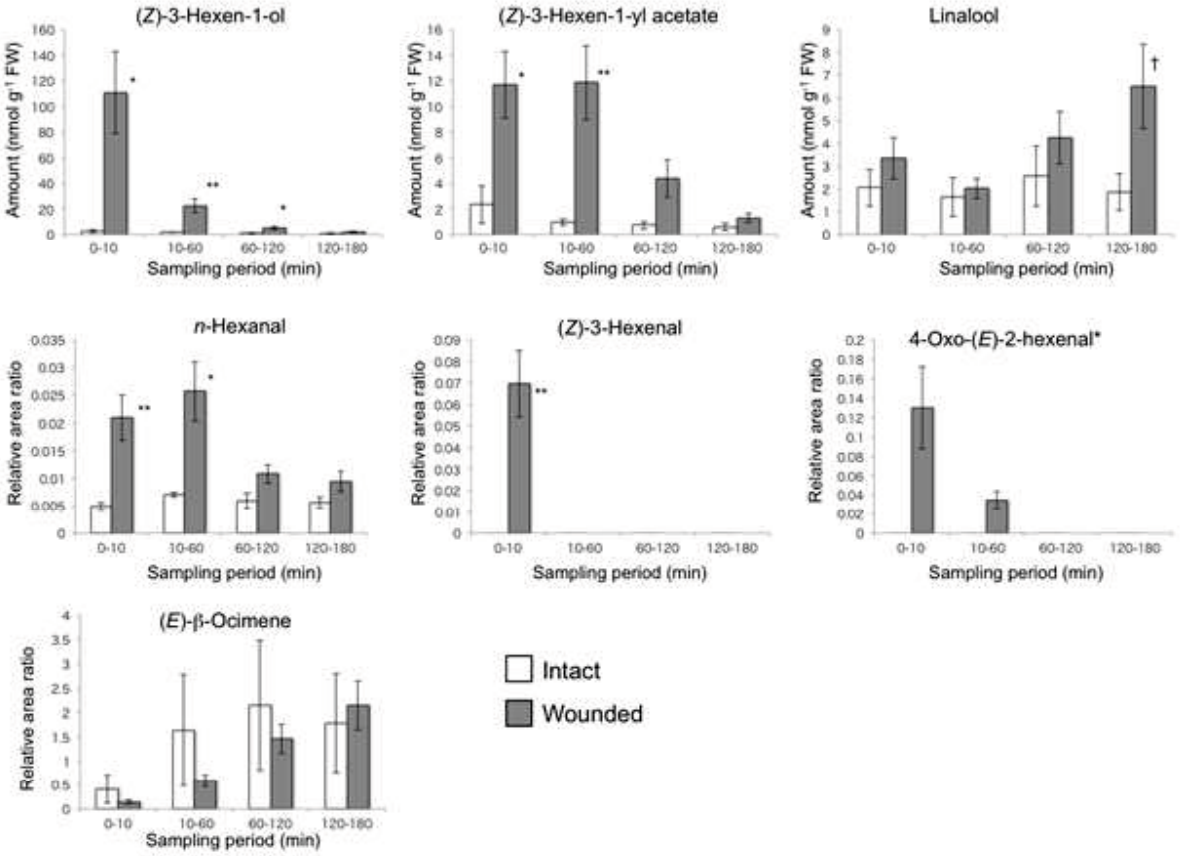


Figure 2-8. Emission of volatiles from intact and mechanically wounded tea leaves. Tea shoots with one newly emerged leaf and two mature leaves were excised from plants, and half of them were mechanically wounded with needles (see figure 2-2). The volatiles emitted out from the leaves were collected with MonoTrap, and subsequently analyzed with GC-MS (see figure 2-6). The most abundant seven peaks were chosen, and among them, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexen-1-yl acetate, and linalool were quantified with calibration curves constructed with authentic compounds. The other four compounds were shown with relative area ration against internal standard (nonanyl acetate).

