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Lipoxygenase Gene Expression in Ripening Kiwifruit in Relation to Ethylene and Aroma Production

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During postharvest ripening of kiwifruit [*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* cv. Bruno] at 20 °C, six lipoxygenase (LOX) genes exhibited different expression patterns. *AdLox1* and *AdLox5* were up-regulated during ripening, and transcript accumulation was delayed by 1-methylcyclopropene (1-MCP), whereas *AdLox2*, *AdLox3*, *AdLox4*, and *AdLox6* were down-regulated with ripening. Levels of two volatiles arising from the LOX pathway, that is, *n*-hexanal and (*E*)-2-hexenal, were highest after harvest and declined during ripening at 20 °C, whereas the characterstic kiwifruit esters ethyl and methyl butanoate levels increased late in the ripening process. Individual fatty acid concentrations underwent little change during ripening, with linoleic (LA) and linolenic (LeA) acids constituting about 40% of the total. Application of LA and LeA to kiwifruit flesh disks promoted LOX activity and *n*-hexanal and (*E*)-2-hexenal generation, whereas inhibitors of LOX, *n*-propyl gallate (*n*-PG) and nordihydroguariaretic acid (NDGA), caused a parallel reduction in enzyme activity and in the production of C6 aldehydes. The six LOX genes showed different sensitivities to the LOX substrates and inhibitors. The ethylene up-regulated genes *AdLox1* and *AdLox5* were induced by LA and LeA and inhibited by n-PG and NDGA. Of the LOX genes that were down-regulated by ethylene, only *AdLox4* and *AdLox6* were stimulated in response to the substrates and retarded by the inhibitors. The possible roles of the six LOX genes in kiwifruit ripening and aroma development are discussed.

KEYWORDS: Ethylene; fruit ripening; gene expression; kiwifruit; lipoxygenase; volatiles

INTRODUCTION

Lipoxygenase (LOX, EC 1.13.11.12) is a nonheme iron-containing dioxygenase that catalyzes the addition of molecular oxygen to fatty acids containing a *cis,cis*-1,4-pentadiene system leading to the formation of hydroperoxide (HPO) derivatives (*1*). In plants, linoleic acid (LA) and linolenic acid (LeA) are the principal substrates for LOX activity. The HPOs produced can be further metabolized through different pathways and show diverse functions (*2*, *3*). LOX is of particular importance in fruit ripening, when fresh fruit taste and quality can be influenced by its activity. In fruit in general, there is a close association of both LOX genes and enzyme activity with fruit ripening and associated food quality properties such as aroma development (*4*−*6*).

LOX-mediated fatty acid oxidation is initiated during the early stages of plant senescence, resulting in membrane breakdown and loss of cellular compartmentalization (*7*). Products derived from the LOX pathway further contribute to oxidative stress during fruit ripening and senescence (*8*). Increased LOX activity has been suggested to be associated with loss of firmness during fruit ripening (*9*), and stimulation of LOX gene expression has been found in senescing *Arabidopsis* leaf tissue (*10*) and ripening fruit such as tomato (*4*), apple (*11*), and watermelon (*12*).

LOX-mediated hydroperoxidation of LA and LeA leads to the production of *n*-hexanal and (*E*)-2-hexenal, respectively (*13*). These C6 aldehydes result in the formation of important fruit flavor components, which contribute to the grassy aroma character of many fruits (*14*). A particular role for LOX in fruit volatile production has arisen from work with transgenic tomato fruit, when specific down-regulation of *TomLoxC* resulted in a significant reduction in the levels of C6 aldehydes (*5*). LOX activity has also been associated with flavor compound generation in strawberry (*15*), olive (*16*), and apple fruit (*17*).

Kiwifruit (*Actinidia* spp.) is another fruit in which the LOX pathway is indispensable for characteristic aroma formation as

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the fruit ripens (*18*). Products such as *n*-hexanal and (*E*)-2-hexenal have been identified as volatile compounds that give the specific kiwifruit aroma (*19*). We have previously identified and cloned six members of the kiwifruit LOX gene family (*6*). *AdLox1*, *AdLox3*, *AdLox4*, and *AdLox6* are grouped into the 13-LOX family, and *AdLox2* and *AdLox5* were proposed to have 9-LOX activity. *AdLox1*, *AdLox2*, and *AdLox4* showed relatively higher transcript abundance than the other three isoforms in vegetative and flesh tissue. The six LOX genes had different expression patterns during kiwifruit ripening, and not all were shown to be responsive to ethylene. In addition, we suggested there could be a relationship between specific LOX genes and fruit aroma generation (*6*). Research to date has not provided any data directly relating specific LOX gene expression levels and enzyme activity with aroma. The suggestion that LOX isoforms may have different roles in kiwifruit ripening and thus on fruit quality has prompted a more detailed analysis of the regulation of LOX genes and production of volatiles during fruit ripening.

