A dual positional specific lipoxygenase functions in the generation of flavor compounds during climacteric ripening of apple

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Lipoxygenase (LOX) is an important contributor to the formation of aroma-active C6 aldehydes in apple (Malus × domestica) fruit upon tissue disruption but little is known about its role in autonomously produced aroma volatiles from intact tissue. We explored the expression of 22 putative LOX genes in apple throughout ripening, but only six LOXs were expressed in a ripening-dependent manner. Recombinant LOX1:Md:1a, LOX1:Md:1c, LOX2:Md:2a and LOX2:Md:2b proteins showed 13/9-LOX, 9-LOX, 13/9-LOX and 13-LOX activity with linoleic acid, respectively. While products of LOX1:Md:1c and LOX2:Md:2b were S-configured, LOX1:Md:1a and LOX2:Md:2a formed 13(R)-hydroperoxides as major products. Site-directed mutagenesis of Gly567 to an alanine converted the dual positional specific LOX1:Md:1a to an enzyme with a high specificity for 9(S)-hydroperoxide formation. The high expression level of the corresponding MdLOX1a gene in stored apple fruit, the genetic association with a quantitative trait locus for fruit ester and the remarkable agreement in regio- and stereoselectivity of the LOX1:Md:1a reaction with the overall LOX activity found in mature apple fruits, suggest a major physiological function of LOX1:Md:1a during climacteric ripening of apples. While LOX1:Md:1c, LOX2:Md:2a and LOX2:Md:2b may contribute to aldehyde production in immature fruit upon cell disruption our results furnish additional evidence that LOX1:Md:1a probably regulates the availability of precursors for ester production in intact fruit tissue.

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INTRODUCTION

Flavor is one of the most important quality attributes of the ripe apple fruit and plays a decisive role in the economic prospects of a cultivar in the market. Among the 350 volatile compounds that are emitted by apple fruit,1 about 20 are considered as key character compounds as they dominate the typical aroma of a particular cultivar.2 Substances with a high impact on the aroma of apple are esters, as well as various C6 alcohols and aldehydes. Although aldehydes are produced at relatively low amounts in mature apples, they determine the flavor of immature fruits.3 During ripening, the aroma profile changes from aldehydes to esters,4 to such an extent that esters can account for more than 80% of all aroma-relevant substances in some cultivars.5

The formation of primary metabolites for inclusion into fruit esters occurs via several pathways.6 Fatty acid degradation is thought to contribute to the formation of short chain fatty acids and aldehydes to yield straight-chain esters.7-9 The leucine/isoleucine/valine pathway provides precursors to branched-chain esters9 and may contribute to the formation of short-chain fatty acids.10 Saturated fatty acids are degraded by the enzymatic steps of β-oxidation whereas linoleic (LA) and linolenic acid (LnA) are either catabolized through β-oxidation or the lipoxygenase (LOX) pathway. The latter involves the sequential action of LOX, hydroperoxide lyase and alcohol dehydrogenase (ADH).6 The resulting alcohols are linked to an acyl moiety by alcohol acyl-CoA transferases (AATs) to form esters. Unsaturated straight-chain esters appear to arise mainly by the LOX pathway in apple.8

LOX (linoleate oxygen oxidoreductase, EC 1.13.11.12) are non-heme iron-containing dioxygenases. They catalyse the regio- and stereospecific introduction of molecular oxygen into polyunsaturated fatty acids, containing a ZZ-1,4-pentadiene system such as LA and LnA to form hydroperoxy octadecadienoic acid (HpODE) and hydroperoxy octadecatrienoic acid, respectively.11 Plant LOX are classified according to the position of oxygenation in the carbon backbone of LA.12 This occurs either at carbon atom 9 (9-LOX) or 13 (13-LOX), forming the corresponding hydroperoxide derivatives, respectively (Figure 1). However, LOX enzymes are not perfectly specific and biocatalysts that produce more than 10% of the alternative regio-isomer are called dual positional specific LOX.14 Both 9- and 13-hydroperoxides can be catabolized to aroma-active volatile aldehydes by hydroperoxide lyase. However, only 13-LOX activity contributes to the apple aroma due to the formation of precursors of C6 volatile compounds.15 Alternatively, LOX genes are classified based on comparison of LOX primary structures.13 Most plant LOX share high sequence similarity of about 70% and are therefore grouped into the so-called type-1 LOX class. Members of the type-2 LOX class show only moderate sequence similarity but exhibit a putative chloroplast transit peptide.12 TomloxC, a chloroplast-targeted LOX from tomato, has been identified as key LOX specifically involved in the generation of fatty acid-derived C6 aldehydes and alcohols upon tissue disruption.16

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Plant LOXs form multi-gene families comprising varying numbers of genes. While five LOX genes have been cloned from the transcriptome of tomato, up to 20 LOXs have been reported for soybean and grapevine. It has been postulated that plant LOX may be involved in plant growth, development, senescence, stress, defense and response to wounding. LOX enzymes catalyze the initial reaction in the biosynthetic pathways of diverse defense molecules such as jasmonates and traumatin, and of volatile compounds such as hexanal, hexenal and hexenol (Figure 1).

LOX genes with potential roles during fruit ripening have been described in tomato, apricot, peach and kiwifruit. Initially, the number of putative LOX genes in apple was determined to be nine but was recently revised to 23 by detailed in silico analysis of the Malus domestica cv. Golden Delicious genome sequence. The identification of genes putatively involved in apple fruit development and flavor formation revealed a LOX gene (expressed sequence tag (EST) cluster MD187410, MdLOX1a) through large-scale statistical analysis of apple EST libraries. This gene was later mapped on the apple linkage group 9 closely to a quantitative trait locus cluster for ester-type volatiles. MdLOX1a transcript level increases during climacteric ripening and storage of apple fruit independent of the tested apple cultivar. The gene is also expressed at high levels in young leaves and is upregulated in response to ethylene consistent with its putative function in the formation of hexanal-derived esters (i.e., hexyl esters) during fruit ripening.

Despite the extensive research on ripening-dependent changes in the aroma profile of apple fruits, very little information is available on the function and regulation of enzymes involved. To identify LOX enzymes contributing to 6-carbon straight-chain ester formation, we biochemically analyzed four LOX enzymes of the MdLOX1 and MdLOX2 gene cluster, as their genes show a ripening-related expression pattern. Here we report the characterization of a dual positional specific (R)-LOX, which is induced by ethylene in fruits and forms both 13(R)- and 9(S)-hydroperoxides from LA and LnA. Site-directed mutagenesis elucidated the sources of enzymatic specificity. The relevance of the candidate gene for fruit ester production in apple fruit is discussed in the context of available information on ripening-dependent changes in aroma composition.

MATERIALS AND METHODS

Chemicals

All chemicals used were standard commercial products of analytical grade from the following companies: linoleic acid (Roth, Karlsruhe, Germany); α-linolenic acid (Roth); arachidonic acid (Sigma-Aldrich, Steinheim, Germany); triphenyl phosphine (Merck Schuchardt, Hohenbrunn, Germany); HPLC solvents (Mallinckrodt Baker, Deventer, Holland); 9(S)-HODE und 13(S)-HODE (Cayman Chemical, Tallinn, Estonia); Hydroperoxy HPLC Mix (Cayman Chemical).

