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Impact of the Suppression of Lipoxygenase and Hydroperoxide Lyase on the Quality of the Green Odor in Green Leaves

JOAQUÍN J. SALAS, CAROLINA SÁNCHEZ, DIEGO L. GARCÍA-GONZÁLEZ, AND RAMÓN APARICIO*

Instituto de la Grasa, CSIC, Avenida Padre García Tejero 4, 41012 Sevilla, Spain

Most of the volatile compounds responsible for the "green" notes to the aroma of fruits and vegetables are produced by the degradation of polyunsaturated fatty acids through the lipoxygenase pathway. The most determinant steps of this pathway are the peroxidation of free linoleic or linolenic acid by the action of lipoxygenase and then the lysis of the resulting hydroperoxides through a reaction catalyzed by the hydroperoxide lyase. This work analyzes the impact of the depletion of these enzymes on the volatile composition of leaves from potato plants. A characterization of the volatile profiles of the different potato mutants, a study of the metabolism of radiolabeled linoleic acid, and a determination of lipoxygenase activity have been carried out. The depletion of hydroperoxide lyase induced an increase in the lipoxygenase activity and the content of C5 volatiles, whereas the lipoxygenase silencing caused a severe decrease in the amount of volatiles produced by the leaves and always in the intensity of their aroma. The changes in the sensory evaluation of leaf aroma, as correlated to depletion of the two enzymes, have been investigated. The perspectives of producing vegetable products with a modified aroma by genetic engineering are discussed in light of the statistical results.

KEYWORDS: ***Solanum tuberosum*; potato; leaf; aroma; lipoxygenase; hydroperoxide lyase**

INTRODUCTION

The so-called "green" odor is present in a variety of food products including fresh vegetables, juices, and other derivatives such as olive oil, being a determinant factor on their quality and acceptance (*1*−*3*). The green odor is caused by a series of volatile aldehydes and alcohols of six carbon atoms—such as hexanal, *E*-2-hexenal, hexanol, or *Z*-3-hexenol (*4*, *5*)—that are typically produced from polyunsaturated fatty acids (linoleic and α-linolenic acids) through the lipoxygenase (LOX) pathway (*6*). This pathway is triggered when the cell tissue is disrupted and involves the hydrolysis of those fatty acids from plant glycerolipids by the action of endogenous acyl-hydrolases (*7*). Free linoleic and α-linolenic acids are then oxidized by the lipoxygenase enzymes to yield their 9- or 13-hydroperoxide derivatives (*8*). These oxygenated intermediates are quickly excised by the action of hydroperoxide lyases (HPLs), which are P450 proteins of a crucial importance in this pathway due to the fact that they determine the chain length of the first volatile compounds that are produced (*9*). The most widespread HPLs display strict specificity toward 13-hydroperoxides, generating a volatile C6 aldehyde (hexanal or *Z*-3-hexenal) and a C12 oxoacid [12-oxo-9(*Z*)-dodecenoic acid] (*10*−*12*). The 9-HPLs, which display activity toward both 9- and 13-hydroperoxides, produce the C9 aldehydes that confer the typical cucumber-like odor to the Cucurbitaceae species (*13*). The C6 aldehydes can be further modified by isomerases or isomerization factors (*14*, *15*) (e.g., the transformation of *Z*-3-hexenal into *E*-2-hexenal) or reduced to their corresponding alcohols by the action of alcohol dehydrogenases (*16*). Finally, the alcohols can be esterified with CoA esters to yield volatile esters in a reaction catalyzed by alcohol acyltransferases (*17*).

The characterization of the LOX pathway, as well as the cloning, sequencing, and expression of the genes encoding their different enzymes, is undoubtedly contributing to the production of tailor-made tastes and aromas of diverse food products. Thus, genetic removal of LOX-2 from soybean greatly contributed to the suppression of off-flavors in soybean meal or milk (*18*), which improved the sensory perception of some food products such as tofu (*19*). Furthermore, the modification of alcohol/aldehyde ratios in the volatile fraction of tomato fruits, by expression of exogenous alcohol dehydrogenases, has resulted in the perception of their particular ripe flavor at earlier stages of ripeness (*20*). Among the different enzymes of the lipoxygenase pathway, special attention has been paid to HPLs due to their impact on the volatile synthesis in fruits, vegetables, and derived products. In this regard, a first attempt to produce cucumber-flavored tomatoes by the expression of a 9-HPL isolated from cucumber seedlings has been carried out unsuccessfully (*21*), indicating that the phytoxylipin metabolism involved in aroma biosynthesis is not as simple as initially supposed. A good approach to establish the impact of the modifications of the LOX pathway on the aroma and flavor of foods would be the study of the volatile fraction of those systems from which enzymatic activities were removed (*22*). The thermal inactivation of these enzymes in plant homogenates does not

* Author to whom correspondence should be addressed (e-mail aparicio@cica.es).

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seem to be an efficient way to undertake this task because there is always a basal activity in the intact tissues that produces a remnant of volatile aldehydes and alcohols not depending on the mashing conditions (*23*). Thus, a blockage of the peroxidation and lysis reactions by silencing or knocking out the gene encoding LOX or HPL would be the best way tofor the totally suppress this pathway. Unfortunately, the number of vegetable species that can be transformed or allow the construction of knockout libraries is relatively small.