The present study was carried out using two approaches. The first was to use whole ripening fruit to follow fruit aroma compounds, LOX gene expression, enzyme activity, and fatty acids in response to the ethylene action inhibitor 1-methylcyclopropene (1-MCP), which allows some modulation of kiwifruit ripening. The second was to use kiwifruit flesh disks to study the effects of substrates and inhibitors on the enzyme activity, C6 aldehyde production, and LOX gene expression, particularly to see whether LOX genes are stimulated by substrates. The findings are discussed in terms of functions of different isoforms in LOX-associated volatile production during fruit ripening.

MATERIALS AND METHODS

Chemicals and Reagents. The reference compounds used for volatiles studies were supplied by Fluka except for 2-octanol, which was purchased from Sigma-Aldrich. Chemical standards used for fatty acid identification were obtained from Sigma-Aldrich. The reagents used for enzyme activity analysis were purchased from Sigma-Aldrich and Bio-Rad. Other chemicals were of analyzed grade and supplied by Shanghai Sangon Engineering and Biotechnology Services Co. Ltd.

Plant Material and Tissue Sampling. Kiwifruits [*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* cv. Bruno] were harvested from an orchard at Wuyi, Zhejiang, China. Medium-sized (80−120 g) fruits, free from visible defects or decay, were selected. Kiwifruit was harvested at commercial maturity with an average total soluble solids concentration (SSC) concentration of 6.8%. Fruits were divided into two batches of 165 fruits each, and for postharvest treatments, each batch was sealed into three 20 L containers (55 fruits per container) and held at 20 °C. The first batch was treated with 10 μL L^{-1} 1-MCP for 24 h with the aim of delaying fruit ripening; the second batch had no treatment. For the 1-MCP treatment, 0.4 g of 1-MCP powder (SmartFresh) was added to 10 mL of water at 40 °C, to give a final concentration in the container of 10 μL L^{-1}. Previous work has shown that 10 μL L^{-1} 1-MCP was an effective concentration to inhibit ethylene production and delay ripening in kiwifruit (*20*). To prevent the accumulation of CO_2 over the 24 h, approximately 300 g of hydrated lime was placed in each container, within a paper bag. Fans were used in the chambers to maintain air circulation. At each sampling time, 10 fruits were used to measure ethylene production, fruit firmness, and SSC, and slices of flesh (without skin, seeds, or core tissue) from the 10 fruits were combined and frozen in liquid nitrogen and stored at −80 °C for further use.

Fruit Ripening. To follow fruit ripening, fruit was held at 20 °C and 92−98% relative humidity for up to 7 days. Ethylene production was determined on three replicates each of 10 fruits at the given sampling times. The 10 fruits were placed in 2 L flasks, which were capped with rubber stoppers for 1 h. Ethylene was measured by withdrawing 1 mL of headspace gas from each flask, which was then analyzed by gas chromatography (GC; model SP 6800, Lunan Chemical Engineering Instrument, Shandong, China) fitted with a GDX-502 column. The injector, detector, and oven temperatures were 110, 140, and 90 °C, respectively. Fruit firmness was measured on each of the 10 fruits at two positions 90° apart at the equator of the fruit, with an Effegi FT-327 (Milan, Italy) hand-held penetrometer with a 7.9 mm diameter head, after the removal of a 1 mm thick slice of skin. SSC was measured after expression of juice from two slices from opposite ends of each fruit using an Atago N20 hand-held refractometer.

Volatile Analysis. Fruit volatile analysis was carried out in two different experiments using whole fruit or flesh disks. For whole fruit, 4 g of frozen flesh tissue was ground in liquid nitrogen and transferred to a 15 mL vial containing 5 mL of saturated sodium chloride solution. The tissues were homogenized with an Ultra Turrax for 1.5 min. Before the vials were sealed, 30 μL of 2-octanol (8690 μg/mL) was added as an internal standard and stirred for 10 s with a vortex. For manual solid-phase microextraction (SPME) analysis, samples were equilibrated at 40 °C for 30 min and then exposed to a 65 μm CAR-DVB fiber (Supelco) (Bellefonte, PA) for 10 min. A GC-FID (Agilent 6890N) equipped with a DB-Wax column (0.32 mm, 30 m, 0.25 μm, J&W Scientific, Folsom, CA) was used for volatile analysis. Chromatograph conditions were as follows: injector, 220 °C; initial oven temperature, 34 °C, held for 2 min, increased by 2 °C min^{-1} to 60 °C, then increased by 4 °C min^{-1} to 180 °C, and held for 2 min. Nitrogen was used as a carrier gas at 1.1 mL min^{-1}. Volatiles were identified by comparison of retention times with those of authentic standards (Sigma). Quantitative determination of compounds was carried out using the peak of the internal standard as a reference value and calculated on the basis of the standard curve of authentic compounds. To determine volatile concentrations in experiments using flesh disks, 1 g of flesh disks was prepared as described below and used for volatile compound analysis as described for whole fruit flesh tissues.

