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Volatile Compound Biosynthesis by Green Leaves from an *Arabidopsis thaliana* Hydroperoxide Lyase Knockout Mutant

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The degradation of polyunsaturated fatty acids through the lipoxygenase pathway is responsible for the production of volatile compounds that confer green sensory notes to the aroma of fruits and vegetables. The peroxidation of free linoleic or linolenic acid by action of lipoxygenase and then the lysis of the resulting hydroperoxides, through a reaction catalyzed by the hydroperoxide lyase, are the most determinant steps of this pathway. This work analyzes the impact of the hydroperoxide lyase depletion on the volatile composition of leaves from *Arabidopsis thaliana* that is used as model system. The work involves the characterization of the volatile profiles of the *Arabidopsis* plants followed by a study of the metabolism of radio-labeled linoleic acid and determinations of lipoxygenase activity. Hydroperoxide lyase-knockout plants show similar levels of C6 compounds, but the total amount of C5 compounds is 4-fold higher in mutant plants. The perspectives of production of vegetable products with a modified aroma by genetically engineering the lipoxygenase pathway were discussed with respect to the statistical results.

KEYWORDS: Arabidopsis; lipoxygenase; knockout mutant; aroma; hydroperoxide lyase; volatiles

INTRODUCTION

The green sensory characteristics of many fresh vegetables and fruits are produced by aldehydes, alcohols, and esters of alcohols of six and five carbon atoms synthesized from polyunsaturated fatty acids by the action of the lipoxygenase (LOX) pathway (*1*−*3*). The first step of this pathway implies the oxygenation of the linoleic or α-linolenic fatty acids by the action of the enzyme lipoxygenase, which introduces an oxygen molecule in the 13 or 9 position of these fatty acids, producing the corresponding 13-*ZE*(*Z*) or 9-*EZ*(*Z*) hydroperoxy derivatives (*4*). These compounds can be cleaved by the action of the enzyme hydroperoxide lyase (HPL), a P450 protein that catalyzes the scission of hydroperoxides at the level of the oxygenated function, releasing a volatile carbonyl fragment of 6-hexanal, *Z*-3-hexenal- or 9-nonanal, *Z*-3-nonenal-carbon atoms from 13- and 9-hydroperoxides, respectively (*5*). 13-Hydroperoxides of α-linolenic acid can also be destroyed by a secondary activity of lipoxygenase, producing alcohols of five carbon atoms like *Z*-2-pentenol or penten-2-ol (*6*). The aldehydes produced in the plant tissues are usually reduced to alcohols by catalysis of alcohol dehydrogenase or cynammic aldehyde dehydrogenase (*7*), soluble enzymes whose activity depends on the supply of reducing equivalents in the form of NADPH. Finally, volatile alcohols are substrates for ester formation catalyzed by alcohol-acyl transferase, the enzyme responsible for most of the sweet and fruity sensory notes found in plant food products, which esterifies a volatile alcohol with acyl-CoA derivatives to produce volatile esters (*8*).

Provided the volatile profile of a given plant product is dependent on the relative activities of the enzymes mentioned previously, the LOX pathway has been a priority target within the plant food biochemistry field. In this regard, changes in the activity levels of the different enzymes of this pathway often affect the final aroma of the products, as Davies et al. (*9*) earlier demonstrated by genetic removal of LOX-2 from soybeans. This opened an interesting field on the production of plant food with tailor-made tastes and aromas. Later investigations revealed the importance of the alcohol dehydrogenase levels on the green/mature sensory perception in ripening tomatoes (*10*) and the direct relationship between the activity level of alcohol-acyl transferase enzyme and organoleptic quality in fruits like strawberry and bananas. On the contrary, the expression of 9-HPL from cucumber in tomato fruits did not affect the final volatile profile of the transformed fruits, which indicated that sometimes the volatile metabolism is not as simple as initially supposed and depends on factors such as the compartment or metabolic channeling besides the enzymatic activity levels (*11*). On the other hand, the studies on the chemical or physical inhibition of enzyme activities have not produced good results since they usually cannot act specifically on a single step but affect all the pathway components (*12*). Thus, a study carried out on olives pointed out the existence of basal enzymatic activity prior to the enzyme inhibition by chemical means (*13*). This inconvenience could be surpassed with an inverse genetic approach by using mutants in which genes encoding for the corresponding enzymes have been removed or suppressed by mechanisms of RNA interaction (antisense RNA, cosuppression) or knocked out by insertion of exogenous DNA. The resulting