Previous works concerning the H57 and H33 mutants represented an excellent approach to determine the physiological roles of different phytoxylipins (*24*, *25*). However, the possible implications of these changes of the volatile metabolism on the quality of the aroma were only partially considered. In the present work, the impact caused on the aroma of the potato leaves by the cosuppression of LOX-H1, or the expression of an antisense HPL construct, was investigated. The metabolism of [1-^{14}C]18:2 by leaf homogenates from these three potato lines was also studied to correlate the changes on the volatile compounds with the accumulation of the nonvolatile intermediates and products of the LOX pathway.

MATERIALS AND METHODS

Plant Material. Three lines of potato plants were studied: wild type (WT) and two transgenic plants, H33 (*24*) and H57 (*25*). The mutants analyzed in this work were produced by transformation of common potato plants (*Solanum tuberosum* cv. Dessireé) with different cDNA constructs. The mutant called H33 was produced by overexpression of a construct containing the cDNA encoding for the lipoxygenase form LOXH1 from potato plants under the regulation of the constitutive 35S promoter. This plant underwent silencing of the transgene expression and cosuppression of the LOX-H1 gene, which was reported to produce a severe decrease of the volatile amounts produced by leaves of that mutant (*24*). The mutant called H57 expresses an antisense cDNA corresponding to the only gene of HPL found in potato, under the control of the cauliflower mosaic virus 35S promoter. This antisense mutant displayed a severe decrease of the HPL gene expression (3% of that found in the WT), which corresponded to a 50-fold decrease of the HPL activity in leaf extracts (*25*). All of these potato plants were kindly supplied by Dr. Jose J. Sánchez-Serrano (CNB, CSIC, Madrid, Spain).

Plants were cultivated in a growth chamber at 15/10 °C (day/night) with a 16-h photoperiod and an electronically controlled fertirrigation. Fully developed nonsenescencing leaves (2−4 weeks old) were used for the homogenate preparations of the metabolism of [1-^{14}C]18:2 and the quantification of the volatile compounds by gas chromatography (leaf homogenates and leaf disks).

Reagents. Methyl vernoleate (12,13-epoxyoctadec-9*Z*-enoic acid), 4-methyl-2-pentanol (internal standard), C6 compounds (hexanal, *E*-2-hexenal, hexyl acetate, *Z*-3-hexenyl acetate, hexan-1-ol, *Z*-3-hexen-1-ol, and *E*-2-hexen-1-ol), and C5 compounds (1-penten-3-ol, *Z*-2-penten-1-ol, pentan-1-ol, and 1-penten-3-one) were obtained from Sigma-Aldrich (St. Louis, MO).

Preparation of Oxoacid Standards. The 12-oxo-dodec-9*Z*-enoic acid was prepared from methyl vernoleate (12,13-epoxyoctadec-9*Z*-enoic acid). A weight of 50 mg of that compound was saponified with 1 mL of 1 M KOH at room temperature for 12 h, then the solution was acidified with 1 M HCl, and free vernolic acid was extracted three times with 1 mL of $CHCl_3$. A weight of 10 mg of vernolic acid dissolved in 1 mL of benzene was treated with 100 mg of the activated acid resin Amberlite XN-1005 for 30 min at room temperature, which opened the epoxy ring to yield a 12,13-dihydroxy derivative. The 12,13-dihydroxyoctadec-9*Z*-enoic acid was then cleaved by reaction with acid ethanolic KIO_4 for 30 min at room temperature. The reaction was quenched with 5 mL of 5% NaCl, and the resulting 12-oxododec-9*Z*-enoic acid was recovered by extraction with 2 mL of $CHCl_3$.

The 13-oxotridec-9*Z*-enoic acid was prepared from 13-hydroperoxyoctadecatrienoic acid by lysis using soybean LOX as reported by Salchs et al. (*26*). Lipids in the reaction solution were isolated by solid-phase extraction in a C18 cartridge previously equilibrated with reaction buffer (KP_i, pH 6.0). The rest of the aqueous phase was eluted with 2 mL of hexane, and lipids were recovered with 3 mL of methanol and fractionated by normal-phase thin-layer chromatography (TLC) using hexane/ethyl ether/formic acid as the solvent. The band corresponding to oxoacids consisted mainly of 13-oxotridec-9*Z*-enoic acid. The nature of both compounds was confirmed by spectral means.

Metabolism of [1-^{14}C]18:2. Leaf crude homogenates were prepared by grinding 0.2 g of leaf tissue in 2.5 mL of 50 mM MES, pH 6.0, in an ice-cooled glass homogenator. Reaction mixtures consisted of 50 mM MES, pH 6.0, 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 1 h at 20 °C with continuous shaking. Aliquots of 0.4 mL of these mixtures were taken at different times, inactivated by the addition of 0.1 mL of acetic acid, and subjected to lipid extraction according to the method described by Hara and Radin (*27*). Lipid extracts were then fractionated by TLC on silica gel plates using hexane/ethyl ether/formic acid 50:50:1 as the mobile phase, including the standards for free fatty acids, hydroperoxides, and oxoacids in the plate. Radioactive bands were found in an Instant Imager Scanner (Packard, Canberra, Australia) 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 <1% of the total label.

LOX Assay. LOX activity was studied in the above-described potato leaf homogenates using a YSI 55 oxygen meter (Yellow Springs Instruments, Yellow Springs, OH). The assays were started by adding 0.05 mL of potato leaf homogenate to 10 mL of buffers (50 mM MES, pH 6.0, 0.5 mM) of linoleic or linolenic acid potassium salts. Reactions were carried out in a hermetic chamber with continuous stirring. LOX activity was measured during the first 5 min of reaction and calculated on the basis of oxygen decrease.