LOX Activity Assay. Total LOX enzyme extraction was carried out by grinding 1 g samples of frozen tissue in 5 mL of 50 mM phosphate buffer (pH 7.0), containing 0.1% (v/v) Triton X-100 and 0.2 g of PVPP (Sigma). The homogenates were centrifuged for 20 min at 15000*g*, and the supernatant was used as crude extract. According to Zhang et al. (*21*), total LOX activity was determined spectrophotometrically by measuring the formation of conjugated diene from hydroxyl fatty acids at 234 nm and 30 °C using LA (Sigma) as substrate. A 3 mL reaction mixture consisted of 2.75 mL of 100 mM acetate buffer (pH 5.4), 50 μL of 0.17 mM LA, and 200 μL of crude extract. The enzyme activity was measured using a DU-800 UV−vis spectrophotometer (Beckman Coulter, Fullerton, CA). The total enzyme activity was expressed as micromoles of HPO per minute per milligram of protein using a molar extinction coefficient of 25000 M^{-1} cm^{-1}. Protein measurements were performed using a Protein Assay kit (Bio-Rad) according to the manufacturer's instructions, using BSA as a standard.

Fatty Acid Analysis. The total fatty acid profile was determined by fatty acid methyl ester (FAME) analysis. Briefly, 6 g of frozen powdered fruit tissue was used to extract total lipids by gentle shaking in a mixture of chloroform/methanol/water (1:2:0.8, v/v/v) at room temperature. After centrifugation at 5000*g* for 15 min at 4 °C, the upper phase was extracted with 8 mL of chloroform and NaCl (0.76%, w/v) by shaking for 15 min; the organic phase containing total lipids was recovered and dried under a nitrogen stream. The residue was dissolved in 2 mL of hexane and stored at −20 °C for further analysis. Total fatty acids of total lipids were transformed into their corresponding FAME by the addition of 2 mL of H_2SO_4 (2.5% in methanol, v/v), and a known amount of heptadecanoic acid (C17:0) was added as internal standard. FAME reaction was terminated by the addition of ice-cold water. Equal volumes of chloroform/water (8:3, v/v) were then added, and the upper phase containing the FAME was removed in an autosampler vial for GC-FID analysis. A GC-FID (Agilent 6890N) equipped with a DB-23 column (0.25 mm, 60 m, 0.25 μm, J&W Scientific) was used for analysis. Conditions for chromatography were injection at 250 °C and intial oven temperature started at 50 °C, increased to 175 °C at 25 °C min^{-1}, increased to 230 °C at 4 °C min^{-1}, and held for 5 min. Identification of compounds was confirmed by comparison with authenticated reference standards (Sigma). Quantitative

Table 1. Ethylene Production and Total LOX Activity of Kiwifruit during Postharvest Ripening (2007 Season)

	ethylene (nL g^{-1} h^{-1})		total LOX activity (μmol of HPO min^{-1} mg^{-1} of protein)	
h after harvest	control	1-MCP	control	1-MCP
0	UD[a]		1.62 ± 0.21	
24	UD		1.74 ± 0.39	1.67 ±0.07
48	1.06 ±0.32	0.75 ±0.33	1.71 ± 0.17	1.32 ±0.13
96	7.96 ±2.20	0.71 ±1.21	1.85 ± 0.25	1.27 ±0.13
120	35.89 ±9.64	3.83 ±2.14	2.15 ± 0.25	1.37 ±0.25
168	88.04 ±5.66	9.26 ±1.57	1.35 ± 0.18	1.50 ±0.26

[a] Fruit was treated with 1-MCP (10 μl L^{-1}) for 24 h at 20 °C or sealed in a similar container of the same volume for 24 h as a control. After treatment, fruit was then allowed to ripen at 20 °C. The harvest time point was set as 0 h. Each value represents the mean ± SE of 10 fruit. [b] UD, under the determination limit.

determination of compounds was carried out using the peak of the internal standard as a reference value and calculated on the basis of the standard curve of authentic compounds.

Fruit Disk Experiments. Fruit disk experiments were carried out in two succeeding seasons (2006 and 2007) to investigate the effects of LOX substrates and enzyme inhibitors on the expression of the six LOX genes. To prepare the disks, 30−40 fruits at preclimacteric stage with firmness of about 15 N in 2006 or 45 N in 2007 were used. Cylinders of flesh were taken using an 8 mm diameter cork borer and 2 mm thick disks cut from these; skin, seeds, and core tissue were excluded. According to Fan et al. (*22*), the disks were briefly rinsed with sterile water to remove the intercellular material and collected in 0.4 M mannitol until transferred to experimental treatments. For each treatment, three replicates of 30 disks were placed into 150 mL conical flasks containing 50 mL of the treatment solutions.