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mutant lines would provide information about the impact of each enzyme on the volatile profile and the final aroma of the plant food products as well as the physiological response of the plant to these modifications. Nevertheless, the number of vegetable species that can be transformed is as limited as the number of libraries of mutants available. In this regard, the green leaves have been broadly used as the system for the biochemical characterization of the volatile biosynthesis through the LOX pathway. Thus, this system could be equally suitable as a model for studying the possible impact of the suppression of the different enzymes of the LOX pathway on the components of aroma. This approach supplied interesting results when the volatiles of the leaves from potato mutants H57 (*14*) and H33 (*15*) were studied. Thus, the LOX3-suppressed mutant H33 showed a very low content of volatiles, with leaves displaying an aroma that was almost imperceivable (*16*), indicating that only certain forms of LOXs are involved in aroma biosyntheses and that the amount of volatiles of a given vegetable is very dependent on the levels of LOX as it is the first enzyme of the enzymatic cascade. In addition, the mutant H57—with HPL enzyme suppressed at the 95% level by antisense techniques—displayed a profile of the volatile composition with lower amounts of C6 aldehydes and alcohols and a much higher content of C5 alcohols so producing a sensory perception in the aroma of the leaves sweeter than in the control ones. Furthermore, the biochemical characterization of the leaf tissues showed that the plant responded to the HPL suppression with a LOX activity increment. In this regard, it would be of interest to confirm if these responses are general to all plant species or are specific for solanaceae. To examine the impact of gene suppression in plant metabolism, *Arabidopsis* (*Arabidopsis thaliana*) is the most appropriate model system. This plant possesses a small genome that has been completely sequenced, and there are several libraries of insertional mutants that allowed studies of genomic reversal (*17*).

In the present work, we have investigated if the results obtained in potato leaves can be reproduced in the green leaves of other species as *Arabidopsis*. In doing so, an insertional knockout (KO) mutant from *Arabidopsis*, already sequenced and reported by TAIR public libraries (http://www.arabidopsis.org/portals/mutants/worldwide.jsp) (*18*), has been used. The profile of volatiles, as well as the HPL and LOX activities of this mutant, were studied in the present work and compared with those in the control ecotype Columbia. The biochemical changes were compared with those found in a HPL-antisense potato mutant previously studied (*14*, *16*). Moreover, data obtained from HPL-depleted *Arabidopsis* and potato leaves were taken as the basis for modeling the aroma of a hypothetical olive oil made with HPL-depleted olives.

MATERIALS AND METHODS

Plant Material. Public databases of *Arabidopsis* insertional mutants were searched for an *A. thaliana* HPL-KO (AtHPL-KO) plant. The line T-DNA_LB.SALK_008351 was found to possess a T-DNA insertion in the second exon of the HPL gene, and so was a virtual null mutant of that enzyme. That line was obtained from the Salk collection service and grown in parallel with control plants of the ecotype Columbia in a chamber at 20/15 °C, with a 16 h photoperiod and electronically controlled fertirrigation. Fully developed nonsenescing leaves from 3−4 week old rosettes were used for DNA extraction, biochemical studies, and the quantification of the volatile compounds by gas chromatography.

PCR Screening for Homozygous AtHPL-KO. Green nonsenescing leaves from candidate plants were cut off from the rosettes, frozen in liquid nitrogen, and then lyophilized overnight. Dry leaf tissue was ground to a fine powder in Eppendorff tubes by vortexing in the presence of glass beads. That powder was resuspended in 0.5 mL of freshly prepared CTAB buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 NaCl, 2% cetylaminopropyl bromide, and 2% β-mercaptoethanol) prewarmed at 65 °C, and cooked for 30 min. The resulting suspensions were extracted with 0.5 mL of trichloromethane/isoamyl acetate 24:1 and centrifuged at 9000*g*. Organic phases were discarded, and the resulting crude DNA extracts were precipitated by the addition of 0.5 mL of 2-propanol for 30 min at −20 °C. DNA was pelleted by centrifugation at 13 000*g* for 10 min, and the supernatants were discarded. Pellets were washed with 70% ethanol and dried under vacuum. Finally, they were resuspended in 0.1 mL of 10 mM Tris-HCl pH 8.0, 1 mM EDTA. The 3.1 Kb DNA genomic fragment corresponding to HPL was amplified using the previously described DNA preps as the template, with 5′-ATAGAAATAGAAGGCGAAGAGATAATTTC-3′ (forward) and 5′-TGTGGTTATGATTATACTACTGAGCTTTG-3′ (backward) oligos. The amplified DNA fragment was cloned into the pCR-2.1 vector using the kit PCR cloning from Invitrogen (Carlsbad, CA) and sequenced to confirm it was the genomic fragment of At HPL. Homozygous plants presented an insert of 2.5 Kb within its DNA fragment corresponding to HPL, so no PCR product was obtained in the conditions in which the reactions were run. Reactions were repeated in all homozygous plants and progenies, amplifying in parallel a 1 kb fragment of the 5′ region of the gene as a positive control.