Intact tea leaves constantly emitted low but substantial amounts of a subset of GLVs, (*Z*)-3-hexen-1-yl acetate, (*Z*)-3-hexen-1-ol, and *n*-hexanal; however, emission of (*Z*)-3-hexenal was not detected. After mechanical wounding, the amounts of volatiles increased significantly. Formation of (*Z*)-3-hexenal was rapidly but transiently enhanced after wounding, and only trace amount could be detected after 10 min of wounding. 4-Oxo-(*E*)-2-hexenal, which was tentatively assigned as an oxidation product of (*Z*)-3-hexenal by examining its mass chromatogram (Figure 2-9), also formed quickly after wounding. Emission of (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-yl acetate was also significantly enhanced after mechanical wounding, and reached to the level of 111 ± 31.8 and 11.9 ± 2.89 nmol g^{-1} Fresh Weight, respectively, at highest. The fate of (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate after wounding largely corresponded to the sequential order of these metabolites in their biosynthetic pathway (D'Auria et al. 2007). Amount of linalool, one of important flavor compounds significant to tea quality (Ohgami et al. 2015), showed a tendency to be increased after 2-3 hr of mechanical wounding.

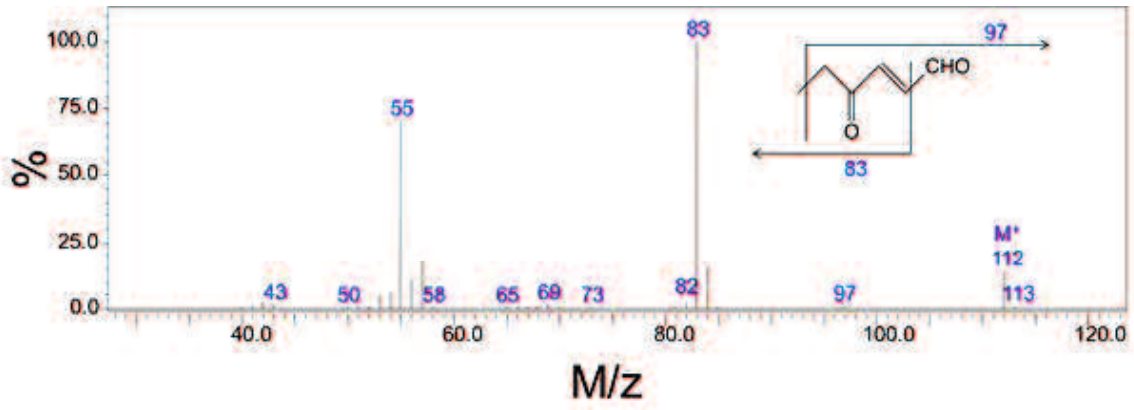


Figure 2-9. Mass spectrum of the compound at retention time of 17.59 min. assigned as 4-oxo-(*E*)-2-hexenal.

Discussion

Through the analyses of phylogeny, gene expression profile as well as biochemical properties, here I demonstrated that *CYP74B24* is a functional 13HPL in *C. sinensis*. CYP74B24 was found to be highly similar to previously reported CsiHPL1 with 96% amino acid sequence identity, but had several nucleotide sequence variations causing amino acid changes (Xin et al. 2014). This structural difference might be derived from different genotypes, i.e., a Japanese variety (cv. Yabukita) in this study and a Chinese variety in the study by Xin et al (Xin et al. 2014).