In the 2006 fruit season, flesh disks were treated with 1.0 mM LA, 0.5 mM LeA, 0.1 mM *n*-propyl gallate (*n*-PG), and 0.1 mM nordihydroguaiaretic acid (NDGA) in 0.4 M mannitol, for 12 h at 28 °C, respectively. The optimal concentrations for each treatment were determined from preliminary experiments. For the substrate treatment, concentrations above 1.0 mM LA and 0.5 mM LeA caused some bleaching of the flesh tissue. For the enzyme inhibitor treatment, concentrations above 0.1 mM caused a noticeable darkening of fruit disk tissues. After the treatment, total LOX activity and gene expression were measured. In the 2007 fruit season, kiwifruit flesh disks were treated with 1.0 mM LA, 0.5 mM LeA, 0.1 mM *n*-PG, 0.1 mM NDGA, LA + *n*-PG, LeA + *n*-PG, LA + NDGA, or LeA + NDGA in 0.4 M mannitol, for 12 h at 28 °C, respectively. Disks treated with 0.4 M mannitol alone were used as a control. After the treatments, volatile compounds and LOX gene expression were analyzed. The experiments were carried out by shaking the discks in the treatment solutions for 12 h at 100 rpm at 28 °C. After 12 h, the disks were blotted on filter paper, frozen in liquid nitrogen, and stored at −80 °C until use.

Real-Time Quantitative RT-PCR Analysis. Total RNA was extracted from frozen fruit tissue according to the method described by Zhang et al. (*6*). Total RNA was treated with DNase I (Fermentas) to remove contaminating genomic DNA. First-strand cDNA was synthesized using 1.0 μg of treated total RNA, 0.5 μg of oligo $d(T)_{18}$, and 200 units of RevertAid M-MuLV reverse transcriptase (Fermentas) to a total volume of 20 μL. PCR reactions were performed in a total volume of 20 μL, 250 μM for each primer, 10 μL of 2× SYBR Green PCR Master Mix (Bio-Rad), and 2.0 μL of the first-strand cDNA in an iCycler iQ real-time PCR instrument (Bio-Rad). The real-time quantitative RT-PCR (qPCR) program included a preliminary step of 5 min at 94 °C, followed by 45 cycles of 94 °C × 10 s and 60 °C × 30 s. No-template controls for each primer pair were included in each run. Kiwifruit actin was used as an internal control to normalize small differences in template amounts. All primers used for qPCR analysis were the same as those described in Zhang et al. (*6*). At least three different RNA isolations and cDNA syntheses were used as replicates for the qPCR. Expression levels produced by qPCR were expressed as a ratio relative to the fruit at the harvest time point for whole fruit or disk control treated with mannitol alone, which was set to 1.

Experimental Design and Statistical Analysis. A completely randomized design was used in the experiment. Standard errors (SE) were calculated by Microsoft Excel, and differences indicated in the table and figures were based on Tukey's test at the 5% level (SAS version 8.0, SAS Institute, Cary, NC). Figures were made by Origin (Microcal Software Inc., Northampton, MA).

RESULTS

Ethylene Production and Total LOX Activity. The climacteric rise in ethylene production of fruit held at 20 °C was initiated at about 96 h after harvest, rising rapidly through 168 h (**Table 1**). Total LOX activity in fruit tended to increase with ripening, peaking at about 120 h after harvest. Treatment of the fruit with 1-MCP for 24 h delayed the climacteric rise in ethylene production by about 3 days, and the total LOX activity was relatively low (**Table 1**). Fruit held at 20 °C softened rapidly from about 70 N at harvest to about 10 N at 120 h after harvest, whereas 1-MCP treatment kept firmness at about 53 N.

Volatile Compound Production during Fruit Ripening. Levels of the C6 aldehydes *n*-hexanal and (*E*)-2-hexenal, potentially derived from LOX activity, were highest at harvest (**Figure 1A**,**B**) and then decreased during fruit ripening. The concentrations of the corresponding C6 alcohols rose at about 96 h for *n*-hexanol and at 24 h for (*E*)-2-hexenol (**Figure 1C**,**D**). There were no significant changes in the level of the alcohols prior to the climacteric rise in ethylene production. However, esters that provide the fruity aromas of ripe kiwifruit, such as methyl butanoate and ethyl butanoate, accumulated as the fruit ripened, increasing particularly with the ethylene climacteric rise at about 96 h after harvest (**Figure 1E**,**F**). The ripening-associated decline in aldehydes and accumulation of esters during ripening are likely to be driven by ethylene, because the above changes were prevented by the 1-MCP treatment (**Figure 1**).

LOX Gene Expression during Fruit Ripening. The relative qPCR results showed that expression of *AdLox1* and *AdLox5* increased with kiwifruit ripening at 20 °C (**Figure 2**). Transcript levels of *AdLox5* increased relatively slowly, concomitantly with the rise in ethylene production at about 96 h after harvest in the control batch. There was an increase in *AdLox1* levels immediately after harvest. By contrast, *AdLox2*, *AdLox3*, *AdLox4*, and *AdLox6* had relatively higher expression levels at harvest and then decreased during fruit ripening.

When fruits were treated with 1-MCP, the expression abundance of *AdLox1* and *AdLox5* was reduced, consistent with their up-regulated response to fruit ripening (**Figure 2**). However, transcript levels of *AdLox2* increased in 1-MCP-treated fruit, peaked at about 96 h after harvest, and then declined at the climacteric stage at 168 h. *AdLox3* showed an increase in relative intensity up to 48 h and then decreased with fruit ripening. *AdLox4* was transiently induced just after the 24 h 1-MCP treatment and then declined. Levels of *AdLox6* were maintained relatively higher in 1-MCP-treated fruit. In the controls, *AdLox2*, *AdLox3*, *AdLox4*, and *AdLox6* were strongly down-regulated with the progress of ripening.