Concentration of Volatile Compounds. Three 5-mm disks cut from a single leaf were placed into 25 mL glass vials tightly capped with PTFE septa 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 microextraction (SPME) needle (*28*) and the fiber exposed to the headspace for 30 min. Then the fiber was loaded with the internal standard following the same procedure with a vial containing a solution of 4-methyl-2-pentanol (1.2 mg/kg). When the process was completed, the fiber was inserted into the injector port of the GC. The same process was applied to the concentration of volatiles from the leaf homogenates (see Metabolism of [1-^{14}C]C18:2).

The SPME fiber was purchased from Supelco (Bellefonte, PA) and was endowed with 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 0.1−2.5 mg/kg. A decrease of ~14%, however, was detected when the concentration was increased to 5 mg/kg (*28*). The response factor for *Z*-2-penten-1-ol, 1-penten-3-ol, and pentan-1-ol was 0.95.

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 mm 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.

The internal standard used for the quantification of volatile compounds was 4-methyl-2-pentanol. This was used to correct possible variations in the concentration step.

Table 1. Volatile Composition of the Aroma Produced by the Leaf Homogenates of the Leaves from WT, H33, and H57 Potato Plants[a]

volatile compound	RT	KI	WT	H57	H33	sensory attributes
pentanal	0.38	935	0.615 ± 0.027	1.931 ± 0.071	0.139 ± 0.003	fruity
1-penten-3-one	0.48	973	0.226 ± 0.028	0.102 ± 0.011	$0.005 \pm 6 \times 10^{-4}$	sweet, strawberry
hexanal	0.67	1024	3.837 ± 0.701	0.078 ± 0.015	0.006 ± 0.005	green, apple
2-pentenal	0.83	1131	0.076 ± 0.009	0.677 ± 0.030	$0.025 \pm 5 \times 10^{-4}$	green, apple, tomato
1-penten-3-ol	0.97	1157	0.097 ± 0.005	0.152 ± 0.005	0.005 ± 0.001	butter, mild green
E-2-hexenal	1.14	1220	9.827 ± 2.596	0.024 ± 0.013	$0.008 \pm 6 \times 10^{-4}$	green, bitter
pentan-1-ol	1.27	1255	0.086 ± 0.003	0.299 ± 0.016	$0.018 \pm 6 \times 10^{-4}$	sweet, pungent
hexyl acetate	1.30	1274	$0.004 \pm 4 \times 10^{-4}$	$0.005 \pm 1 \times 10^{-4}$	$0.002 \pm 76 \times 10^{-5}$	green, fruity
Z-3-hexenyl acetate	1.46	1316	0.017 ± 0.003	0.025 ± 0.002	trace	banana, green
Z-2-penten-1-ol	1.48	1320	0.052 ± 0.006	0.061 ± 0.003	$0.003 \pm 5 \times 10^{-4}$	banana
hexanol	1.59	1360	1.016 ± 0.085	0.054 ± 0.008	0.020 ± 0.004	fruity
Z-3-hexenol	1.65	1396	0.132 ± 0.018	0.008 ± 0.001	$0.002 \pm 4 \times 10^{-4}$	green, banana
E-2-hexenol	1.72	1417	0.204 ± 0.022	0.012 ± 0.001	$0.006 \pm 4 \times 10^{-4}$	green, fruity

[a] Compounds were identified by standards and quantified in mg/kg, using 4-methyl-2-pentanol as the internal standard. Contents are given in mg/kg. Values of chemical compounds are given as their mean ± standard error of the mean. RT, relative retention time; KI, Kovats retention index; WT, wild-type or control plant; H57, transgenic plant with HPL-depleted enzyme; H33, transgenic plant with LOX H1-depleted enzyme.

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 12% of the initial concentration of each volatile was quantified in the second chromatogram in all of the selected samples. The relative standard deviation of the internal standard, investigated by consecutively analyzing 12 samples of a refined sunflower oil spiked with 1.2 mg/kg of the standard, was <10%.

Identification of Volatiles. The C5 and C6 volatile compounds were identified using the relative retention times of the standards with respect to the internal standard (4-methyl-2-pentanol). **Table 1** shows the relative retention times and Kovats retention indices of the C5 and C6 volatile compounds identified as well as the sensory attribute characterizing them.

Sensory Analysis. Ten disks of potato leaves were presented to eight assessors for sensory evaluation. Assessors, with more than 10 years of experience evaluating olive oils, were requested to detect the presence of the following sensory notes: cut green lawn, green leaf, wild flowers, green tomato, sweet-fruity, green-fruity, fruity, and roots. The selection of these sensory notes was based on the previous experience of the authors evaluating virgin olive oils with predominant C6 and C5 compounds (*29*). A unipolar structured scale of intensity was used to evaluate the sensory notes as it produces better judgments of single samples than when comparative judgments are made, and it also requires less time for the assessment. The intensity of each sensory note was ranked into a sequence of six designations: not detectable, just detectable, slightly strong, moderately strong, strong, and very strong (*30*).

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 groups of potato plants (WT, H33, and H57).

The statistical study of the differences among the potato 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, and it is particularly suited for quickly and simultaneously analyzing univariate designs with three or more groups (*31*). However, the volatile profile of the potato 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 statistical study was carried out with samples from the leaf disks and the lipid extracts of the potato plants (WT, H33, and H57). Twenty-seven samples were collected from different leaves having a similar color after visual inspection; 11 and 8 samples were analyzed from the modified plants H57 and H33, respectively, and 8 samples from the control plant (WT). With respect to the leaf homogenates, 4 samples were analyzed from each potato mutant. At least 2 plants of each line were used in the statistical study.