The HPL activity of the recombinant CYP74B24 observed in a series of *in vitro* assay was consistent with the results obtained with the enzyme purified from tea leaves in terms of molecular weight (50-55 kDa), substrate specificity (13-HPOT > 13-HPOD, and little activity with 9-isomers), and optimum pH (6.0-6.5) (Matsui et al. 1991). This also led to an assumption that an enhancement of GLV formation, especially, (*Z*)-3-hexenal and (*Z*)-3-hexen-1-ol, after overexpressing *CsiHPL1* in tomato (Xin et al. 2014) was accountable to the biochemical activity of the gene product. Considering the observation in the structural analysis of *Arabidopsis thaliana* AOS (CYP74A1) that the double mutation in F137L and S155A in AOS resulted in conferring strong HPL activity (Lee et al. 2008), amino acid residues at the two positions in CYP74 are crucial for determining its product specificity directing either to be HPL or AOS (Figure 2-3). In the case of *C. sinensis HPL,* CYP74B24, it has Leu (L105) and Ala (A123) at the equivalent position to

Phe (F137) and Ser (S155) residue of *A. thaliana* AOS, respectively. Thus, these residues would contribute to the HPL activity.

The length of N-terminal amino acid sequences upstream of the first highly conserved L(A)PxR(K)xIPGxYG motif (started from A18 in CYP74B2) varied among the products of three genes identified in this study. CYP74A52 had the longest stretch among the three gene products, and it might be involved in translocation of CYP74A52 into plastids as found with 13AOS from tomato and Arabidopsis (Froehlich et al. 2001; Knopf et al. 2012). ChloroP analysis supported this assumption. CYP74A51 and CYP74B24 have 28 and 17 amino acid extension at their N-termini, respectively. The stretches are highly S- and P-rich (seven S and six P within 17 amino acid stretch of CYP74B24), as mostly the case with the other HPLs. This feature might be prerequisite for localization of the gene product in plastids, especially at the outer membrane of envelope (Froehlich et al. 2001, Mwenda et al. 2015), even though detailed analysis on their subcellular localization must be carried out.

In tea plant, C6-alcohols among GLVs are known to accumulate as water-soluble glycosides, hexenyl glucoside and hexenyl primeveroside, which are considered as storage form of bioactive volatile alcohols (Ohgami et al. 2015) (Figure 2-1). The low but substantial formation of a subset of GLVs in intact tea leaves might be important to the accumulation of these glycosides in intact tea leaves. This is at least partly supported by constitutive expression of 13HPL gene in leaves and stems found in this study. Attacks of tea leaves by herbivores would result in release of C6-alcohols with repellant activity against several herbivores via hydrolysis by βPD (Mizutani et al. 2002). The same hydrolysis proceeds during tea processing and is also important for intense green note of green tea (Ravichandran & Parthiban 1998). At the same time, *de novo* synthesis of GLVs from endogenous fatty acids is also activated after disruption of tissues (Ravichandran & Parthiban 1998; Matsui 2006). This was also the case with tea leaves, and (*Z*)-3-hexenal was transiently formed essentially through *de novo* manner after wounding, and subsequent reduction and acetylation afforded (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-yl acetate (Figure 2-7). This transient GLV-burst happened within 10 min after mechanical wounding when transcript level of 13HPL showed no change. Therefore, the wound-induced GLV-burst should be regulated by activations of pre-existed components in the biosynthetic pathway. The amounts of (*Z*)-3-hexen-1-ol reached at the level of 111 nmol g^{-1} FW, that was much higher than the amounts of hexenyl glycosides

found in tea leaves (ca. 13 nmol g^{-1} FW) (Ohagami et al. 2015). Therefore, in the case of tea leaves, the transient formation of GLVs after mechanical wounding is supported essentially by *de novo* biosynthesis but the hydrolysis of hexenyl glycosides contributed only partly if any. The hydrolysis of volatile-glycosides might become dominant later at 2-3 hr after mechanical wounding as found with linalool. When considering the flavor quality of tea, hydrolysis of hexenyl and linalyl glycosides during tea processing is important (Kobayashi et al. 1994; Hattori et al. 2005). The results shown here would be useful to control the quality of tea by improving pre and post-harvest treatment (Ravichandran & Parthiban 1998).