Cloning of full-length apple LOX cDNAs

Total RNA was isolated from fruit peel of domesticated apple (Malus domestica cv. Golden Delicious) cultivar Golden Delicious by CTAB extraction. Reverse transcription was performed on 5 μg of total RNA with Superscript III reverse transcriptase (Invitrogen, Karlsruhe, Germany) and a GeneRacer oligo-dT primer (5'-GCT GTC AAC GAT ACG CTA CGT AAC GGC ATG ACA GTG T(18')-3'). The primers for gene amplification were designed based on bioinformatically selected LOX sequences from the apple genome described by Vogt et al. In total, primer pairs for six full-length apple LOX genes were used.

Figure 1. The LOX pathway. The dioxygenation of PUFAs by 9- and 13-LOX activity forms precursors for important phytoxylipins with functions in plant defense, wound signaling, senescence and fruit ripening. The formation of fruit esters from 13-hydroperoxide occurs by the HPL pathway and the action of AAT. AAT, alcohol acyl-CoA transferase; ADH, alcohol dehydrogenase; AOS, allene oxidase synthase; DES, divinyl ether synthase; HPL, hydroperoxide lyase; POX, peroxynase; PUFAs, polyunsaturated fatty acids. Adapted from Feussner and Wasternack.
at corresponding annealing temperature (Supplementary Table S1), 90 s at 72 °C, 35 cycles; final extension at 72 °C for 5 min. PCR products were then gel-purified and A-tailed with Taq-DNA polymerase (New England Biolabs, Frankfurt, Germany) for ligation into the pgEM-T easy vector (Promega, Mannheim, Germany). Sequencing was used to confirm the identity of the amplified fragments.

Characterization of ripening apple fruit

‘Jonagold’ (Malus × domestica Borkh.) apples from a commercial orchard in Sparta, MI were used to investigate the relationship between stage of development, the volatile profile and the expression of putative LOX genes as described7 (Supplemental Figure S10).

RNA isolation and reverse transcription PCR (RT-PCR)

The expression of putative LOX genes was initially evaluated using RT-PCR to screen candidate genes for further analysis. A hot borate extraction procedure was used24 with some adaptation using a commercial purification kit (RNaseasy Mini Kit Qiagen). Two micrograms of total RNA was pretreated with Rnase-free DNase (Qiagen Inc., Valencia, CA, USA) to remove contaminating genomic DNA. The concentration of total RNA was measured using a spectrophotometer (Nanodrop; Thermo Scientific). First-strand cDNA was synthesized from 2.0 μg of treated total RNA, using a commercial kit as described by the manufacturer (SuperScript II; Invitrogen, Carlsbad, CA, USA) to a total volume of 20 μL. Expression of the LOXs was determined relative to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using semi-quantitative PCR analysis. cDNAs (1 μL) were used as templates in a 25-μL reaction containing 10 μM of the forward and reverse gene-specific primer (Supplementary Table S2). Specific and not conserved regions from each of 22 putative LOX genes were amplified. Amplification sizes ranged from 196 to 409 bp. For each PCR, two biological and two technical replicates were analyzed. PCR primers were designed using Primer3.29 Primer sequences, the expected size of the PCR product, optimum cycle number and optimum temperature for primer binding are listed (Supplementary Table S2). The PCR reaction was performed as follows: (i) 2 min at 94 °C, (ii) 30 s at 94 °C, (iii) 30 s at 55–61 °C, and (iv) 45 s at 72 °C, repeating 19–33 cycles from steps 2 to 4, and final elongation 5 min at 72 °C. Two genes, MdLOX7d and MdLOX1b, were amplified using Touchdown-PCR with the following steps: (i) 5 min at 94 °C, (ii) 30 s at 94 °C, (iii) 30 s at 72 °C (lowering 1 °C every cycle), (iv) 45 s at 72 °C, (v) repeating nine cycles from steps 2 to 4, (vi) 30 s at 94 °C, (vii) 30 s at 62 °C, (viii) 45 s at 72 °C, and (ix) repeating 31–33 cycles from steps 6 to 8, and final 5-min elongation at 72 °C. The amplified PCR products were separated by electrophoresis on a 1.2% (w/v) agarose gel, visualized with UV fluorescence of ethidium bromide, and photographed. Relative light density of the bands was quantified by a digital imaging system (EagleEye II; Stratagene, La Jolla, CA, USA). To identify the fraction to normalize relative to changes in expression during ripening. In addition, an additional 13-LOX selected for qPCR analysis based on RT-PCR results indicating significant changes in expression during ripening. In addition, an additional 13-LOX candidate gene (MdLOX2a) was subjected to qPCR based on its apparent ripening-independent RT-PCR expression pattern in order to further validate RT-PCR results. For the selected LOX genes, as well as for the housekeeping gene, GAPDH (GAPDH) PCR primers (Supplementary Table S3) were designed using Primer Express Software for Real Time PCR version 3.0 (Applied Biosystems, Grand Island, NY, USA) according to the strategies set up by Yokoyama and Nishitani.30 The high level of sequence similarity between the apple LOX members was taken into account when designing quantitative real-time PCR (qPCR) primers to avoid cross-annealing of primers. For this, the gene-specificity of primer sets was tested using the following procedure: (i) Genome Database for Rosaceae NCBI BLAST searches of apple genome database were performed for each primer to confirm that no other sequence in the apple genome was similar to any primer; and (ii) the specificity of PCR amplification was examined by monitoring the dissociation curves for qPCR reactions using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). PCR amplification efficiencies for each primer pair were determined in a semi-log regression plot, all primers efficient primers were in a range of 3.32 ± 10% slope (~3.32 indicates a PCR reaction with 100% efficiency). The length of all PCR products ranged from 50 to 70 bp. Final primer sets are listed in Supplementary Table S3. Serial dilutions of cDNA (10, 1, 0.1 and 0.01 ng μL−1) were made with water for generating standard curves to test primer efficiency. PCR reactions were performed in a total volume of 10 μL, using 3 μL of the two primers (1 μM), 5 μL of 2× SYBR Green PCR Master Mix (Applied Biosystems) and 2 μL of the cDNA template. qPCR analysis was an ABI PRISM 7900HT sequence detection system (Applied Biosystems). The qPCR program included a preliminary step of 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. No-template controls for each primer pair were included in each run. Apple GAPDH gene was used as an internal control to normalize small differences in template amounts. The real time data generated was analyzed applying the ΔΔCt method.31 At least two different RNA isolations and cDNA syntheses were used as biological replicates and three technical replicates for the qPCR. Expression levels produced by qPCR were expressed as a ratio relative to the fruit stage one to emphasize ripening-dependent behavior.