RESULTS AND DISCUSSION

The potato leaves are not a homogeneous material, and despite the care taken in their selection, the repeatability studies of the volatile compounds showed high values of relative standard deviation. Furthermore, the disruption of the tissues during the preparation of the leaf homogenates can cause changes in the LOX pathway that might affect the results; therefore, both leaf homogenates and leaf disks were used in this work. **Figure 1** shows the box−whisker plot of the volatile compounds quantified in three leaf disks of the potato plants: wild-type plant (WT), LOX H1-depleted transgenic plant (H33), and HPL-depleted transgenic plant (H57). Because the amount of the volatile compounds varies from parts per billion (e.g., *Z*-2-hexenol) to part per million (e.g., *E*-2-hexenal), 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 three kinds of potato plants. The figure shows clear differences in the amounts of C5 compounds quantified in the potato plant H57 with respect to the other potato plants (WT and H33). There are no overlaps between the values (mean ± std) of the C5 compounds 1-penten-3-ol and *Z*-2-pentenol quantified in each potato line, being different even when their confidence interval is taken into account. These results agree with those of other authors (*24*, *25*), although only partial results were reported in these works (no information was given on C5 compounds of the line H33).

More overlaps were detected in the concentrations of C6 compounds, although their highest values corresponded to WT immediately followed by H57, and the minimum concentration corresponded to line H33. The variability of the concentrations of hexanol and hexanal in line H33 and the behavior of hexyl acetate, the values of which overlap in all of the potato plants, are remarkable.

A similar study of volatile compounds was carried out with the leaf homogenates (**Table 1**) in order to compare their results with those obtained from the leaf disks (**Figure 1**). The concentrations of C5 compounds were also higher in the HPL-depleted transgenic plant H57 with the exception of 1-penten-3-one, which has not been described in the LOX cascade (*32*). Concerning the concentrations of C6 compounds, they were

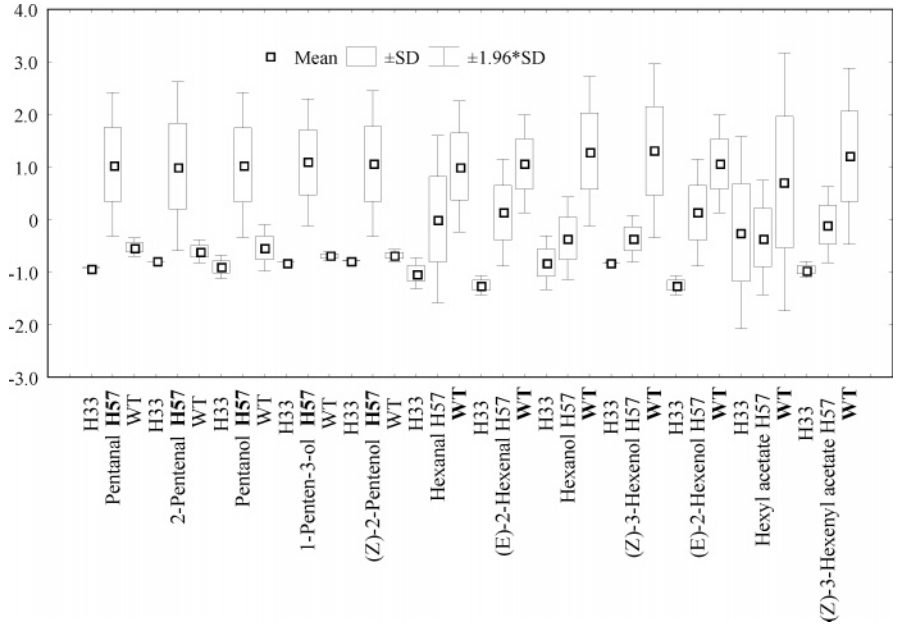

Figure 1. Box–whisker plots of the C5 and C6 volatile compounds quantified in the leaf disks from the potato plants (WT, H57, H33) (mean and standard deviation of the analyzed compounds).

higher in the WT plant with the exception of the C6-acetate compounds. The differences in the concentrations of C6 and C5 compounds between WT and line H33 (LOX H1-depleted transgenic compounds) are also shown in **Table 1** (leaf homogenates) and **Figure 1** (leaf disks).

The volatile profiles of the three analyzed lines considerably differ from each other. The profiles from all of the potato plants showed a similar number of identified compounds but differing in their relative proportions. Furthermore, the H33 mutant showed a profile in which most volatile compounds were barely detectable (except pentanal), confirming the impact of the suppression of that form of LOX on the synthesis of volatile aldehydes. A previous work on the difference in volatile composition between WT and H33 potato plants has already been published (*24*), but this study involved only a few volatile compounds, applying an analytical method based on direct analysis by headspace gas chromatography that has poor sensitivity and selectivity in comparison with the method based on SPME-GC.