It should be also noted that *CYP74B24* gene is induced by mechanical wounding and also highly expressed in flower. The characteristic expression profile of *CYP74B24* was resembled to that of a type 1-class 9/13-lipoxygenase (*CsLOX1*), which produces 13-hydroperoxides of plastid-derived C18-fatty acids (Liu and Han, 2010). Thus, coordinated expression of *LOX* and *HPL* genes would accelerate GLV biosynthesis, indicating other ecological aspects of GLV metabolism in flowers and defense response. Future investigation will clarify these issues.

Considering the commercial impact of GLVs on characteristic green note in tea products, identification of CYP74B24 as a *C. sinensis* HPL herein provides the promising molecular marker for controlling green note by development of cultivating and manufacturing techniques of tea leaves as well as for breeding of tea plant.

References

Arimura G, Matsui K, Takabayashi J. Chemical and molecular ecology of herbivore-induced plant volatiles: proximate factors and their ultimate functions. Plant Cell Physiol. 2009; 50: 911–923.

D'Auria J C, Pichersky E, Schaub A, Hansel A, Gershenzon J. Characterization of a BAHD acyltransferase responsible for producing the green leaf volatile (*Z*)-3-hexen-1-yl acetate in *Arabidopsis thaliana*. Plant J. 2007; 49: 194–207.

Edgar R C. MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 2004; 32: 1792–1797.

Feussner I, Wasternack C. The lipoxygenase pathway. Ann. Rev. Plant Biol. 2002; 53: 275–297.

Frey M, Schullehner K, Dick R, Fiesselmann A, Gierl A. Benzoxazinoid biosynthesis, a model for evolution of secondary metabolic pathways in plants. Phytochemistry. 2009; 70: 1645–1651.

Froehlich J E, Itoh A, Howe G A. Tomato allene oxide synthase and fatty acid hydroperoxide lyase, two cytochrome P450s involved in oxylipin metabolism, are targeted to different membranes of chloroplast envelope. Plant Physiol. 2001; 125: 306–317.

Grabherr, M G, Haas B J, Yassour M, Levin J Z, Thompson D A, Amit I. et al. Full-length transcriptome assembly from RNA-Seq data without a reference genome. Nat. Biotechnol. 2011; 29: 644–652.

Gouy M, Guindon S, Gascuel O. SeaView version 4: a multi- platform graphical user interface for sequence alignment and phylo- genetic tree building. Mol. Biol. Evol. 2010; 27: 221–224.

Hattori S, Takagaki H, Fujimori T. Identification of volatile compounds which enhance odor notes in Japanese green tea using the OASIS (Original Aroma Simultaneously Input to the Sniffing port) method. Food Sci. Technol. Res. 2005; 11: 171–174.

Knopf R R, Feder A, Mayer K, Lin A, Rozenberg M, Schaller A, Adam Z. Rhomboid proteins in the chloroplast envelope affect the level of allene oxide synthase in *Arabidopsis thaliana*. Plant J. 2012; 72: 559–571.

Kobayashi A, Kubota K, Joki Y, Wada E, Wakabayashi M. (*Z*)-3-Hexenyl β-D-glucopyranoside in fresh tea leaves as a precursor of green odor. Biosci Biotechnol. Biochem. 1994; 58: 592–593.

Koeduka T, Ishizaki K, Mwenda C M, Hori K, Sasaki-Sekimoto Y, Ohta H, Kohchi T, Matsui K. Biochemical characterization of allene oxide synthases from the liverwort *Marchantia*

polymorpha and green microalgae *Klebsormidiumu flaccidum* provides insight into the evolutionary divergence of the plant CYP74 family. Planta. 2015; 242:1175–1186.

Langmead B, Trapnell C, Pop M, Salzberg S L. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol. 2009; 10: R25.

Lee D-S, Nioche, P, Hamberg M, Raman C S. Structural insights into the evolutionary path of oxylipin biosynthetic enzyme. Nature. 2008; 455: 363–370.

Liu S, Han B. Differential expression pattern of an acidic 9/13-lipoxygenase in flower opening and senescence and in leaf response to phloem feeders in the tea plant. BMC Plant Biol. 2010; 10: 228.