Metabolism of [1-^{14}C] 18:2. Studies of the metabolism of radiolabeled linoleic acid were run as described by Salas et al. (*16*). Leaf homogenates were prepared from 0.25 g of leaf tissue that were ground in 2.5 mL of 50 mM MES, pH 6.0 in an ice-cooled glass homogenator. Reactions were run in the same buffer having 0.5 mM 18:2, 30 MBeq of [1-^{14}C] 18:2 ammonium salt (55 Ci/mol), and 0.25 mL of leaf homogenate in a final volume of 2.5 mL. They were incubated for 40 min at 20 °C with continuous shaking. Aliquots of 0.4 mL of these mixtures were taken at different times and inactivated by the addition of 0.1 mL of acetic acid before lipid extraction by the method described by Hara and Radin (*19*). The lipids extracted from each replicate were fractionated by thin-layer chromatography on silica gel plates using hexane/ethyl ether/formic acid 50:50:1 as the mobile phase, including in the plate the standards for free fatty acids, hydroperoxides, and oxoacids prepared as previously described in Salas et al. (*16*). Radioactive bands were quantified in an Instant Imager scanner (Packard, Canberra) and identified by comparison of their retention times with standards. The radioactivity present in the bands corresponding to the untransformed substrate, hydroperoxides, and oxoacids was determined. Unidentified oxidation products accounted typically for less than 1% of the total label.

LOX Assay. LOX activity was assayed in *Arabidopsis* leaf homogenates similar to those described for studies of linoleate metabolism using a YSI 55 oxygen meter (Yellow Springs Instruments, Yellow Springs, OH). The assays were started by adding 0.05 mL of leaf homogenate to 10 mL of buffer MES 50 mM pH 6.0 and 0.5 mM linoleic or linolenic acid potassium salts after baseline stabilization. Reactions were carried out in a hermetic container with continuous stirring. LOX activity was measured during the first 2.5 min of reaction and calculated on the basis of oxygen decrease. The enzymatic nature of the oxygenation reaction was assessed using homogenates inactivated at 80 °C for 10 min.

Kinetics of Conjugate Diene Destruction. 13-Hydroperoxy linoleic acid was prepared from linoleic acid by oxidation with commercial LOX from soybeans (Sigma). Reactions were run as described in Salas and Sánchez (*20*). Oxidation products were extracted in C^{18} cartridges and purified by normal-phase TLC as described for the reaction products in the studies of radio-labeled linoleate mechanism. Purified 13-*EZ*-hydroperoxide was converted in the ammonium salt and resuspended in 50 mM potassium phosphate buffer to a final concentration of 1 mM. The activity of diene destruction of the Columbia and AtHPL-KO plants was measured in leaf homogenates similar to those described previously. The reaction mixture contained 50 mM potassium phosphate buffer pH 7.2, 50 μM 13-*EZ*-hydroperoxylinoleic acid, and 0.01 mL of homogenate in a final volume of 1 mL. The destruction of the conjugated diene of hydroperoxides was monitored continuously for

Table 1. Volatile Composition of Aroma Produced by Leaves of *Arabidopsis*[a]

volatile	KI	BF	sensory attribute[b]
pentanal	935	0.050	fruity
1-penten-3-one	973	0.026	sweet, strawberry
hexanal	1024	0.903	green, apple
2-pentenal	1131	0.029	green, apple, tomato
1-penten-3-ol	1157	0.033	butter, mild green
E-2-hexenal	1220	0.393	green, bitter
pentan-1-ol	1255	0.008	sweet, pungent
hexyl acetate	1274	0.006	green, fruity
Z-3-hexenyl acetate	1316	0.072	banana, green
Z-2-penten-1-ol	1320	0.027	banana
hexanol	1360	0.387	fruity
Z-3-hexen-1-ol	1396	0.867	green, banana
E-2-hexenol	1417	0.001	green, fruity

[a] Compounds were identified by standards (Sigma-Aldrich, St. Louis, MO). KI, Kovats retention index and BF, *p*-level by applying the Brown–Forsythe test. [b] Sensory attributes determined in refs *16* and *23*.

2 min as A_{234} of the reaction mixture decreased. The enzymatic nature of the reaction was assessed using extracts inactivated at 80 °C for 10 min.

Concentration of Volatile Compounds. Leaves were cut up to reach approximately 0.20 g. All the leaves were immediately submerged into liquid N_2 and ground to a fine powder inside a mortar. The resulting sample was placed into 25 mL glass vials tightly capped with PTFE septum and left for 10 min at room temperature to allow the equilibrium of volatiles in the headspace. The septum covering each vial was then pierced with a solid-phase micro-extraction (SPME) needle (*16*), and the fiber was exposed to the headspace for 30 min. When the process was completed, the fiber was inserted into the injector port of the GC.

The SPME fiber was purchased from Supelco (Bellefonte, PA) and was endowed with a Stable Flex stationary phase (50:30 μm film thickness) of divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS). The fiber was previously conditioned following the supplier's instructions. The linearity of the response of this fiber as a function of concentration was >0.95 for all of the C6 compounds and most of the C5 compounds for the range of 0.1−2.5 mg/kg; the response factor for *Z*-2-penten-1-ol, 1-penten-3-ol, and pentan-1-ol was 0.95. A decrease of 14%, however, was detected when the concentration was increased to 5 mg/kg (*16*).