Among the volatile compounds produced by the LOX pathway in the WT plant, the C6-aldehyde *E*-2-hexenal, synthesized via HPL from 13-hydroperoxylinolenic acid, diminished >400-fold in the HPL-depleted transgenic plant and >1200-fold in the LOX H1-depleted transgenic plant (**Table 1**). Concerning hexanal, the other C6-aldehyde, its concentration in WT was 50- and 600-fold more abundant than in the transgenic plants H57 and H33, respectively. C6 alcohols (hexanol, *Z*-3-hexenol, and *E*-2-hexenol) were 20-fold more abundant in the WT line than in the HPL-depleted H57 line, differences that were 1 magnitude order higher when WT was compared with the H33 line; these compounds are synthesized from the aldehydes via alcohol dehydrogenase (*32*). Furthermore, the content of C5 compounds was generally higher in the H57 line, above all when C5-aldehydes were considered. Thus, contents 10- and 3-fold higher of 2-pentenal and pentanal, respectively, were found in H57 when compared with the untransformed WT line. C5-alcohols were also in higher proportion in H57, although differences were not so remarkable. Finally, the H33 line presented only traces of these compounds. In this regard, it is important to remark that the C6/C5 ratio in WT was very similar to the C5/C6 ratio in H57 (16.5 vs 14.9), which discloses an inverted production of volatile compounds of the transgenic plant with respect to the WT. This behavior has also a definitive influence on the aroma perception of these kinds of plants.

Table 2. Relationships between C6 and C5 Volatile Compounds Produced by the Leaf Homogenates and Leaf Disks from the Leaves from the Three Lines of Potato Plants under Study[a]

		volatile composition (mg/kg)				ratio of
	line	C6	C5	total	%	C6 to C5
leaf homogenates	WT	15.18	0.92	16.10	100	16.5
	H33	0.04	0.19	0.23	0.01	0.21
	H57	0.21	3.12	3.33	0.21	0.07
leaf disks	WT	15.95	1.05	17.00	100	15.2
	H33	0.03	0.27	0.30	0.02	0.11
	H57	0.69	4.12	4.81	0.29	0.17

[a] Given values are the mean of triplicate analyses.

Among these C5 volatiles induced in H57, 1-penten-3-ol and *Z*-2-penten-1-ol were associated with the homolytic cleavage of 13-hydroperoxylinolenic acid by the action of LOX (*26*), pointing to a scenario in which, due to the low HPL activity, the fatty acid hydroperoxides are accumulated in the plant tissue and then excised by endogenous LOXs that would compete for the substrates with the residual lyase enzyme. In this regard, pentan-1-ol was probably a product of the lysis of the 13-hydroperoxylinoleic acid, a process that has been reported to yield *n*-pentane as the main volatile product (*33*). This theory is in agreement with the higher content of lipid peroxides found in the H57 potato leaves (*34*). **Table 2** shows that the total content of volatiles is lower in line H57 than in WT. This agrees with the fact that H57 displayed a strong inhibition of HPL. On the other hand, the contents of these volatiles in the H33 line were, as expected, much lower than those in the lines having an active LOX H1.

Observations on the ratios C6/C5 versus C5/C6 were checked by analyzing the volatiles not only from the leaf homogenates but also from leaf disks, although the content of C6 compounds from the latter material was higher (mostly due to *Z*-3-hexenol)

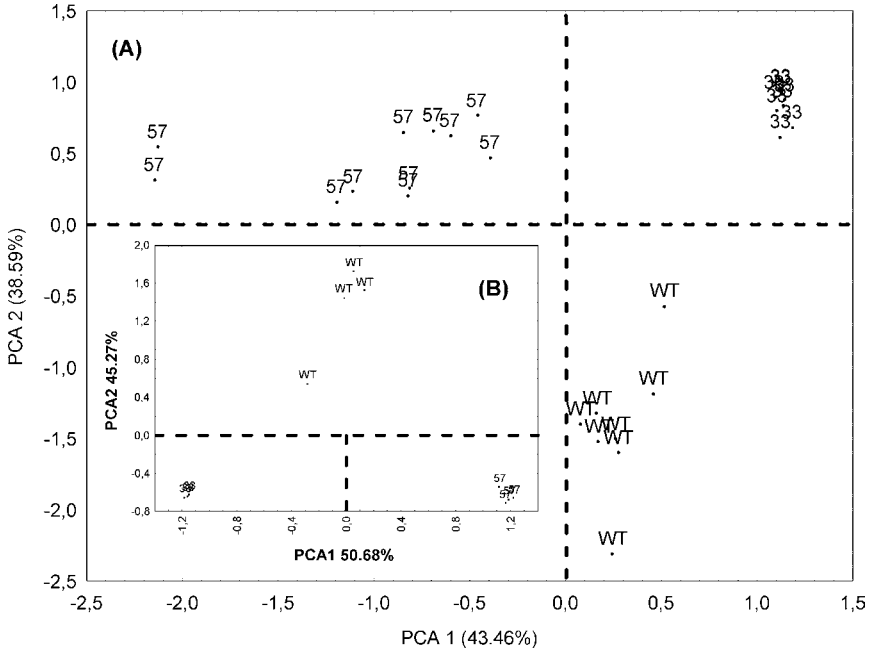

Figure 2. Principal component analysis: differentiation among potato plants by means of the C5 and C6 volatile compounds produced by the samples of leaf disks (**A**) and leaf homogenates (**B**). Percentages in parentheses indicate the variance explained by each principal component. WT, wild-type or control plant; 33, LOX H1-depleted transgenic plant; 57, HPL-depleted transgenic plant.

than from leaf homogenates. Although all of the potato leaves were selected after a careful physical inspection, the set of samples from H57 transgenic plant was not homogeneous in terms of their chemical composition. Fortunately, **Figure 2** shows that, using the whole set of compounds described in **Table 1**, the lack of homogeneity did not mask a clear differentiation among the potato plants. Besides application of the multivariate statistical procedure of principal components, the univariate statistical analysis based on the Brown−Forsythe test also allowed determination of the volatile compounds that were able to discriminate between the potato plants simultaneously. Thus, all of the C5 compounds plus *Z*-3-hexenyl acetate, quantified from the leaf disks, discriminated the three potato lines, whereas only three compounds (2-pentenal, hexanal, and *Z*-3-hexenol) were able to discriminate the potato plants from the volatiles of the leaf homogenates.