Expression of recombinant LOX protein in yeast

Heterologous expression of four LOX genes in Saccharomyces cerevisiae was achieved by cloning in the pYES2 expression vector (Invitrogen, Karlsruhe, Germany). The full-length open reading frames of the genes were amplified from the pgEM-T easy vector constructs described above with Phusion® High-Fidelity DNA Polymerase. Since the pYES2 vector does not exhibit a tag for protein purification, the PCR products were subcloned in the bacterial pRSET B vector (Invitrogen, Karlsruhe, Germany). This vector enables in frame fusion to a sequence that encodes an N-terminal fusion peptide including a histidine tag and an enterokinase recognition site for subsequent removal of the fusion peptide from the purified recombinant protein. The sequences of the fusion proteins were then amplified from the pRSET B vector with oligonucleotides containing the yeast translation initiation site required for heterologous expression in yeast and cloned in the pYES2 expression vector. Oligonucleotides applied during this procedure are depicted in Supplementary Table S5. The resulting vector constructs were transformed into the S. cerevisiae INVSc1 strain for expression of recombinant protein, which was performed as described by12 Cells were harvested 24 h after induction in aliquots corresponding to 25 mL culture by centrifugation (5000g, 4 °C, 5 min). Cell pellets were washed with water once, frozen in liquid nitrogen and kept at ~80 °C until further use.

Partial purification of recombinant protein

Cell pellets corresponding to 200 mL culture were resuspended in 4 mL of breaking buffer (50 mM sodium phosphate buffer, pH 7.5, 1 mM EDTA, 5% (v/v) glycerol, 10 mM imidazole, 1 mM PMSF) to obtain an OD_{600} of 50. Lysis of cells was achieved by addition of glass beads and repeated vortexing for 30 s followed by 30 s incubation on ice. After 20 min of treatment cell debris was removed by centrifugation (14,800g, 4 °C, 10 min). Protein crude extract was loaded on a gravity column with 1 mL Profilin™ IMAC Ni-charged resin (Bio-Rad Laboratories, Munich, Germany) and incubated for 2 h with orbital shaking at 4 °C. After washing with 10 mL of 50 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl and 30 mM imidazole, elution was performed with 600 μL of 50 mM sodium phosphate buffer (pH 7.5) containing 300 mM NaCl and 500 mM imidazole. The elution was repeated three more times. Fractions with LOX activity were pooled, dialyzed overnight against 50 mM sodium phosphate buffer (pH 7.0) and concentrated by ultrafiltration using Vivaspin 500 centrifugal concentrators (Sartorius), Invitrogen Biotech, Germany). The protein was dialyzed against the protein buffer (pH 7.0) with an OD_{600} cutoff of 50,000. Samples were analyzed via SDS–PAGE and protein concentration was determined by the Bradford method using bovine serum albumin as standard.33 Protein was stored in small aliquots at ~80 °C until required.

Determination of enzyme properties

LOX activity was determined by continuously monitoring the formation of conjugated dienes at 234 nm. The standard assay mixture consisted of

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500 μL 50 mM sodium phosphate buffer (pH 7.5) and the appropriate amount of purified protein (0.2 to 2 μg). Reactions were started by addition of 0.3 mM substrate and incubated at 25 °C with constant shaking for 1 h. The reaction products were extracted with dichloromethane, reduced by addition of 400 nmol triphenyl phosphine and evaporated to dryness under a constant flow of nitrogen. After resuspension in 30% (v/v) methanol samples were submitted to HPLC analysis. Reaction buffers were prepared with 0.5 pH unit increments as follows: 50 mM sodium citrate buffer (pH 3.0–6.0), 50 mM sodium phosphate buffer (pH 6.0–8.0) and 50 mM Tris HCl buffer (pH 8.0–10.0). For determination of the pH optimum of the recombinant proteins, the reaction was run across a pH range of 3.0–10.0 at 25 °C. The optimum temperature was determined by assaying LOX activity rates at temperatures of 5–55 °C with 5 °C increments at a pH of 7.0. Kinetic properties of recombinant LOX were determined by monitoring hydroperoxide formation across a range of concentrations of LA and LnA with a Niccolot evolution 100 spectrophotometer (Thermo, Cambridge, UK). The standard assay was performed at 45 °C with 0–100 μM substrate in 50 mM sodium citrate buffer (pH 6.0) for LOX1:Md:1a and Gly567Ala and 50 mM sodium phosphate buffer (pH 7.5) for LOX1:Md:1c, respectively. Each reaction was run in triplicate. The first order kinetic rates were estimated as changes in absorbance at 234 nm s⁻¹ and converted into μmol min⁻¹ mg⁻¹ using the extinction coefficient ε = 23 000 M⁻¹ cm⁻¹ for LA and LnA hydroperoxides. One unit of LOX activity is defined as the amount of enzyme catalysing the formation of 1 μmol of hydroperoxide per minute under optimal reaction conditions. The data were then fitted using Michaelis–Menten equation to determine the kinetic parameters for all enzymes.

Identification of LOX products
Hydroxy fatty acids were separated by straight phase-HPLC (SP-HPLC) using a LiChrospher® 100 Diol column (Merck, Darmstadt, Germany; 125 × 4 mm, 5 μm particle size). The solvent used was hexane/2-propanol/formic acid (100:2:0.1, by vol) and a flow-rate of 2 mL min⁻¹, with detection at 234 nm. Fractions of the 9- and 13-enantiomers were collected and submitted to Chiral phase-HPLC (CP-HPLC) for analysis of product stereochemistry. This was carried out on a Chiralpak® IA column (Daicel Chem. Industries, distributed by Alltmann Analytik (München, Germany); 250 × 4.6 mm, 5 μm particle size) with a solvent system of hexane/ethanol/acidic acid (95:5.0:0.2, by vol) and a flow-rate of 0.75 mL min⁻¹. The enantiomers of 9- and 13-HODE as well as 15(S)-, 12(S)- and 5S)-HETE were identified by coelution with authentic standards. 9- and 13-HOTE as well as 11-, 9- and 8-HETE products were identified via their mass and fragmentation pattern in LC-MS analysis based on data for fatty acyl standards derived from the LIPID MAPS Lipidopedia Gateway (http://www.lipidmaps.org/data/standards/index.html). LC-MS analysis was performed as described by Huang et al. The HPLC system consisted of a quaternary pump and a variable wavelength detector, all from Agilent 1100 (Bruker Daltonics, Bremen, Germany). The column was a Luna® C18 (Phenomenex, Aschaffenburg, Germany; 150 × 2 mm, 100 A, 5 μm particle size). Absorbances were recorded at 234 nm for the detection of hydroxy fatty acids.

Site-directed mutagenesis of MdLOX1a
Two approaches were chosen to decide on amino-acid positions for site-directed mutagenesis: (i) the available literature was searched for mutagenesis studies on single amino acids of plant and mammalian LOXs. The primary sequences of characterized enzymes were aligned to LOX1:Md:1a using Geneious, version 5.6.6 (Biomatters; http://www.geneious.com/) to identify corresponding positions in the apple LOX. Amino acids, which are likely to be responsible for stereo- or regiospecificity in LOX, were chosen for mutagenesis; and (ii) homology models of apple LOXs were created by the SWISS-MODEL server using the X-ray coordinates of soybean L-3 (PDB ID 1IK3) as template and several structures of the dual positional specific LOX1:Md:1c, and a closely related 9-LOX (LOX1:Md:1c) were visualized with the PyMOL Molecular Graphics System, version 1.3 (Schrödinger, LLC; http://www.pymol.org) and inspected for substitutions in the active site region. Each of these positions was mutated separately in the MdLOX1a gene.