Gas−Liquid Chromatography (GLC) System. The volatiles were thermally desorbed in the hot injection port and deposited onto the column, where the chromatographic analysis was run. GC analyses were performed on a Varian 3900 gas chromatograph with a flame ionization detector (FID). The carrier gas was hydrogen at a flow rate of 1.5 mL/min. The temperature of desorption of analytes inside the GC injector port was 260 °C. Separation of compounds was carried out on a TR-WAX capillary column (60 m × 0.25 mm i.d., 0.25 μm coating, Teknokroma). The column temperature was held at 40 °C for 10 min and then increased to 200 °C at a rate of 3 °C/min. The detector temperature was 260 °C. Desorption time of the fiber into the injection port was 10 min. The signal was recorded and processed with the WorkStation (version 5.5.2) software.

Randomly selected samples were desorbed twice, according to the protocol already described, to determine the remnant of the volatiles after the first desorption process. Less than 8% of the initial concentration of each volatile was quantified in the second chromatogram in all of the selected samples.

Identification of Volatiles. The C5 and C6 volatile compounds were fully identified by standards. The standards, purchased from Sigma-Aldrich (St. Louis, MO), were hexanal, hexanol, *E*-2-hexenal, *E*-2-hexen-1-ol, *Z*-2-hexen-1-ol, *E*-3-hexen-1-ol, *Z*-3-hexen-1-ol, *E*-2-pentenal, 1-pentanol, 1-penten-3-ol, 1-penten-3-one, 3-pentanone, 2-pentanone, hexyl acetate, and 3-hexenyl acetate. The volatiles were also checked using the relative retention times with respect to hexanal according to the experience of authors working with volatiles produced by olive oils (*21*) and dry-cured hams (*22*). **Table 1** shows the relative retention times (with respect to hexanal) and Kovats retention times of the C5 and C6 volatile compounds identified as well as the sensory attribute characterizing them (*23*).

Statistical Analyses. Univariate and multivariate algorithms have been used by means of Statistica version 6.0 (Statsoft, Tulsa, OK). Thus, the box-whisker plot has been used to show the ranges or distribution characteristics of the volatile compounds. The variables were plotted separately for the plant groups: wild-type (WT) and mutant (KO).

The statistical study of the differences among the plants was carried out by independently analyzing each volatile compound. The Brown−Forsythe test was used to perform the analysis on the deviations from the group medians; it gives quite accurate error rates even when the underlying distributions for the raw scores deviate significantly from the normal distribution (*24*). However, the volatile profile of the plants is multivariate, and principal component analysis (PCA) was also applied to determine if there were clear differences by taking into account all of the quantified volatile compounds simultaneously.

The statistics were carried out by analyzing the leaves from eight different WT plants and eight different HPL-KO plants.

RESULTS AND DISCUSSION

Previous studies on the volatile profiles of mutants of potato with silenced HPL and LOX showed that these enzymes have a very important role in determining the leaf aroma (*16*). Results indicated that the chloroplastic form LOX 3 was the one involved in the volatile biosynthesis among the different LOX copies present in the potato genome. Thus, the silencing of this enzyme by a mechanism of cosuppression dramatically decreased the total amount of volatile compounds produced by the leaf tissue. Furthermore, the HPL enzyme depletion affected both the amount of volatile compounds and the balance of C6 and C5 volatiles. In addition, the silencing of the HPL enzyme increased the LOX activity in the leaf tissue. To confirm if these responses are general to other plants, the effect of HPL suppression was studied in *A. thaliana*. This species has a single copy of the HPL gene in its genome, so several mutant libraries were screened to find the insertional mutant of this enzyme (T-DNA_LB.SALK_008351). That plant was ordered, and the insertion of T-DNA in the HPL was confirmed by PCR. Then, a homozygous line was selected and studied for volatile synthesis and linoleate metabolism. This line should not have, in theory, any HPL activity due to the insertion interrupting the transcription of the gene within the coding sequence.

Volatile Compounds. As *Arabidopsis* leaves are not homogeneous material, the first study was to set up a procedure that avoided increasing the standard deviation in the repeated experiments due to external variables. Thus, all the leaves were selected after a visual inspection, and the chromatographic values were normalized by the sample weight (approximately 0.20 g per sample). All the samples were submerged into liquid N_2 prior to being ground to reach a fine powder to avoid the disruption of the tissues during the preparation that might affect the results (*25*).

The repeatability study, however, shows high values of relative standard deviation. **Figure 1** shows the box-whisker plot of the volatile compounds. Because the chromatographic area of the volatile compounds showed high variation from one another, the values were standardized to better show their variation. The plot shows the standardized values of the mean, the standard deviation (±std), and the 95% confidence interval (defined as mean ± 1.96 × std) of each chemical compound quantified in the *Arabidopsis* plants. Six volatile compounds showed values higher than 20% in one of the plants (WT and KO)—pentanal (KO), 1-penten-3-one (KO), 1-penten-3-ol (KO), *Z*-2-penten-1-ol (KO), hexanol (WT), and *Z*-3-hexen-1-ol (KO)—while hexanal presented high values in both plants.