An analysis of the metabolism of the nonvolatile compounds of the linoleic acid degradation through the LOX pathway could contribute to the understanding of the particular profile of volatiles of H57 transgenic plant. Previous studies have pointed out that the preferential place for the metabolism of oxylipins is the plastids or chloroplasts (*35*). Thus, leaf homogenates from the three potato lines, not subjected to any differential centrifugation step, were incubated with [1-^{14}C]18:2. Linoleic acid was a substrate metabolized in the same way as its homologous linolenic acid, producing the same nonvolatile products after oxygenation and cleavage by the action of LOX and HPL. This precursor had the advantage of being more stable against autoxidation, which avoids possible artifacts in the labeling experiments. The formation of the nonvolatile intermediates of this pathway, hydroperoxides and oxoacids, was monitored to assess the effect caused by depletion of the different enzymes. The exogenous linoleic acid was efficiently metabolized by the homogenates prepared from H57 or WT plants (**Figure 3A**). Only 5% of the total radiolabeled precursor was consumed by the homogenate of the H33 line after a 60 min incubation. The generated products, at similar amounts, were hydroperoxides and the 12-oxododec-9(*Z*)-enoic acid (**Figure 3A**,**B**). The profile obtained from the WT line was quantitatively different from the one displayed by the LOX-deficient H33 line. Thus, the linoleic acid precursor was readily oxidized by WT homogenates to yield fatty acid hydroperoxides as the main product, accounting for >35% of the label after incubation for 60 min. The fraction of oxoacids, which consisted of 12-oxododec-9(*Z*)-enoic acid, increased during the incubation time up to a final incorporation of 5% of the initial precursor (**Figure 3B**). The line metabolizing the 18:2 precursor at a higher rate was, unexpectedly, H57 (**Figure 3A**). This line oxidized the fatty acid precursor at a rate that was 4-fold higher than that in the WT line at short incubation times. This phenomenon paralleled an equivalent increment in the hydroperoxides, which reached >50% of the total label at long incubation times. The oxoacid profiles displayed by line H57 were also different from those of WT and H33 lines. The band corresponding to these compounds appeared to be split into two, one matching the 12-oxododec-9(*Z*)-enoic acid standard and the other matching the 13-oxotridecadi-9(*Z*),11(*E*)-enoic acid that is produced via LOX homolytic cleavage of 13-hydroperoxides. As expected, the incorporation into 12-oxododec-9(*Z*)-enoic acid was lower in the H57 line than in the WT, where no labeling into 13-oxoacids was found. The most remarkable information obtained from this experiment was the important increase of LOX activity that took place when HPL was depleted. In this regard, LOX activity in the leaf homogenates of the three potato lines was also assayed to discard any artifact in the labeling experiments such as a possible chemical autoxidation of the precursor. LOX in leaf tissue was mainly bound to a particulate fraction that hampered the classical spectrophotometric method of LOX assay. Nevertheless, it was possible to measure the activity of this enzyme by means of an oxygen electrode that was not altered by the turbidity of the assay mixture. The LOX activity found in the leaf homogenates was in agreement with the labeling experiments. This activity was barely detectable in the H33 line, whereas it reached values on the order of 12 nkat/g of fresh weight in the WT line. Furthermore, this line displayed similar activities toward the two fatty acids assayed (**Table 3**). The