Site-directed mutagenesis was carried out by using the methods described by Horton or the QuikChange Site-Directed Mutagenesis Kit (Stratagene, Amsterdam, The Netherlands). The mutants Gly567Ala and lle566Ph and Val582Ph were created using overlap extension PCR (oePCR) producing overlapping gene fragments with the desired mutation. In order to minimize the risk of hairpin and self-folding the apple oligonucleotides contained additional conservative base exchanges apart from the appropriate mutations. The mutants Gly567Ala, Leu572Ile, ile578Leu and Thr775Leu were created following the instructions of the QuikChange™ protocol. All oligonucleotides used in this experiment are depicted in the Supplementary Table S6. Mutagenesis of the apple LOXs was confirmed by sequencing with gene-specific primers.

Transient expression of yellow fluorescent protein (YFP) fusions in tobacco plants
Subcellular localization of deduced LOX proteins was predicted through the identification of signal peptide sequences using several prediction programs such as ProtComp (http://www.softberry.com/berry.phtml), PSORT (psort.ibbb.jp/), TargetP (http://www.cbs.dtu.dk/services/TargetP/), ChloroP (http://www.cbs.dtu.dk/services/ChloroP/), Signal-3L (http://www.csbio.sjtu.edu.cn/bioinf/Signal-3L/) and Predotar (http://urgi-versailles.sina.fr/predotar/predotar.html). Chimeric YFP constructs were produced for MdLOX2a, MdLOX4a and MdLOX6b in the 13-LOX group and MdLOX1a, MdLOX7a and MdLOX7c in the 9-LOX group. All were designed to express the first 166–300 amino-acid residues of the protein including, where present, the putative chloroplast transit peptide. The YFP sequence was fused to the C-terminus of each LOX sequence. Phusion® High-Fidelity DNA polymerase (New England Biolabs, Burlington, MA, USA) was used for PCR and cloning applications. Directional cloning was performed using the P-ENTR- D-TOPO entry vector kit (Life Technologies, Carlsbad, CA, USA). After sequencing, purified plasmids were digested with MluI restriction enzyme (New England Biolabs) and a LR clonase recombination reaction between the entry and destination vector (3SS-Gateway-YFP-HA tag-OC3’, ABRC stock no. CD3-683 at The Ohio State University) was performed as described by the manufacturer (Life Technologies). Tobacco (Nicotiana tabacum cv Petit havana) plants, grown for five weeks at 22 °C in a greenhouse, were used for Agrobacterium tumefaciens-mediated transient expression. Bacterial culture was incubated at 28 °C with agitation for 16 h. An aliquot (0.2 mL) of bacterial culture then was pelleted in a 1.5-mL Eppendorf tube by centrifugation at 4000g, for 5 min at room temperature. The pellet was resuspended in 0.5 mL of infiltration medium (50 mM MES, pH 5.6, 2 mM NaHPO₄, 200 μM aceto-syringone and 5 mg mL⁻¹ glucose). The bacterial suspension was diluted with infiltration buffer to adjust the inoculum concentration to a final OD600 value range of 0.05–0.1. The bacterial suspension was infiltrated using a 1-mL syringe without a needle by gentle pressure through the stomata on the abaxial surface. Transformed plants then were incubated under normal growth conditions for 72 h.

Confocal imaging was performed using an inverted laser scanning microscope (IX81 model FV1000, Olympus, Melville, NY, USA) with ×20 and ×40 air objectives. Excised plant tissues were mounted in water between a microscope slide and cover slip and examined. To detect YFP fluorescence, an argon ion laser was used to produce excitation at a wavelength of 488 nm and fluorescence was detected in the range of 505–530 nm. Chlorophyll fluorescence was detected in the range of 670–730 nm using an excitation laser with a wavelength of 633 nm. All images were acquired from single optical sections. Post-acquisition image processing was done using Olympus Fluoview FV1000 software.

Sequence data: The nucleotide sequences reported in this paper are annotated in the GenBank database at NCBI (http://www.ncbi.nlm.nih.gov) under the accession numbers KC706480 MdLOX1a, KC706481 MdLOX1c, KC706482 MdLOX1d, KC706483 MdLOX2a and KC706485 MdLOX2b.

RESULTS

Cloning and sequence analysis of LOXs from apple
The oligonucleotides for amplification of MdLOX1 and MdLOX2 genes were designed based on manually curated Malus × domestica predicted protein sequences. The MdLOX1 gene cluster comprises five putative functional LOX sequences, whereas the MdLOX2 gene cluster seemed to consist of only two LOX isoforms. Six candidate genes (MdLOX1a, MdLOX1b, MdLOX1c, MdLOX1d, MdLOX2a and MdLOX2b) contained all functional motifs of plant LOX enzymes and were chosen for primer design (Supplementary Table S1). The bioinformatically predicted open reading frame of MdLOX1e (MDP0000312394) exhibited large sequence deletions and lacked the stop codon immediately downstream of the highly conserved

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C-terminal motif. Also, manual evaluation of the Golden Delicious genome did not succeed in a functional full-length LOX sequence for MdLOX1.

The PCR amplification of LOX genes on apple-derived cDNA resulted in the successful cloning of three members of the MdLOX1 gene cluster (MdLOX1a, MdLOX1c and MdLOX1d) and both genes of the MdLOX2 group. Nucleotide sequences of MdLOX1a, MdLOX1c and MdLOX1d share an identity of 76%–86% and display highest similarity to the almond PdLOX1 gene (AJ404331) and the hazelnut CaLOX1 gene (AJ417975) with up to 87% and 79%, respectively (Supplementary Figure S1). Both LOX genes have been categorized as type-1 9-LOX.

MdLOX2a and MdLOX2b share an identity of 93% and exhibit highest similarity towards the grapevine VvLOXO gene (71%, FJ858257), which encodes a functional type-2 13-LOX. Sequences of apple LOX genes were submitted to the GenBank database at NCBI (http://www.ncbi.nlm.nih.gov, accession numbers KC706480 MdLOX1a, KC706481 MdLOX1c, KC706482 MdLOX1d, KC706485 MdLOX2a and KC706486 MdLOX2b). The nomenclature of the deduced LOX proteins was employed as described by Vogt et al. Thus, the resulting protein of the MdLOX2a gene which is a type-2 LOX was named LOX2:Md:2a.

For comparison of protein primary structures, we created a multiple sequence alignment of the deduced amino-acid sequences of all five candidate genes (Supplementary Figure S2). The predicted sizes of LOX2:Md:2a (906 amino acids) and LOX2:Md:2b (905 amino acids) (Supplementary Table S1) were larger compared to the MdLOX1 group, except of LOX1:Md:1d, due to the presence of a putative chloroplast transit peptide, thus designating them as type-2 LOX. The deduced proteins LOX1:Md:1a and LOX1:Md:1c lacked an amino-terminal extension and were therefore classified as type-1 LOX. Earlier attempts to predict sub-cellular localization of LOX1:Md:1d has not been unequivocally possible since the gene encodes for an unusually long N-terminus harboring a potential signal peptide. However, due to the high sequence similarity to genes of the MdLOX1, MdLOX3 and MdLOX7 clusters, it was designated as type-1 LOX.