Matsui K, Toyota H, Kajiwara T, Kakuno T, Hatanaka A. Fatty acid hydroperoxide cleaving enzyme, hydroperoxide lyase, from tea leaves. Phytochemistry. 1991; 30: 2109–2113.

Matsui K. Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. Curr. Opin. Plant Biol.2006; 8: 274–280.

Matsui K, Sugimoto K, Mano J, Ozawa R, Takabayashi J. Differential metabolisms of green leaf volatiles in injured and intact parts of a wounded leaf meet distinct ecophysiological requirements. PLoS ONE. 2012; 7: art. no. e36433.

Mizutani M, Nakanishi H, Ema J, Ma S J, Noguchi E, Inohara-Ochiai M, Fukuchi-Mizutani M, Nakao M, Sakata K. Cloning of β-primeverosidase from tea leaves, a key enzyme in tea aroma formation. Plant Physiol. 2002; 130: 2164–2176.

Mwenda C M, Matsui K. The importance of lipoxygenase control in the production of green leaf volatiles by lipase-dependent and independent pathways. Plant Biotech. 2014; 31: 445–452.

Mwenda C M, Matsuiki A, Nishimura K, Koeduka T, Matsui K. Spatial expression of the Arabidopsis hydroperoxide lyse gene is controlled differently from that of the allene oxide synthase gene. J. Plant Interact. 2015; 10: 1–10.

Natsume S, Takagi H, Shiraishi A, Murata J, Toyonaga H, Patzak J, Takagi M, Yaegashi H, Uemura A, Mitsuoka C, Yoshida K, Krofta K, Satake H, Terauchi R, Ono E. The Draft Genome of Hop (*Humulus lupulus*), an Essence for Brewing. Plant Cell Physiol. 2015; 56: 428–441.

Nelson D R. The Cytochrome P450 Homepage. Human Genomics. 2009; 4: 59–65.

Noguchi A, Fukui Y, Iuchi-Okada A, Kakutani S, Satake H, Iwashita T, Nakao M, Umezawa T, Ono E. Sequential glucosylation of a furofuran lignan, (+)-sesaminol, by *Sesamum indicum* UGT71A9 and UGT94D1 glucosyltransferases. Plant J. 2008; 54: 415–427.

Ohgami O, Ono E, Horikawa M, Totsuka K, Murata J, Toyonaga H, Ohba Y, Dohra H, Asai T, Matsui K, Mizutani M, Naoharu W, Ohnishi T. Volatile glycosylation in tea plants: Sequential glycosylations for biosynthesis of tea aroma primeverosides are catalyzed via two glycosyltransferases in *Camellia sinensis*. Plant Physiol. 2015; 168: 464–477.

Rabichandran R, Parthiban R. The impact of processing techniques on tea volatiles. Food Chem. 1998; 62: 347–353.

Shiojiri K, Kishimoto K, Ozawa R, Kugimiya S, Urashimo S, Arimura G, Horiuchi J, Nishioka T, Matsui K, Takabayashi J. Changing green leaf volatile biosynthesis in plants: an approach for improving plant resistance against both herbivores and pathogens. Proc. Natl. Acad. Sci. USA. 2006; 103: 16672–16676.

Sugimoto K, Matsui K, Iijima Y, Akakabe Y, Muramoto S, Ozawa R, Uefune M, Sasaki R, Alamgir K M, Akitake S, Nobuke T, Galis I, Aoki K, Shibata D, Takabayashi J. Intake and transformation to a glycoside of (*Z*)-3-hexenol from infested neighbors reveals a mode of plant odor reception and defense. Proc. Natl. Acad. Sci. USA. 2014; 111: 7144–7149.

Xin Z, Zhang L, Zhang Z, Chen Z, Sun X, A tea hydroperoxide lyase gene, CsiHPL1, regulates tomato defense response against *Prodenia Litura* (Fabricius) and *Alternaria Alternata* f. sp. Lycopersici by modulating green leaf volatiles (GLVs) release and jasmonic acid (JA) gene expression. Plant Mol. Biol. Rep. 2014; 32: 62–69.