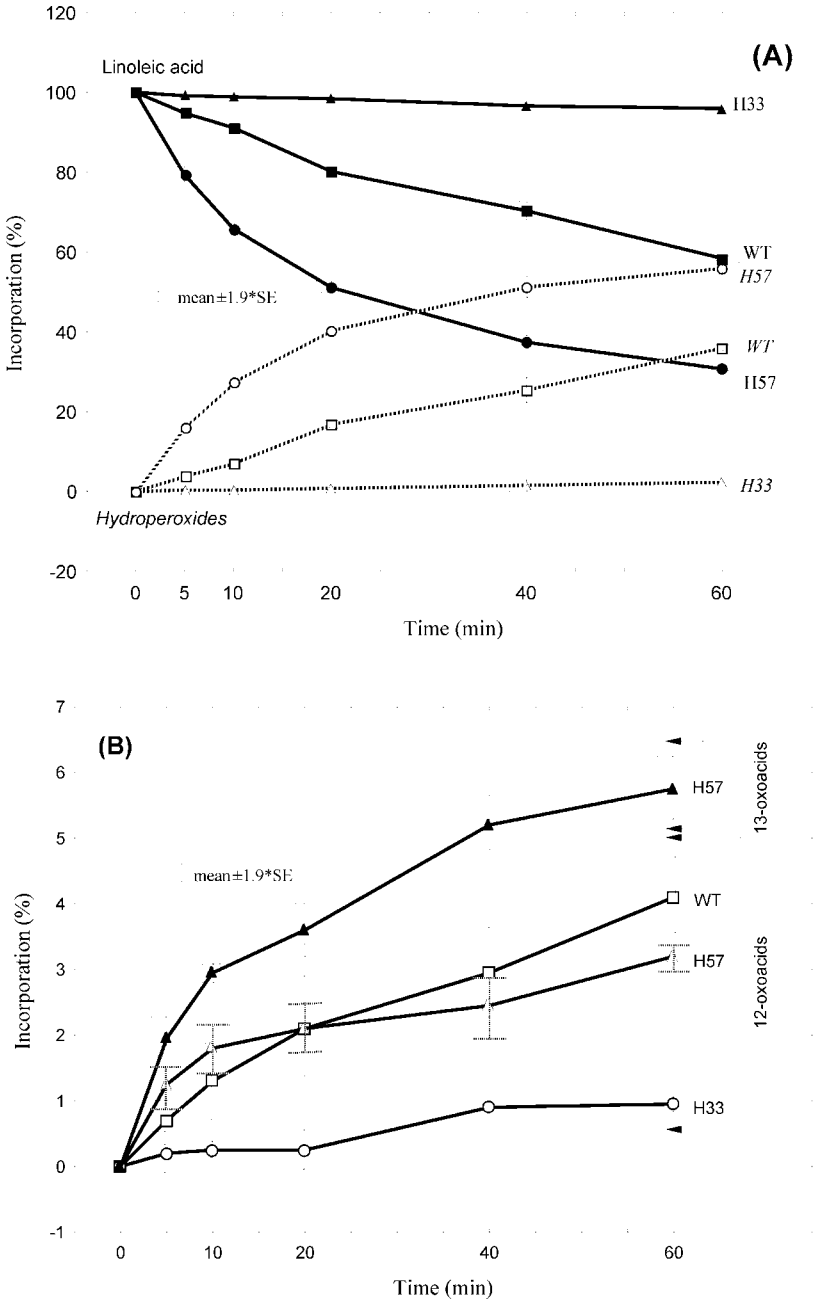

Figure 3. Courses of radiolabeled linoleate metabolization by leaf homogenates from the H33, WT, and H57 potato plants: (**A**) percentages of remnant substrate linoleic acid and fatty acid hydroperoxides; (**B**) percentages of 12-oxododec-9(*Z*)-enoic and 13-oxotridecadi-9(*Z*),11(*E*)-enoic acids.

Table 3. Lipoxygenase Activity (Mean ± Standard Error) in Leaf Homogenates from H57, WT, and H33 Potato Mutants with Linoleic and Linolenic Substrates

	substrate	LOX activity (nkat/g of fresh wt)
H57	18:2	93.7 ± 3.6
	18:3	252.3 ± 8.55
WT	18:2	12.6 ± 0.3
	18:3	12.9 ± 0.3
H33	18:2	1.2 ± 0.1
	18:3	1.1 ± 0.1

activity in the H57 homogenates was the highest among the lines assayed. It was 7-fold higher than in WT homogenates when the linoleic acid was used as substrate and 20-fold higher when the substrate was the linolenic acid. These results confirm that there was an induction of LOX activity when the HPL expression was suppressed and also the preference for the linolenate peroxidation displayed by LOX induced in the H57 plant. This induction of LOX, which was not reported in the molecular characterization of the mutant (*25*), gives information about a concerted regulation of HPL and the different LOXs present in the potato leaves. The increment of LOX also contributed to the increment of the concentration of hydroperoxides in the tissues of the potato H57 mutant described previously (*34*). It could have induced an increment of the rate of homolytic cleavage of hydroperoxides, which would explain the unexpectedly high content of C5 volatile compounds found in that line.

The depletion of LOX H1 and HPL enzymes also affects the aroma of the potato leaves through the changes in the concentration of the volatile compounds. The small amounts of volatile compounds that are generated in the H33 line meant that the aroma of the leaves stayed below the perception threshold (*36*),

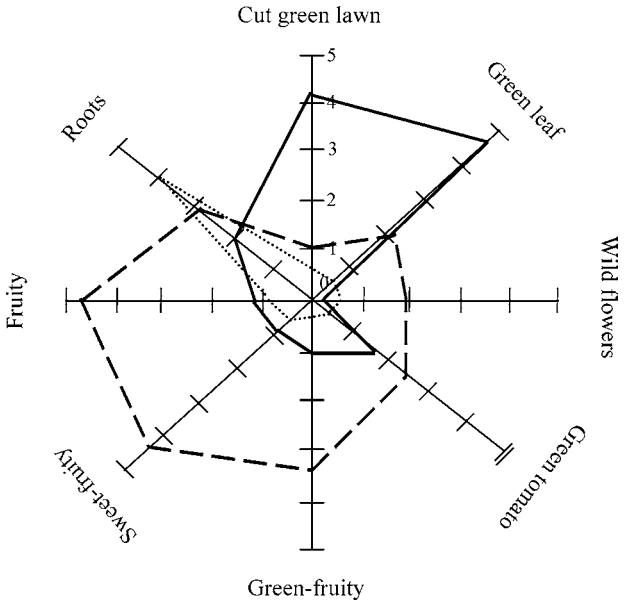

Figure 4. Scores of the sensory notes evaluated by assessors in the disks of potato leaves of WT (solid line), H33 (dotted line), and H57 (dashed line) potato plants. Each axis shows the detection level of each selected sensory attribute expressed in a numerical interval from 0 to 5: 0, not detectable; 1, just detectable; 2, slightly strong; 3, moderately strong; 4, strong; 5, very strong.