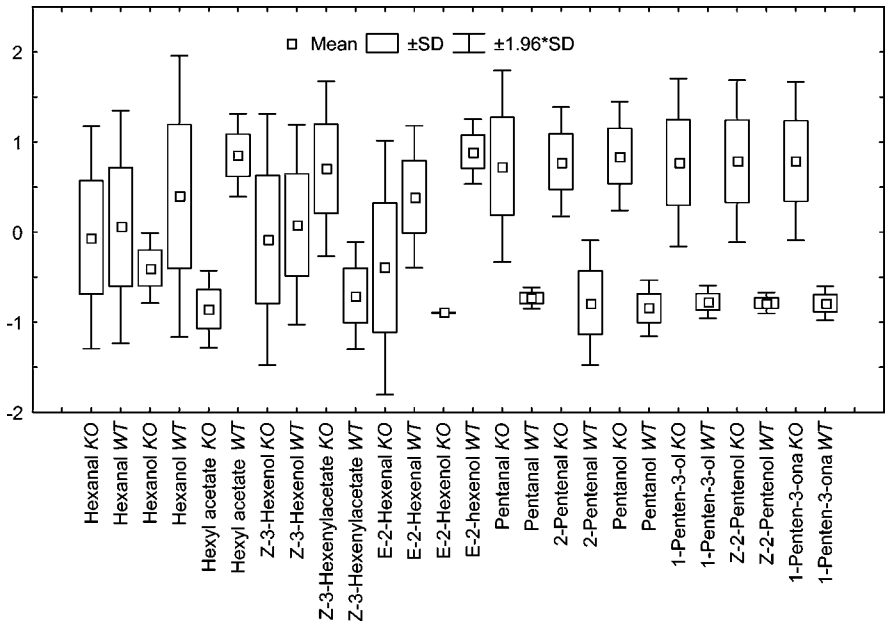

Figure 1. Box-whisker plot of the C5 and C6 volatile compounds quantified in the wild-type (WT) and mutant (KO) plants (mean and standard deviation of the analyzed compounds).

Despite the variability in the volatile quantification, the figure shows clear differences in all the amounts of C5 compounds and two C6 compounds—hexyl acetate and *E*-2-hexenol (**Figure 1**); in fact, there are no overlaps between the values (mean ± 1.96 × std). This graphic result was corroborated with the Brown−Forsythe test that found significant differences between the C5 volatile compounds of the KO and WT plants while there were no differences in the production of the C6 compounds except *E*-2-hexenol and hexyl acetate (**Table 1**). These results agree with those already found working with potato plants (*16*) and soybean (*6*), although the production of C5 compounds in the KO *Arabidopsis* plants was lower than that reported in the cited papers.

The difference in the amount of C5 compounds between wild-type and mutant plants explains the multivariate statistical procedure of principal components, applied to the whole set of volatile compounds quantified in the samples, which neatly distinguished all the WT and KO plants (**Figure 2**). That figure, however, shows a notorious lack of homogeneity among WT plants that are split into two clusters. This set of samples is not homogeneous, in terms of chemical composition, mostly due to the volatiles produced from the linoleic acid. In fact, the relative standard deviation of hexanol in WT samples is high enough (32%). **Figure 3** shows that the variation of hexanol depends on the amount of hexanal. It seems that the activity of the enzyme alcohol deshydrogenase (ADH) varies from a WT plant to another, although the amount of volatile compounds is still almost invariable. Thus, the amount of hexanol is low when the amount of hexanal is high (WT5, WT1, and WT7), which means a low enzymatic activity of ADH, while, in the opposite situation, a low amount of hexanal and a high of hexanol (WT2 and WT3) means a high enzymatic activity. The same behavior occurs in the HPL-KO plants but at very low levels. Similar results were found in the WT plants described in **Figure 3** when the linolenic acid enzymatic pathway by ADH activity was studied. Fortunately, this lack of homogeneity did not mask a clear differentiation between WT and KO plants.

Among the volatile compounds produced by the LOX pathway in the WT plants, the amount of each individual C6 compound was of the same order of magnitude as the KO plants,

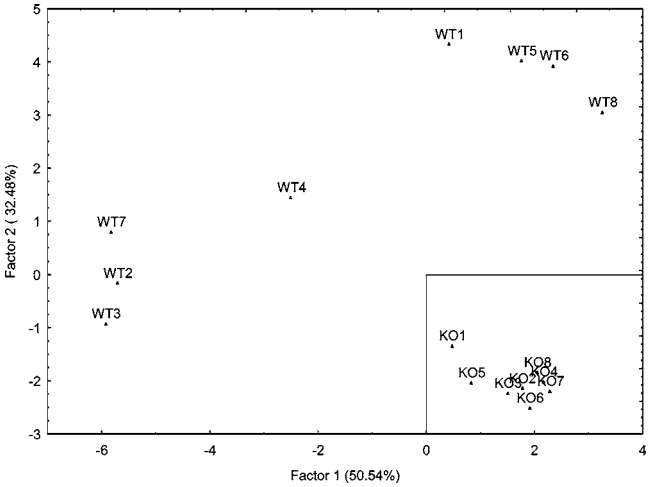

Figure 2. Principal component analysis. Differentiation between *Arabidopsis* plants by means of C5 and C6 volatile compounds. Percentages in parentheses indicate the variance explained by each principal component. Note: WT, control plant and KO, HPL-KO plant or mutant plant.