Furthermore, all MdLOX1 proteins exhibit the TV motif in Sloane position (Supplementary Figure S2; LOX1:Md:1a Thr581/Val582; LOX1:Md:1c Thr580/Val581; LOX1:Md:1d Thr670/Val671) that is indicative of LOX enzymes with 9-LOX function. Whereas both members of the MdLOX2 group harbor the more space filling amino-acid cysteine and phenylalanine typical for linoleate 13-LOX (LOX2:Md:2a Cys623/Phe624; LOX2:Md:2b Cys622/Phe623). In addition, stereospecificity of LOX enzymes is determined by a conserved alanine residue in close proximity to this motif. All characterized apple LOX proteins possess an alanine at this position (LOX1:Md:1c Ala566; LOX1:Md:1d Ala656; LOX2:Md:2a Ala609; LOX2:Md:2b Ala608) suggesting either 9(S)- or 13(S)-LOX activity. Only LOX1:Md:1a exhibits a glycine residue (Gly567) responsible for dual positional specificity and (R)-LOX activity.

Ethylene, respiration rate and aroma volatiles
To link changes in gene expression with fruit ripening and the concomitant synthesis of hexyl esters (i.e., esters derived from hexanol), we determined progressive changes in the internal ethylene concentration, respiration and ester formation (Figure 2). Eight developmental stages were discernible based on changes in these three indices. Hexyl ester (the sum of hexyl acetate, hexyl propanoate, hexyl butanoate, hexyl pentanoate, hexyl hexanoate, hexyl 2-methylpropanoate and hexyl 2-methylbutanoate) emissions were
first detected after stage 3, while internal ethylene levels were low (less than 0.1 μL L⁻¹), but steadily increasing. The onset of autocatalytic ethylene occurred after stage 4 and coincided with the onset of climacteric respiration and a rapid increase the emissions of hexyl esters.

RT-PCR and real-time qPCR assays

Semi-quantitative RT-PCR of 22 putative LOX genes revealed that 17 were expressed in apple peel (Supplementary Figure S3). The expression of most genes exhibited no discernable pattern during ripening; however, at least six were highly expressed and ripening-dependent. In the putative 13-LOX group, MdLOX4a showed a marked peak at developmental stage 5, which coincided with the rise in autocatalytic ethylene. The expression of MdLOX6a and MdLOX6b declined during ripening. In the putative 9-LOX group, MdLOX3a expression declined during ripening, while MdLOX7a and MdLOX7c increased (Supplementary Figure S3). No visible PCR bands were detected from MdLOX1d, MdLOX4a, MdLOX5d, MdLOX5e and MdLOX9a during fruit development.

qPCR was performed on the 6 LOX genes that showed ripening-dependent patterns, and additionally included MdLOX2a due to its lack of variation during ripening to provide additional support for the accuracy of the RT-PCR results. In each instance, the RT-PCR and qPCR results yielded similar results (Figure 3). In the putative 13-LOX group, MdLOX2a exhibited little change or a slight decrease in expression during ripening. again, MdLOX4a evidenced a sharp increase at stage 5, and MdLOX6a and MdLOX6b were downregulated during ripening. Within the putative 9-LOX group, MdLOX7c was downregulated and MdLOX7a and MdLOX1a were upregulated as ripening progressed. MdLOX1a expression increased more than 100-fold throughout the course of ripening, with the initial increase occurring between developmental stages 3 and 4, concomitant with the first detectable emissions of hexyl esters.

Biochemical analysis of four recombinant apple LOX

The full-length open reading frame sequences of MdLOX1a and MdLOX1c (type-1 9-LOX) as well as MdLOX2a and MdLOX2b (type-2 13-LOX) were cloned into the pYES2 vector for heterologous expression in yeast. His-tagged apple LOX proteins were partially purified from protein crude extracts by affinity chromatography. SDS–PAGE analysis revealed proteins with a molecular mass of about 105 kDa, in accordance with the predicted values for recombinant LOX1:Md:1a (101.5 kDa), LOX1:Md:1c (101.6 kDa), LOX2:Md:2a (106.5 kDa) and LOX2:Md:2b (106.6 kDa) proteins (Supplementary Figure S4). Both members of the MdLOX1 group exhibited highest enzymatic activity with LA at 45 °C (Supplementary Figure S5a and S5b). Recombinant LOX1:Md:1a showed a rather narrow optimum at 45 °C with rapidly decreasing activity at lower or higher temperatures, but high residual activity at 55 °C (54%). LOX1:Md:1c displayed high catalytic activity in a broad range of temperatures between 15 and 50 °C, with a sharp decrease above 50 °C. In contrast, LOX2:Md:2a and LOX2:Md:2b had lower optimum temperatures of 35 °C and 25 °C, respectively (Supplementary Figure S5c and S5d). Both enzymes showed negligible activity at temperatures higher than 45 °C. However, it should be mentioned, that LOX2:Md:2b displayed considerably high activity at 5 °C (60%). The members of the MdLOX1 group (Supplementary Figure S5e and S5f) showed an optimum pH of 6.0 (LOX1:Md:1a) and 7.5 (LOX1:Md:1c). Both enzymes exhibited high activity (>80%) in a broad pH range with sharply decreasing relative activity below 5.0 and above 7.5. The members of the MdLOX2 group had an optimum pH of 7.0. They showed moderate enzyme activity (greater than 60% of relative activity) in a pH range of 4.5–8.0 (LOX2:Md:2a) and 6.0–9.5 (LOX2:Md:2b), but only a rather narrow range of high activity around neutral pH (Supplementary Figure S5g and S5h).

Regio- and stereochemistry of reaction products

Reaction products of LA, LnA and arachidonic acid (AA) formed by the four proteins were analyzed to characterize regio- and stereospecificity of the apple LOXs. Partially purified LOX1:Md:1a, LOX1:Md:1c, LOX2:Md:2a and LOX2:Md:2b proteins converted all three tested substrates to their corresponding hydroperoxy fatty acids, as was confirmed by LC-MS analysis (Supplementary Figure S6). Additionally, reaction products with LA were reduced to their corresponding hydroxy fatty acids and analyzed using SP-HPLC (Figure 4). Fractions of 9- and 13-HODE were collected and analyzed for enantiomer composition by CP-HPLC (Figure 4 insets). Both, LOX1:Md:1a and LOX1:Md:1c produced 9(S)-HpODE from linoleic acid (Figure 4a and 4b), but LOX1:Md:1a formed primarily 13(R)- HpODE (about 77% of total hydroperoxides). LOX1:Md:1a acted as a dual positional specific enzyme producing 13- and 9-HpODE in a ratio of 8:1 (Table 1). In contrast, 9(S)-HpODE was the major product of LOX1:Md:1c activity with LA, as only trace amounts of the regiosomer (0.5% of total hydroperoxides) and enantiomer (2.3%) was formed. Similar results were obtained for LOX activity of both
enzymes with LnA (Supplementary Table S4). LOX2:Md:2a and LOX2:Md:2b converted LA and LnA predominantly to 13-hydroperoxides (Figure 4c and 4d). However, the main product of LOX2:Md:2b reaction with LA was identified as 13(\textit{S})-HpODE, whereas LOX2:Md:2a formed 13(\textit{R})- and 9(\textit{S})-HpODE in a ratio comparable to LOX1:Md:1a. Accordingly, LOX1:Md:1a and LOX2:Md:2a were classified as (\textit{R})-LOX with a 13(\textit{R})/9(\textit{S})-specificity. LOX1:Md:1c and LOX2:Md:2b proved to be (\textit{S})-LOX enzymes and were classified as linoleate 9(\textit{S})- and 13(\textit{S})-LOX, respectively.