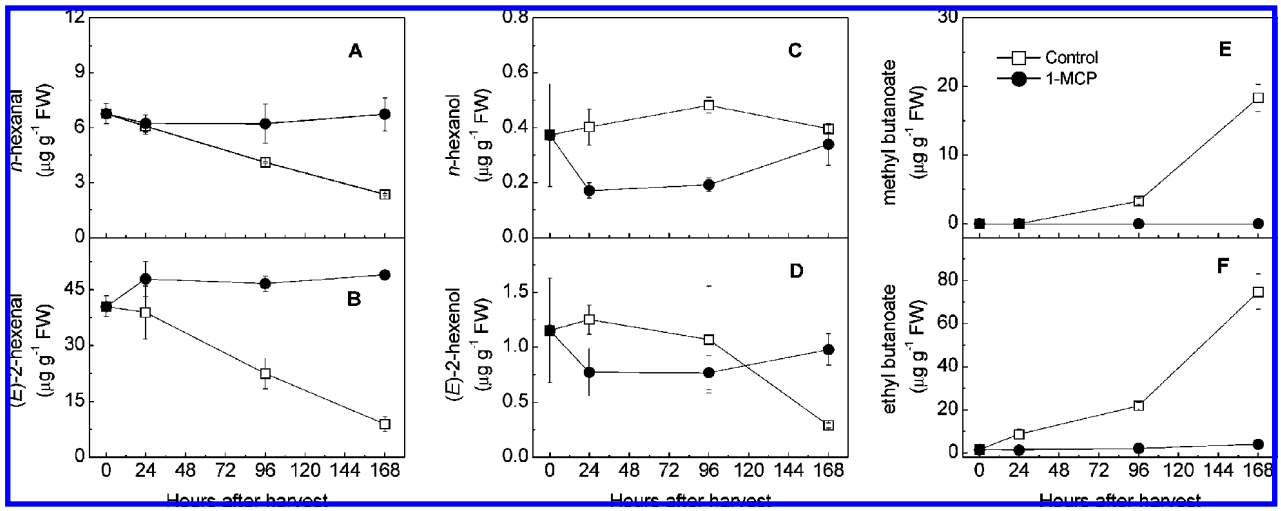

Figure 1. Production of *n*-hexanal (**A**), (*E*)-2-hexenal (**B**), *n*-hexanol (**C**), (*E*)-2-hexenol (**D**), methyl butanoate (**E**), and ethyl butanoate (**F**) from ripening kiwifruit (2007 season). Fruit was pretreated for 24 h with 10 μL L^{-1} 1-MCP (●) held at 20 °C for 168 h as a control (□). After treatment, fruit was then allowed to ripen at 20 °C. Fruit aroma compounds were determined from headspace analysis by SPME and GC. Error bars indicate SE from three replicates.

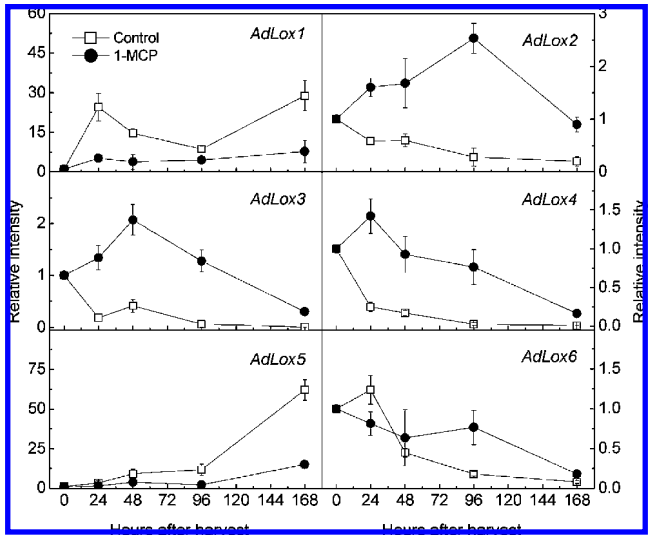

Figure 2. Expression of LOX family transcripts in ripening kiwifruit by real-time quantitative RT-PCR analysis (2007 season). Fruit was held at 20 °C for 168 h as a control (□) or pretreated for 24 h with 10 μL L^{-1} 1-MCP (●) and then allowed to ripen at 20 °C. Error bars indicate SE from three replicates. Expression levels of each gene are expressed as a ratio relative to the harvest time (0 h), which was set at 1.