with the exception of *E*-2-hexenol that was more abundant in WT plants (**Figure 1**). However, the total amount of C6 compounds in KO plants was very similar to WT (**Table 2**). The contents of C6 aldehydes and C6 acetates were also similar in both types of plants, while the total content of C6 alcohols (hexanol, *Z*-3-hexenol, *E*-2-hexenol, and *E*-3-hexenol) was 3-fold more abundant in the WT plants mainly due to *E*-2-hexenol.

The main difference between WT and KO plants is due to the content of C5 compounds that are higher in KO plants (**Figure 1**). The contents of pentanal, 1-penten-3-ol, and 1-penten-3-one are 4 times lower in the untransformed WT plants, the content of *Z*-2-penten-1-ol is 5 times higher in KO plants, while, surprisingly, pentan-1-ol is 3-fold more abundant in WT plants. The total content of C5 alcohols in KO plants was 4 times higher than WT plants, while the difference between both plants was not remarkable in C5 aldehydes (**Table 2**). In this regard, it is important to remark that the C6/C5 ratio in

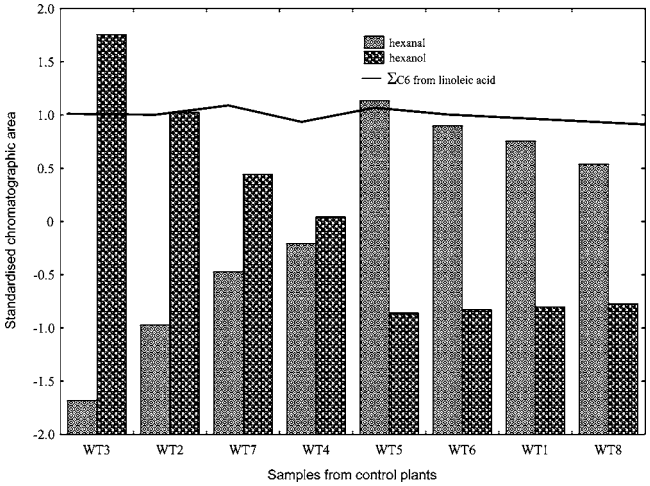

Figure 3. Chromatographic areas of volatiles produced in LOX pathway of the linoleic acid: hexanal, hexanol, and sum of all the volatiles (hexanal, hexanol, and hexyl acetate).

Table 2. Relationship between C6 and C5 Volatile Compounds Produced in *A. thaliana* Wild-Type Plants (WT) and Hydroperoxide Lyase Knockout Plants, HPL-KO (KO)

	WT[a]	KO[a]	WT/KO
all C6 compounds	1144.6 ± 74.5	1081.3 ± 52.7	1.1
all C5 compounds	896.0 ± 131.6	3342.5 ± 662.7	0.3
C6/C5	1.31 ± 0.13	0.34 ± 0.04	3.9
C6 from linoleic acid	177.4 ± 34.7	434.8 ± 104.86	0.4
C6 from linolenic acid	967.2 ± 61.4	646.5 ± 89.1	1.5
C6-aldehydes	1641.1 ± 255.0	1518.1 ± 280.7	1.1
C5-aldehydes	284.0 ± 28.5	683.6 ± 87.7	0.4
C6/C5-aldehydes	1925.2 ± 260.8	2201.7 ± 200.6	0.9
C6-alcohols	418.8 ± 57.2	148.3 ± 25.6	2.8
C5-alcohols	460.3 ± 75.3	2019.6 ± 457.2	0.2
C6/C5-alcohols	0.92 ± 0.05	0.08 ± 0.01	12.1
C6-acetates	28.9 ± 2.3	33.3 ± 2.7	0.9

[a] Sum of peak areas/mg of FW.

WT was 4-fold higher than KO mostly due to the ratio of C6 alcohols/C5 alcohols that was 12-fold higher in WT plants.