Additionally, we tested for substrate conversion of AA by all four apple LOXs. Arachidonic acid is of minor physiological importance in plants and is therefore often a poor substrate for plant LOXs. Even so, all wild-type apple LOX enzymes were able to convert AA at least to some extent to hydroperoxy eicosatetraenoic acid (HpETE). LOX2:Md:2b showed highest product specificity as it formed solely 15-HpETE (Supplementary Table S4). LOX1:Md:1a and LOX2:Md:2a produced considerable amounts of 15-HpETE (42%–45%), but also 12-HpETE (16%–17%), 9-HpETE (12%–16%) and small amounts of 11-, 8- and 5-HpETE from AA. The major AA products formed by LOX1:Md:1c was 11-HpETE (53%) and 5-HpETE (18%).

Site-directed mutagenesis of \textit{MdLOX1a}

To further evaluate the potential for the involvement of LOX1:Md:1a in autonomous aroma formation in ripe apple fruits, we investigated the determinants of LOX1:Md:1a specificity by site-directed mutagenesis of the \textit{MdLOX1a} gene and subsequent biochemical analysis of the heterologously expressed mutant proteins. To compare our findings on enzymatic activity, we chose LOX1:Md:1c as a reference. Both apple LOXs share a sequence identity of 85% and a highly conserved active site with the putative 9-LOX-specific TV motif (Supplementary Figure S2, LOX1:Md:1a Thr581/Val582; LOX1:Md:1c Thr580/Val581) at the so-called Sloane position.

Table 1. Linoleic acid products formed by wild-type apple LOX enzymes. Proportion of hydro(peroxy)xy fatty acids is given as percent of total product amount obtained from specific LOX activity with linoleic acid.

| Enzyme    | 9-H(p)ODE (%) | 13-H(p)ODE (%) |
|-----------|---------------|----------------|
| LOX1:Md:1a | 11.2          | 88.8           |
| LOX1:Md:1c | 99.5          | 0.5            |
| LOX2:Md:2a | 12.4          | 87.6           |
| LOX2:Md:2b | 6.0           | 94.0           |

Chiral analysis (%)

|           | 9\textit{R} | 9\textit{S} | 13\textit{R} | 13\textit{S} |
|-----------|-------------|-------------|-------------|-------------|
| LOX1:Md:1a | 29.1        | 70.9        | 86.8        | 13.2        |
| LOX1:Md:1c | 2.3         | 97.7        | racemic     |              |
| LOX2:Md:2a | 8.8         | 91.2        | 97.9        | 2.1         |
| LOX2:Md:2b | racemic     | 13.6        | 86.4        |              |

Figure 4. HPLC analysis of hydroxy fatty acids formed by wild-type apple LOX enzymes followed by chemical reduction. Product profiles for LOX activity with linoleic acid were obtained via SP-HPLC. Ratios of (\textit{R})- and (\textit{S})-enantiomers were determined by CP-HPLC (Insets). (a) Wild-type LOX1:Md:1a. (b) Wild-type LOX1:Md:1c. (c) Wild-type LOX2:Md:2a. (d) Wild-type LOX2:Md:2b. A LiChrospher® 100 Diol column (125×4 mm, 5 µm) was eluted at 2 mL min$^{-1}$ with hexane:2-propanol:formic acid (100:2:0.1, by vol.) with UV detection at 234 nm. Chiral phase HPLC analysis of enantiomer composition was conducted on a Chiralpak® IA column (250×4.6 mm, 5 µm) with an elution at 0.75 mL min$^{-1}$ in hexane/ethanol/acetic acid (95:5:0.02, by vol.) and UV detection at 234 nm.
However, only LOX1:Md:1c produced exclusively 9(S)-hydroperoxides from LA and LnA. Comparison of their primary structures revealed four positions that differed in the enzyme active sites of both proteins (Supplementary Figure S2). Thus, Gly567, Leu572, Ile578 and Thr775 in LOX1:Md:1a were individually replaced by the corresponding amino acids of LOX1:Md:1c, resulting in the single mutants Gly567Ala, Leu572Ile, Ile578Leu and Thr775Leu (Figure 5). Three additional positions were mutated to elucidate the role of an inverse head-to-tail orientation of the substrate in the active site on the production of 13-hydroperoxides by LOX1:Md:1a.40 In the straight substrate orientation, free fatty acids enter the hydrophobic cavity with their methyl end first. The carboxyl group is then supposed to form salt bridges with polar residues near the cavity entrance.50 In LOX1:Md:1a, the positions Arg268 and Lys283 were candidates for such a salt bridge formation. Their effect on product regiochemistry was analyzed by substitution of Arg268 with a hydrophobic alanine residue (Figure 5). In addition, Ile566 at the cavity entrance was substituted by the more space-filling phenylalanine residue of LOX1:Md:1c to analyze a potential steric hindrance of salt bridge formation. A similar effect should be observed for substitution of Val582 at the Sloane position by a phenylalanine residue. Arg268Ala and Ile566Phe should interfere with the interaction of the carboxyl group and polar residues at the cavity entrance, leading to a preference of inverse substrate orientation and thereby 9(S)-LOX activity, whereas Val582Phe should hinder salt bridge formation of the carboxyl group and the conserved arginine residue (Arg732) at the bottom of the pocket, resulting in a straight substrate orientation and 13(S)-LOX activity. In total, seven positions were chosen for site-directed mutagenesis (Figure 5).