Fatty Acid Contents in Ripening Fruit. We analyzed the levels of all major fatty acids to compare changes in specific LOX substrates with general patterns of fatty acid changes during fruit ripening. The major fatty acid component in kiwifruit at harvest (percentages give the range of contents) was linolenic acid (LeA, C18:3) (25−30%), followed by oleic acid (OA, C18:1) (15−28%), palmitic acid (PA, C16:0) (20−26%), linoleic acid (LA, C18:2) (8−16%), stearic acid (SA, C18:0) (7−10%), palmitoleic acid (PoA, C16:1) (3−4%), and arachidic acid (AA, C20:0) (2−3%). As the main substrates for LOX-catalyzed oxidation, LA and LeA showed different profiles as the fruit ripened. Levels of LA increased by 40% after 168 h at 20 °C, whereas the 1-MCP treatment inhibited this increase (**Figure 3F**). LeA showed a decrease as the fruit ripened for 168 h, and this was reduced by 1-MCP (**Figure 3G**). Other major fatty acids such as OA, AA, and PoA underwent a decrease, whereas PA and SA concentrations were relatively unchanged.

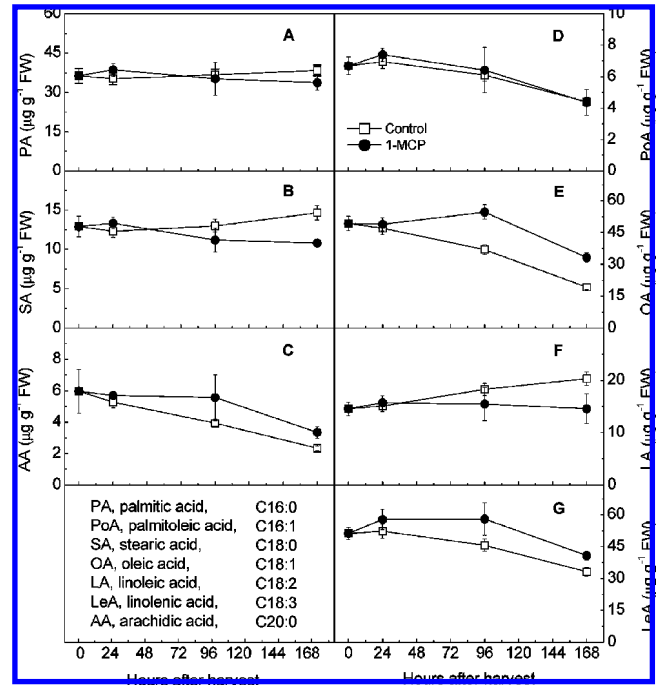

Figure 3. Changes in palmitic (**A**), stearic (**B**), arachidic (**C**), palmitoleic (**D**), oleic (**E**), linoleic (**F**), and linolenic (**G**) acid during kiwifruit ripening at 20 °C (2007 season). Fruit was pretreated for 24 h with 10 μL L^{-1} 1-MCP (●) or held at 20 °C for 168 h as a control (□). After treatment, fruit was then allowed to ripen at 20 °C. Error bars indicate SE from three replicates.

C6 Aldehyde Production in Fruit Disks Treated with LOX Substrates and Inhibitors. Treatment of fruit disks with the two LOX substrates LA and LeA resulted in increases in production of *n*-hexanal and (*E*)-2-hexenal (**Figure 4**). By contrast, the inhibitors *n*-PG and NDGA both reduced the volatile production. Such data confirmed the specific effect of the substrates on their relevant end-products such as LA to *n*-hexanal and LeA to (*E*)-2-hexenal. The presence of slightly increased *n*-hexanal in LeA-treated disks and accumulated production of (*E*)-2-hexenal in LA-treated disks suggest the possible transformation of *n*-hexanal and (*E*)-2-hexenal.

LOX Enzyme and Gene Expression in Fruit Disks. Both LA and LeA treatments significantly increased total LOX

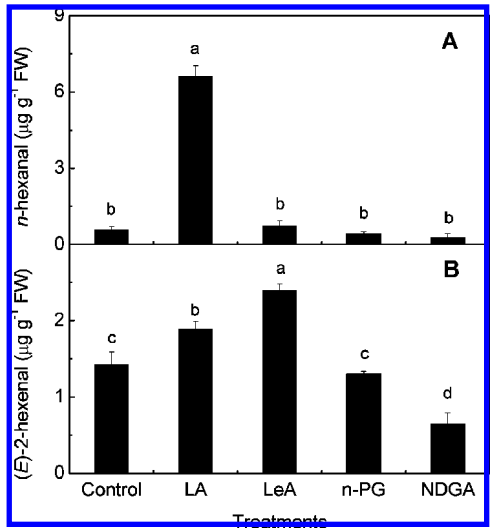

Figure 4. Production of *n*-hexanal (**A**) and (*E*)-2-hexenal (**B**) from kiwifruit flesh disks (2007 season). Disks were treated with 1.0 mM linoleic acid (LA), 0.5 mM linolenic acid (LeA), 0.1 mM *n*-propyl gallate (*n*-PG), or 0.1 mM nordihydroguariaretic acid (NDGA) in 0.4 M mannitol, for 12 h at 28 °C, respectively. Disks treated with 0.4 M mannitol alone were used as a control. Aldehyde levels were determined from headspace analysis by SPME and GC. Error bars indicate SE from three replicates. Values with different letters for *n*-hexanal and (*E*)-2-hexenal production are different at $P < 0.05$, respectively.