LIST OF ABBREVIATIONS

GLV: Green leaf volatile

RNA-Seq: RNA-Sequencing

HPL: Hydroperoxide lyase

HPOT: linolenic acid hydroperoxide

HPOD: linoleic acid hydroperoxide

NGS: Next Generation Sequencer

VOCs: volatile organic compounds

GENERAL CONCLUSION

Plants produce and release a diverse array of volatile organic compounds. These volatiles are assumed to benefit plants through positive and negative interactions with biotic and abiotic factors in the environment. GLVs, for instance, attract pollinators and seed dispersers and protect plants directly from attacking herbivores and pathogens. The GLVs follow biosynthetic pathway with two important enzymes, LOX and HPL, which were considered to be the key enzymes for formation of C6 aldehydes.

In this thesis, considerable effort has been done to understand the function of two enzymes, LOX and HPL, in GLV formation in two plant species *Marchantia polymorpha* and *Camellia sinensis*. In order to get evidence for their functions, I analyzed and quantified the volatiles by GC/MS, and the expression level of *MpLOX7* and *CYP74B24* have been examined using RT-PCR. Furthermore, I analyzed enzymatic properties with recombinant enzymes.

I conclude my achievement on two questions. The first question addressed in my study was; how the liverwort *M. polymorpha* produces GLVs, while there is no *HPL* gene in the genome sequence. To answer the question, I investigated a LOX enzyme that might has an activity to form C6 aldehydes. *M. polymorpha* genome has sixteen LOX genes. Therefore, I performed deep search in RNA-sequencing data and phylogenetic tree analysis. As a result, cytosolic MpLOX7 located in non-seed plant clade in the phylogenetic tree with unique conserved domain in between L298 to F308 was chosen. After cloning and expression, the purified recombinant protein showed enzyme activity to form *n*-hexanal and (*Z*)-3-hexenal. *MpLOX7* in *M. polymorpha* might at least partly account for the formation of C6 aldehydes especially after mechanical wounding.

The second research question addressed in this thesis was; how the VOCs composition in tea plant *C. sinensis* is regulated. CYP74B24 was demonstrated to encode a functional 13HPL in *C. sinensis*. It was also noted that expression of *CYP74B24* was induced at two hours after mechanical wounding. The mount of GLVs increase by 10 min after mechanical wounding, then, decrease to low level by two hours. Thus, I conclude that, the possibility of other factors like *LOX* or lipase is in the regulating step of GLVs formation in *C. sinensis*.

LIST OF PUBLICATIONS

This thesis is based on the following two studies, which are shown as in chapter 1 and 2.

CHAPTER 1

1. *n*-Hexanal and (*Z*)-3-hexenal are generated from arachidonic acid and linolenic acid by a lipoxygenase in *Marchantia polymorpha* L.

Moataz M. Tawfik, Katsuyuki T. Yamato, Takayuki Kohchi, Takao Koeduka and Kenji Matsui.

Bioscience, Biotechnology, and Biochemistry (2017), in press.

CHAPTER 2

2. CYP74B24 is the 13-hydroxyperoxide lyase involved in biosynthesis of green leaf volatiles in tea (*Camellia sinensis*)

Eiichiro Ono, Taiki Handa, Takao Koeduka, Hiromi Toyonaga, Moataz M. Tawfik, Akira Shiraishi, Jun Murata, and Kenji Matsui.

Plant Physiology and Biochemistry, Volume: 98, Issue: January 2016, pages: 112-118.

Academic symposium

6th International Singapore Lipid Symposium, National University of Singapore, Singapore (Postal presentation). Title: Cloning and expression of a lipoxygenase gene from *Marchantia polymorpha* L.

Moataz M. Tawfik, Katsuyuki T. Yamato, Takayuki Kohchi, Takao Koeduka and Kenji Matsui.