Of these seven analyzed single mutants only Arg268Ala, Gly567Ala, Ile578Leu and Val582Phe showed high substrate conversion rates of LA, LnA and AA (60%–100% of LOX1:Md:1a activity with LA). The Thr775Leu mutant exhibited significant lower activity (33% of LOX1:Md:1a activity with LA), whereas LOX activity of the Ile566Phe and Leu572Ile mutants was nearly completely abolished. Hydroperoxy products formed by the different mutant LOX pro-

Figure 5. Overview of the substrate binding pocket of LOX1:Md:1a. Homology model showing positions of amino-acid residues described to be essential for LOX activity and specificity (gray, blue) as well as positions chosen for site-directed mutagenesis (orange, blue). A 13(S)-HpODE molecule (green) illustrates position and depth of the substrate binding pocket. The bottom of the cavity is marked by Arg732, which is essential for enzyme activity and highly conserved among plant LOX enzymes. However, the catalytic iron atom (red sphere) is coordinated by five conserved residues (His524, His529, His715, Asn719, Ile863) near the entrance. The residues Thr581 and Val582 align with the so-called Sloane position described for mammalian LOX and are known to control regiospecificity of fatty acid oxygenation. In addition, Gly567 has been described to influence both regio- and stereoselectivity of LOX enzymes.
led to a catalytic efficiency of $2.78 \mu M^{-1} s^{-1}$ for LA- and $1.62 \mu M^{-1} s^{-1}$ for LnA-hydroperoxidation and demonstrate that LA is the preferred substrate of the Gly567Ala mutant. The 9(S)-LOX LOX1:Md:1c showed a lower affinity to LA ($K_m = 40.61 \mu M$) and LnA ($K_m = 70.85 \mu M$) than LOX1:Md:1a, but exhibited a higher substrate turnover rate comparable to the Gly567Ala mutant. Also, the catalytic efficiency for LA was 2.8-fold higher compared to LnA.

**Table 2.** Linoleic acid products formed by MdLOX1a mutants. Proportion of hydro(pero)xy fatty acids is given as percent of total product amount obtained from specific LOX activity with linoleic acid

| Enzyme     | 9-H(p)ODE (%) | 13-H(p)ODE (%) |
|------------|---------------|---------------|
| Arg268Ala  | 10.9          | 89.1          |
| Gly567Ala  | 97.3          | 2.7           |
| Ile578Leu  | 22.6          | 77.4          |
| Val582Phe  | 8.7           | 91.3          |

| Chiral analysis (%) | 9R | 9S | 13R | 13S |
|---------------------|----|----|-----|-----|
| Arg268Ala           | 37.5 | 62.5 | 82.5 | 17.5 |
| Gly567Ala           | 10.1 | 89.9 | racemic |
| Ile578Leu           | 22.2 | 77.8 | 86.5 | 13.5 |
| Val582Phe           | racemic | 79.1 | 20.9 |

**Figure 6.** HPLC analysis of hydroxy fatty acids formed by MdLOX1a mutants followed by chemical reduction. Product profiles for LOX activity with linoleic acid were obtained via SP-HPLC. Ratios of (R)- and (S)-enantiomers were determined by CP-HPLC (insets). (a) Arg268Ala mutant. (b) Gly567Ala mutant. (c) Ile578Leu mutant. (d) Val582Phe mutant. A LiChrospher® 100 Diol column (125 × 4 mm, 5 μm) was eluted at 2 mL min⁻¹ with hexane/2-propanol/formic acid (100:20:1, by vol.) with UV detection at 234 nm. Chiral phase HPLC analysis of enantiomer composition was conducted on a Chiralpak IA column (250 × 4.6 mm, 5 μm) with an elution at 0.75 mL min⁻¹ in hexane/ethanol/acetic acid (95:5:0.02, by vol.) and UV detection at 234 nm.

**DISCUSSION**

The C6 volatiles such as hexanal, Z-3-hexenal, E-2-hexenal and Z-3-hexenol are important for the characteristic aroma of different fruit products and are formed by LOX action and subsequent enzymatic reactions during maceration of leaf and fruit tissue. The unsaturated alcohols and aldehydes are abundant in the headspace or extracts of fruit juice or pulped tissue, whereas saturated esters predominate in the headspace volatiles above intact fruits such as apples.5,22 In most plant species (except tomato), aldehydes are more abundant in unripe fruit and esters in ripe fruit as esters.
accumulate when the fruit undergoes a climacteric rise in ethylene production. Thus, in apples and other fruits, most of the studies on aroma formation have focused on the last step of ester formation, in which AAT enzymes have a key role. Esters biosynthesis in apple is also limited by the levels of alcohols, which implies that a critical step for ester production may be regulated at the level of ADH and LOX. In the following, we provide strong evidence that LOX1:Md:1a is involved in the formation of C6 precursor substrates for AAT and is essential for ester production during climacteric ripening of apples whereas LOX1:Md:1c, LOX2:Md:2a and LOX2:Md:2b probably contribute to C6 aldehyde production in immature fruit after decompartmentation.

MdLOX1a is highly expressed during final fruit ripening after picking. Initially, the involvement of the LOX pathway in the generation of fruit esters was discovered by feeding experiments using unlabeled and isotopically labeled precursors. Although β-oxidation of fatty acids has been established as one of the primary processes contributing to the pool of alcohols and acyl-CoA for ester production, unsaturated straight-chain esters appear to arise only by the LOX pathway. Besides, precursors for butyl and possibly also propyl and pentyl ester may be formed from unsaturated fatty acids by sequential activity of LOX/HPL followed by β-oxidation. As ester composition and levels differed in apples treated with different signal molecules such as salicylic acid, ethephon (ethylene precursor) and methyl jasmonate, it was supposed that regulation of the pathway upstream of AAT activity depends on the regulation of LOX and ADH activity by the signal molecules.

Consistent with this, the suppression of ethylene biosynthesis and perception in apple caused a major reduction in all group of volatiles, especially in esters and alcohols. Exposure of apple fruit (in which ethylene synthesis had been suppressed) to ethylene led to a recovery in the levels of alcohols and aldehydes. While AAT enzyme activity displayed a clear pattern concomitant with ethylene regulation, total LOX enzyme activity (mixture of all LOX) seems to be independent of ethylene modulation. However, MdLOX1a is the only LOX gene that underwent a sustained increase in expression level concomitant with the onset of the rise in ripening-related ethylene and the climacteric rise in CO2 production and hexyl ester formation. Given that no Z-3-hexenal, Z-3-hexenol or esters derived from Z-3-hexenal are detected in emissions from intact fruit, LOX metabolism of fatty acid substrates is apparently limited to LA in intact tissues, which would yield hexanal, and subsequently, hexanol and hexyl esters. This conclusion is consistent with the observations that free LnA levels are quite low in ripening apple fruit, while LA levels are substantially higher. Given the low expression of MdLOX1a in immature fruit, other LOX genes are more likely to contribute to C6 aldehyde synthesis prior to ripening.

Table 3. Kinetic parameters of purified wild-type apple LOX and the Gly567Ala mutant of MdLOX1a

| Substrate | $K_m$ (µM) | $V_{max}$ (µmol min⁻¹ mg⁻¹) | $k_{cat}$ (s⁻¹) | $k_{cat}/K_m$ (µM⁻¹ s⁻¹) |
|-----------|------------|-------------------------------|----------------|--------------------------|
| LOX1:Md:1a | LA | 10.35±0.28 | 1.57±0.07 | 2.67±0.13 | 0.26 |
| | LnA | 26.65±0.99 | 3.05±0.14 | 5.16±0.24 | 0.19 |
| LOX1:Md:1c | LA | 40.61±3.10 | 30.21±0.93 | 51.14±1.58 | 1.26 |
| | LnA | 70.85±5.48 | 18.22±2.69 | 30.86±4.56 | 0.44 |
| Gly567Ala | LA | 11.43±1.21 | 18.74±0.86 | 31.75±1.45 | 2.78 |
| | LnA | 15.53±0.87 | 14.82±0.46 | 25.10±0.77 | 1.62 |

MdLOX1a is associated with a quantitative trait locus cluster for ester-type volatiles

Large-scale statistical analysis of apple EST libraries revealed an apple LOX gene (EST cluster MD187410) as potentially important in volatile ester formation in ripening fruit. Transcripts of this LOX gene were barely detectable at 123 days after full bloom (DAFB), increased in abundance before the onset of significant volatile production, peaked at 162 DAFB and remained at high levels 183 DAFB. This gene was later mapped on the apple linkage group 9 closely to the chloroplast, much the same as MdLOX6b. Furthermore, the pH of the juice of disrupted apple tissue, which is typically near 3.5, would be expected to differentially affect LOX enzyme activity. Based on the pH-dependent activity profiles demonstrated herein, LOX2:Md:2b activity would be completely suppressed, for instance, while that of the other three LOX enzymes would not.