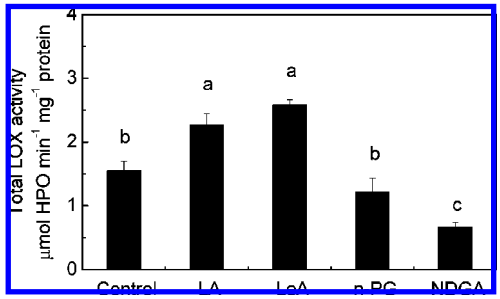

Figure 5. LOX enzyme activity in kiwifruit flesh tissue disks (2006 season). Disks were treated with 1.0 mM linoleic acid (LA), 0.5 mM linolenic acid (LeA), 0.1 mM *n*-propyl gallate (*n*-PG), or 0.1 mM nordihydroguariaretic acid (NDGA) in 0.4 M mannitol, for 12 h at 28 °C, respectively. Disks treated with 0.4 M mannitol alone were used as a control. All of the data for LOX activity are means ± SE of three replicates. Values with different letters for LOX activity are different at $P < 0.05$.

activity in the flesh disks by 38 and 65%, respectively (**Figure 5**), in agreement with C6 aldehyde production stimulated by the fatty acid treated tissues (**Figure 4**). As LOX inhibitors, NDGA treatment significantly inhibited total LOX activity, whereas *n*-PG treatment partially inhibited the enzyme activity as compared with controls (**Figure 5**).

We studied transcript levels of all six kiwifruit LOX genes in these fruit disk experiments for both 2006 and 2007 kiwifruit seasons. *AdLox1*, *AdLox4*, *AdLox5*, and *AdLox6* transcripts were substantially up-regulated by the addition of both LA and LeA to the disks (2006 data, **Figure 6**). Transcripts of *AdLox2* and *AdLox3* did not show any response to fatty acid treatments. These results were confirmed in a second set of experiments carried out in the succeeding season, although the levels of stimulation were not so great, with the highest increase of 1.5−2-fold found for *AdLox6* among the six genes. Transcript levels of LOX genes were inhibited by application of the inhibitors *n*-PG and NDGA.

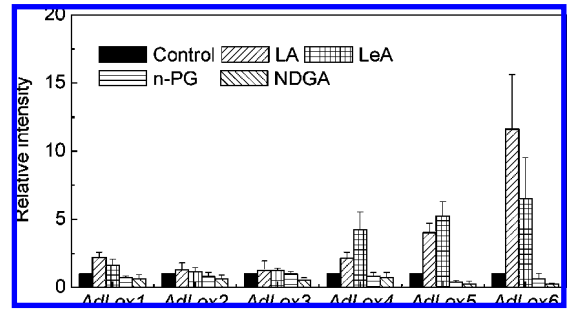

Figure 6. LOX gene expression in kiwifruit flesh tissue disks (2006 season). Disks were treated with 1.0 mM linoleic acid (LA), 0.5 mM linolenic acid (LeA), 0.1 mM *n*-propyl gallate (*n*-PG), or 0.1 mM nordihydroguariaretic acid (NDGA) in 0.4 M mannitol, for 12 h at 28 °C, respectively. Disks treated with 0.4 M mannitol alone were used as a control. All of the data for LOX gene expression are means ± SE of three replicates. Expression levels of each gene are expressed as a ratio relative to a control, which was set at 1.

DISCUSSION

The six LOX genes fall into two groups in relation to kiwifruit ripening. Expression of *AdLox1* and *AdLox5* was driven by ethylene-dependent ripening, confirmed by the retardation of an increase in expression by 1-MCP. The other group, *AdLox2*, *AdLox3*, *AdLox4*, and *AdLox6*, was down-regulated as ripening proceeeded, again ethylene-driven as shown by the effect of 1-MCP in preventing or retarding this decline. Our previous results (*6*) suggested this grouping, and the new data from the 1-MCP treatment provide confirmation. Ethylene up-regulated expression of LOX genes has also been found for tomato *TomLoxB* and *TomLoxC* (*4*), apple *MdLox1* and *MdLox7* (*11*), and watermelon *CiLox* (*12*) during fruit ripening and senescence.

There was a similar set of patterns in the volatile production. The two aldehydes we measured, *n*-hexanal and (*E*)-2-hexenal, declined during ripening, and the other characteristic group for kiwifruit, the butanoate esters, increased in the later stages. Thus, there were similar trends with the declining expression of the four LOX genes and the reduction in aldehyde production and, similarly, with the increases in *AdLox1* and *AdLox5* and ester production in the late stages of senescence.