Studies of Linoleate Metabolism. The analytical characterization of the mutant was completed with studies of metabolism of linoleate by homogenates prepared from leaf tissue from both the HPL-KO and the WT plants. In these experiments, the crude homogenates from leaf were fed with radio-labeled fatty acids, and the incorporation into the nonvolatile compounds of the LOX pathway (hydroperoxides and oxoacids) was monitored with respect to the time. Results in **Figure 4A** indicated that both control and At-HPL-KO plants were able to oxidize linoleic acid to hydroperoxides and C12 and C13 oxoacids. The rate of linoleate oxidation was about 3 times faster in HPL-KO than WT. Thus, the homogenate from HPL-KO leaves consumed 30% of the substrate in 40 min of reaction, whereas the WT plants only reached a 10% of substrate oxidation. This indicates that the insertion into the HPL gene induced the LOX activity in *Arabidopsis* as it happened in potato plants (*16*). That increase in the rates of oxidation induced a higher accumulation of hydroperoxide and oxoacid derivatives. In this regard, the complete knockout of the HPL gene should have resulted in the absence of C12 oxoacids typically produced through HPL cleavage of fatty acid hydroperoxides. These derivatives were, however, present as the products of the linoleate metabolism even at higher concentrations than in the WT plants, so the At-HPL-KO mutant incorporated 5.6% of the label into C12 oxoacids after 40 min, which was higher than 2.3% of

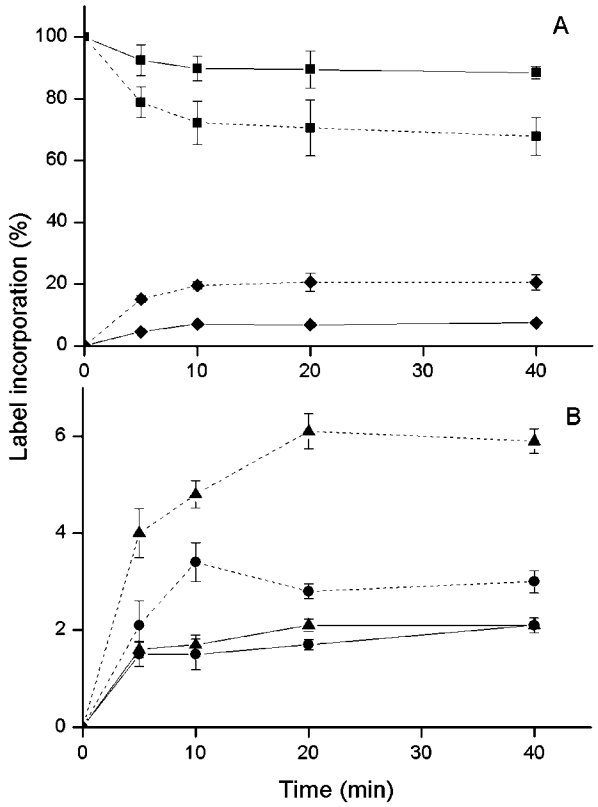

Figure 4. Course of degradation of labeled linoleate through the LOX pathway by homogenates from control (solid lines) and HPL-KO (dashed lines) plants. Incorporation into hydroperoxides (◆), C12 oxoacids (▲), C13 oxoacids (●), and the remaining substrate (■) are shown as a function of time. Results correspond to the average of three determinations.

compounds found in the WT plants (**Figure 4B**). This result agrees with the analysis of the volatile fraction, where the C6 aldehydes and alcohols produced via HPL were present in both KO and WT plants at similar concentrations. Nevertheless, the relative incorporation into C13 oxoacids, which were compounds derived from the production of C5 volatiles, was slightly higher in the mutant, a result that was expected due to the increment of the LOX activity, the one responsible for the homolytic cleavage of 13-hydroperoxides. This profile of linoleate metabolism was different from that found in potato leaves (*16*), given that the rates of linoleate oxidation were several-fold lower in *Arabidopsis*, which indicates that the level of LOX activity was lower in this plant. Incorporation rates into oxoacids, on the other hand, were similar in both species.

Hydroperoxide Destruction and LOX Activity. Since the studies of linoleate metabolism indicated that there is still HPL activity in the KO-HPL mutant, some measurement of the rates of hydroperoxide cleavage by crude extracts from *Arabidopsis* leaf were fulfilled. HPL acts by degrading the conjugated double-bond system present in the hydroperoxide molecules; therefore, it can be monitored by measuring the decrease of A_{234} of the assay media associated with the scission of the mentioned group, although other hydroperoxide-consuming activities present in the leaf tissue could also cause destruction of the conjugated diene. In the case of the antisense mutant from potato leaves H57, the HPL activity was 95% depleted, which caused an important decrease of the C6 volatile compounds (*16*). In *Arabidopsis*, however, the rates of hydroperoxide cleavage were similar in control and mutant plants, being even higher in KO (**Table 3**), which agreed with the experiment of linoleate metabolism (**Figure 4**) that displayed higher amounts of C12

Table 3. Lipoxygenase and Hydroperoxide Destruction Activity in Homogenates from Control (WT) and HPL-KO (KO) Plants[a]

	LOX (nkat/g of FW)		hydroperoxide destruction (nkat/g of FW)
	linoleic acid	linolenic acid	
WT	48 ± 5	43 ± 6	165 ± 10
KO	75 ± 8	156 ± 25	232 ± 11