LOX1:Md:1a is a dual specific lipooxygenase

The first LOX enzyme isolated from apple fruit was a membrane-bound protein, which preferably converted LA to hydroperoxy products. This enzyme displayed optimal activity at pH 6.9 and reacted to changing pH values similar to LOX2:Md:2a. Another purified apple LOX was found to exhibit a pH optimum of 6.0 and preferably converted linolenic acid. The product mainly consists of the 13-hydroperoxide, which was in agreement with LOX activity found in apple homogenates. Chiral analysis revealed 13(R)-HODE, 9(S)-HODE and their ketones as major metabolites of total LOX activity with LA in stored apple fruits. Regio- and stereochemistry of the reported metabolites showed a striking similarity to the products gained from the LOX1:Md:1a and LOX2:Md:2a reaction with LA. Therefore, we propose a significant contribution of LOX2:Md:2a and LOX1:Md:1a to hydroperoxide formation during
Figure 7. Proposed metabolism of fatty acids in the intact apple fruit. Saturated and mono-saturated fatty acids are degraded to precur- sor molecules for straight-chain esters via β-oxidation. For PUFAs, this process seems to be partially impaired. Precursors for straight chain esters might be formed via the action of the LOX pathway, instead. PUFA, polyunsaturated fatty acid.

In our study, three out of four characterized apple LOX were able to form 13-hydroperoxides from LA and LnA. The major fraction of 13-HpODE detected for LOX2:Md:2b was in (S)-conformation, whereas LOX1:Md:1a and LOX2:Md:2a formed mainly 13(R)-HpODE. In contrast to the in silico prediction, LOX1:Md:1a was identified as (R)-specific dual positional LOX. (R)-LOX specificity is a rare trait among plant LOXs and has so far only been described for type-2 LOX from soybean and pea as well as an olive 9/13-LOX. Similar to LOX1:Md:1a, olive 9/13-LOX harbors a glycine residue (Gly568) in the sequence position determining stereoselectivity of fatty acid oxygenation. In contrast, LOX2:Md:2a harbors alanine (Ala609), which is conserved among (S)-LOXs. It has been suggested that type-2 LOXs with an alanine residue are able to mimic (R)-LOX specificity depending on varying substrate concentrations and pH.

Factors that determine regio- and stereospecificity of LOX1:Md:1a were discussed in Supplementary Figure S9. However, taking recent theories on stereo- and regiospecificity of LOXs into account, this would lead to the formation of 9(R)- and 13(S)-hydroperoxides. Based on the current knowledge it is difficult to predict which mode of substrate binding into the active site would cause the unexpected product specificity of LOX2:Md:2a. Factors that determine regio- and stereospecificity of LOX1:Md:1a are discussed in Supplementary Figure S9.

Furthermore, HPLs have been shown to exhibit a high degree of substrate specificity. Most HPLs specifically convert either 9(S)- or 13(S)-hydroperoxides of LA and LnA, or both. This, however, raises the question, how 13(R)-hydroperoxides are metabolized in apple fruits. There is only limited information available on HPL from apple. The enzymatic conversion of LA and LnA, respectively, to hexanal and E-2-hexenal has been reported in homogenates prepared from apple fruits. Later an enzyme with HPL activity was partially purified and shown to convert 13-hydroperoxides to hexanal, E-2-hexenal and Z-3-hexenal, whereas 9-hydroperoxides were not accepted as potential substrates.

MdLOX1a contributes to aroma formation in apples

The typical flavor profile of an intact apple fruit, which consists mainly of short-chain esters, develops during ripening with maximum volatile formation near the peak of the respiratory climacteric and the rise in ethylene production. Aldehydes that are produced at only low amounts in intact fruits are formed in copious amounts by the action of LOX enzymes upon cell disruption. While LOX enzymes are present in intact systems prior to disruption, it is not known if LOX contribute to the synthesis of aroma active compounds (C6 aldehydes and esters) in non-disrupted tissue, although its involvement has been proposed.

It is evident that all four analyzed apple LOXs are catalytically active and reaction specificity as well as developmental changes in gene expression suggest in vivo functions for each isoenzyme during apple ripening. Likewise, a shift in LOX pathway activity was observed during ripening of apples. For aroma biosynthesis in mature apples, (aldehyde and ester formation), MdLOX1a was identified as the most promising candidate as recent investigations on LOX gene expression revealed an ample rise in MdLOX1a transcript abundance upon ethylene-induced fruit ripening. Ripening-dependent changes in the LOX pathway have already been discussed to account for the formation of aroma-relevant alcohol and ester volatiles.

We propose that MdLOX1a is responsible for the majority of LOX activity found in intact apple fruits during the late stages of the ripening process based, among others, on the peculiar conformity of the regio- and stereochemistry of the hydroperoxide products formed by the recombinant and endogenous enzyme. Thus, LOX1:Md:1a is suggested to be involved in the generation of C6 aldehydes (primarily hexanal in the case of apple) and the formation of hexyl and possibly other straight-chain esters in intact, ripening fruit (Figure 7) whereas other LOXs primarily take part in the production of ‘green note’ aldehydes. In particular, the coincident ripening-dependent decline in the synthesis of unsaturated C6 aldehydes in disrupted tissue and associated esters and the decline in two of the putative 13-LOX genes, MdLOX6a and MdLOX6b, reported herein is also suggestive of the involvement of specific LOX genes in regulating the synthesis of disruption-dependent aldehydes.

In stored apple fruits, it seems that the degradation of LA by the β-oxidation pathway is impaired as high amounts of the intermediates escape as octane-1,3-diol (Figure 7). Likewise, apple fruit do not accumulate octanoic acid derived esters from LA but form abundant amounts of hexyl acetate and hexanoic acid derived esters. We assume that saturated and mono-unsaturated fatty acids are primarily degraded by β-oxidation in intact fruit, thus providing precursors for short, straight-chain esters, whereas the late steps of the β-oxidation process for polyunsaturated fatty acids such as LA and LnA are impaired (Figure 7). MdLOX1a activity might contribute to ester formation in intact fruit as it circumvents the metabolic block and channels short chain aldehydes into the biosynthetic pathway of aliphatic esters.
COMPETING INTERESTS
The authors declare no conflict of interest.

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