Our current and previous results also show that total LOX activity also increased during the early ripening phase and then decreased as kiwifruit softened to the eating-ripe stage (*21*). It has long been suggested that LOX activity is associated with membrane degradation during fruit ripening, for instance as seen in strawberry fruit (*15*). In kiwifruit, the contents of the total fatty acids of the lipids declined from 176.90 μg g^{-1} of fresh weight (FW) at harvest to 127.63 μg g^{-1} of FW at 168 h when the fruit had proceeded into the ethylene climacteric stage. LA and LeA constituted up to 40% of the total complement, and LA was notable for increasing slightly during fruit ripening. Breakdown of thylakoid membranes with transition of the chloroplasts to chromoplasts is initiated by LOX as tomato fruits ripen (*23*). Higher tomato LOX activity at the mature green stage was capable of oxygenation of fatty acids esterified in membrane phospholipids, and free radicals derived from LOX action can directly attack lipids, contributing to membrane structure turnover and other aspects of fruit ripening (*4, 23*). For example, *TomLoxA* strongly expressed at the breaker stage has been suggested to be involved in lipid metabolism during tomato fruit ripening (*4*). *AdLox2*, *AdLox3*, *AdLox4*, and *AdLox6* exhibited expression patterns similar to that of tomato *TomLoxA*, and the latter three genes possess chloroplast transit peptides (*6*). It is possible that peroxidation of fatty acids mediated by

these ethylene down-regulated LOX genes may also contribute to fruit aroma formation, because fatty acid-derived C6 and C9 aldehydes are basically formed by the LOX pathway (*24*).

C6 aldehydes such as *n*-hexanal and (*E*)-2-hexenal endow kiwifruit with intense green and grassy flavor notes and are particularly associated with under-ripe kiwifruit (*25*), with their levels decreasing with fruit softening. In contrast, ripe fruit volatiles such as ethyl butanoate and methyl butanoate accumulate. This highlights the question of the relationship between LOX and production of these volatiles. Aldehydes could be substrates to generate the corresponding alcohols. However, apart from a decline in (*E*)-2-hexanol at the late stage in ripening (**Figure 1**), there were no particularly significant change in C6 alcohols in our present data and in previous papers on ripening kiwifruit (*25*, *26*). A decrease in LOX activity and gene expression has been suggested to be associated with less aldehyde production as fruits ripen. Reduction of *n*-hexanal and (*E*)-2-hexenal during kiwifruit ripening accompanied the decline in expression of *AdLox2*, *AdLox3*, *AdLox4*, and *AdLox6*. Inhibition of LOX activity in pulped kiwifruit preparations during collection of headspace volatiles resulted in a marked reduction in (*E*)-2-hexenal (*18*). In tomtatoes, antisense *TomLoxC* caused a marked reduction in tomato fruit C6 aldehydes, to as little as 1.5% that of wild-type fruit (*5*). There is little evidence from analytical data to suggest that changes in precursor fatty acids may account for the changes in aldehyde production. LA levels tended to increase significantly after kiwifruit harvest, and similar results have been reported in mango fruit (*27*). The decrease in LeA with kiwifruit ripening has also been observed in apple flesh tissues (*17*). It seems unlikely that the decline in aldehyde aromatics is due to depletion of fatty acid precursors (*28*).

The disk experiments provide a new set of data that confirm the differentiation of the LOX genes and show them to be susceptible to substrate stimulation. The fatty acid treatments of the disks resulted in accumulation of *n*-hexanal and (*E*)-2-hexenal, suggesting that kiwifruit tissues are able to metabolize LA and LeA into C6 aldehyde through the LOX pathway in vivo. Among the four LOX genes that are down-regulated as kiwifruit ripen, only transcripts of *AdLox4* and *AdLox6* were induced by LA and LeA treatments. It is interesting to note that *AdLox4* and *AdLox6* have been suggested to contain putative chloroplast transit peptides with 13-LOX activity (*6*) and clustered with potato *StLoxH1* and tomato *TomLoxC* (*6*), both of which have been identified as specific LOX gene family members responsible for C6 aldehyde generation in transgenic potato (*29*) and tomato (*5*). These results suggest that *AdLox4* and *AdLox6* may be potential LOX isoforms involved in C6 aldehyde production as kiwifruit ripens. The two LOX genes, *AdLox1* and *AdLox5*, which were up-regulated during the progress of ripening and are ethylene-stimulated, both also responded to the fatty acid substrates (**Figure 6**) and are clearly more associated with late ripening/senesence events. In apple fruit, ripening-induced LOX genes have been suggested to be responsible for aroma generation (*11*).

The above findings taken together suggest that LOX genes may have different roles during kiwifruit ripening. Ethylene up-regulated *AdLox1* and *AdLox5* seem to be associated with later ripening events such as fruity aroma ester release, whereas ethylene down-regulated *AdLox2*, *AdLox3*, *AdLox4*, and *AdLox6* were sensitive to ethylene-driven fruit ripening and may contribute to grassy aroma C6 aldehyde generation. Transcript stimulation by substrates suggests a loop whereby ethylene-stimulated lipid breakdown induces LOX activity, which in turn produces reactive oxygen species. These will stimulate further membrane degradation as the fruit tissue moves into postripening senescence. Initial LOX-derived aldehyde production is replaced by ester production as the aroma profile changes with ripening.

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