[a] 13-Hydroperoxy linoleic acid was used as the substrate in the hydroperoxide destruction determination. Results correspond to independent triplicates.

oxoacids in the KO mutant at long incubation times. Results indicated that the insertion of the T-DNA in one exon of the only copy of the HPL gene of *Arabidopsis* did not cause changes in the rates of degradation of hydroperoxides but that activity stayed at a similar level than in WT plants. This indicates that another HPL active form, responsible for the C6 and C12 oxoacid production, could not exist as being accounted as a HPL. This form of HPL, if it is also present in potato leaves, could be sensitive to silencing by the expression of antisense constructs, which would explain why the H57 potato mutant displayed lower C6 volatile contents (*16*). Another result found in that paper was an increment of the LOX activity in the HPL antisense mutant of potato. Results in **Figure 4** suggested that there was a similar response in *Arabidopsis*, provided crude extracts from At-HPL-KO plants displayed higher rates of linoleate oxidation than extracts from control plants. Therefore, measurements of LOX activity were carried out in fresh homogenates of both control and mutant *Arabidopsis* plants. LOX activity was significantly higher in the At-HPL-KO mutant when using both linoleic and linolenic fatty acids. Activity in the At-HPL-KO leaves was 50% higher than in the control Columbia plants, whereas that difference was even more evident when using linolenic acid (**Table 3**). Thus, similar to potato leaves, when HPL genes were silenced, the plant response consisted of an increment in the LOX activity, maybe for compensation of the decrease of C6 aldehydes with the C5 alcohols produced by scission of hydroperoxides by LOX. The mechanism that rules this response is still unknown.

The depletion of HPL enzyme also affects the aroma through the changes in the concentration of the volatile compounds, which agrees with previous works in which off-flavors of soybean products were removed by the suppression of the soybean LOX-2 activity (*9*), whereas the modification of the activity level of LOX enzymes in potato produced a strong variation in the aroma of its leaves (*16*). Thus, the higher concentration of C5 alcohols presents in HPL-KO plants (ratio of WT/KO is 0.2) should increase the sweet−fruity sensory notes of the aroma generated by the leaves, which should contrast with the predominantly green sensory notes of WT leaves due to a higher presence of C6 alcohols (ratio of WT/KO is 2.8) (*16*, *26*).

This study shows that it is possible to change the flavor by modifying the activity level of the enzymes, but the modification of the enzymatic activity is not always possible as, for instance, in olives. Mathematical models, however, can assist to emulate the basic aroma profile of a virgin olive oil obtained from hypothetical HPL-KO olives, although this kind of model might have certain speculative components since we applied the results to another plant and tissue. The statistical sensory wheel (*23*) was the mathematical model used to emulate the olive oil aroma under the assumption that the changes produced in the HPL-KO *A. thaliana* plants and in hypothetical HPL-KO olives, with respect to the wild plants, were similar. **Figure 5** shows the basic sensory profile of a virgin olive oil produced from

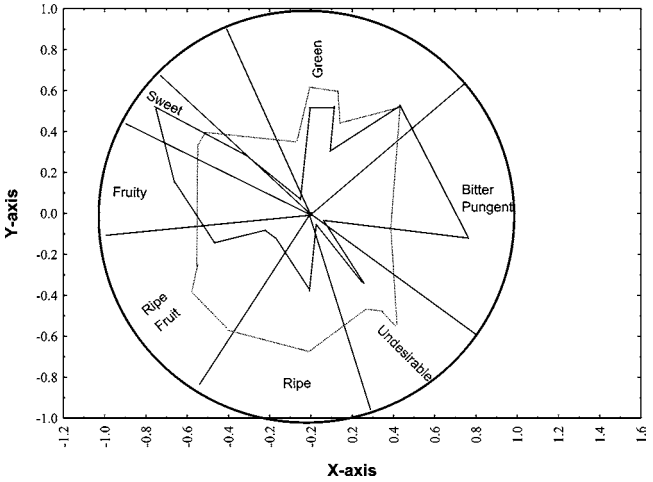

Figure 5. Statistical sensory wheel of virgin olive oils based on the concentration of 65 volatile compounds. Area of sensory sectors covered by an average of 48 diverse virgin olive oils (dotted line) and the emulated oil obtained from hypothetical HPL-KO olives (solid line). The wider the area covered in a sector, the more intense the sensory note.

hypothetical HPL-KO olives. The resulting oils would be more sweet−fruity and bitter, less green, undesirable, and ripe than oils representing an average of 48 virgin olive oils from several single varietal olives extracted at different maturity levels (*23*). The current result agrees with a previous study on potato leaves (*16*) in which a panel test was used to evaluate the sensory notes.

In conclusion, the mechanism of HPL enzyme suppression in *Arabidopsis* seems to be affected by other forms of HPL as some results have pointed out. However, the suppression of this enzyme had similar effects in *Arabidopsis* and potato leaves concerning the increase of C5 volatile concentrations and LOX activity.

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