giving way to odorless potato leaves. This result showed that, in general, LOX activity in the tissues limits the total amount of volatile presents in them, and it agrees with previous works in which off-flavors of soybean products were removed by the suppression of the soybean LOX-2 activity (*18*, *19*). These studies confirm that there are specific forms of LOX involved in the aroma biosynthesis, whereas others contribute only to the metabolism of jasmonic acid (*24*). The other studied lines produced aromas with important differences concerning the sensory notes. The higher concentration of C5-alcohols present in H57 increased the sweet-fruity notes of the aroma generated by the leaves (**Figure 4**), which contrasted with the predominantly green notes found in the aroma of the WT line. Therefore, this study showed that it is possible to change the flavor of the vegetables by modifying the activity level of the different enzymes of the LOX pathway. The reduction of LOX H1 activity was especially determinant on the concentration of the chemical compounds responsible for the aroma, whereas HPL activity depletion exerted an important impact not only on the GC peak sizes but also on the strength of the green sensory notes. Nevertheless, the studies of the linoleic acid metabolism and the measurements of LOX activity in the three lines indicated that there was an important increase of the LOX activity of the leaf tissue concomitant with the HPL depletion, which could be a physiological response of the plant intended to increase the performance against pathogen attack caused by the depletion of HPL (*25*). In this regard, it would be of great interest to investigate the level of regulation at which this induction occurs, as well as the form of LOX induced.

LITERATURE CITED

(1) Buttery, R. G.; Teranishi, R.; Ling, L. C. Fresh tomato aroma volatiles: A quantitative study. *J. Agric. Food Chem.* **1987**, *35*, 540−544.

(2) Dimick, P. S.; Hoskin, J. C. Review of apple flavour—state of the art. *Crit. Rev. Food Sci. Nutr.* **1983**, *18*, 387−409.

(3) Morales, M. T.; Alonso, M. V.; Rios, J. J.; Aparicio, R. Virgin olive oil aroma: relationship between volatile compounds and sensory attributes by chemometrics. *J. Agric. Food Chem.* **1995**, *43*, 2925−2931.

(4) Hatanaka, A.; Kajiwara, T.; Sekiya, J. Biosynthetic pathway for C6-aldehydes formation from linolenic acid in green leaves. *Chem. Phys. Lipids* **1987**, *44*, 341−361.

(5) Morales, M. T.; Aparicio, R.; Calvente, J. J. Influence of olive ripeness on the concentration of green aroma compounds in virgin olive oil. *Flavour Fragrance J.* **1996**, *11*, 171−178.

(6) Hatanaka, A. The biogeneration of green odour by green leaves. *Phytochemistry* **1993**, *34*, 1201−1218.

(7) Matsui, K.; Kurishita, A.; Hisamitsu, A.; Kajiwara, T. A lipid-hydrolysing activity involved in hexenal formation. *Biochem. Soc. Trans.* **2000**, *28*, 857−860.

(8) Vick, B. A. Oxygenated fatty acids of the lipoxygenase pathway. In *Lipid Metabolism in Plants*; Moore, T. S. J., Ed.; CRC: Boca Raton, FL, 1993; pp 167−191.

(9) Noordermeer, M. A.; Veldink, G. A.; Vliegenthart, J. F. Fatty acid hydroperoxide lyase: a plant cytochrome P450 enzyme involved in wound healing and pest resistance. *Chembiochem.* **2001**, *2*, 494−504.

(10) Matsui, K.; Toyota, H.; Kajiwara, T.; Kakuno, T.; Hatanaka, A. Fatty acid hydroperoxide cleaving enzyme, hydroperoxide lyase, from tea leaves. *Phytochemistry* **1991**, *30*, 2109−2113.

(11) Shibata, Y.; Matsui, K.; Kajiwara, T.; Hatanaka, A. Purification and properties of fatty acid hydroperoxide lyase from green bell pepper fruit. *Plant Cell Physiol.* **1995**, *36*, 147−156.

(12) Bate, N. J.; Sivasankar, S.; Moxon, C.; Riley, J. M. C.; Thompson, J. E.; Rothstein, S. J. Molecular characterization of an *Arabidopsis* gene encoding hydroperoxide lyase, a cytochrome P-450 that is wound inducible. *Plant Physiol.* **1998**, *117*, 1393−1400.

(13) Matsui, K.; Ujita, C.; Fujimoto, S.; Wilkinson, J.; Hiatt, B.; Knauf, V.; Kajiwara, T.; Feussner, I. Fatty acid 9- and 13-hydroperoxide lyases from cucumber. *FEBS Lett.* **2000**, *481*, 183−188.

(14) Phillips, D. R.; Matthew, J. A.; Reynolds, J.; Fenwick, G. R. Partial purification and properties of a *cis*-:*trans*-2-enal isomerase from cucumber fruit. *Phytochemistry* **1979**, *18*, 401−404.

(15) Hatanaka, A.; Kajiwara, T.; Matsui, K.; Matsunaga, T. Non-enzymatic isomerization of 12-hydroxy-3(*Z*)-dodecenal to the 2(*E*)-isomer after enzymatic cleavage of 13-hydroperoxylinoleil alcohol in tea chloroplasts. *Z. Naturforsch.* **1989**, *44*, 161−164.

(16) Salas, J. J.; Sánchez J. Alcohol dehydrogenases from olive (*Olea europaea*) fruit. *Phytochemistry* **1998**, *48*, 35−40.

(17) Harada, M.; Ueda, Y.; Iwata, T. Purification and some properties of alcohol acetyltransferase from banana fruti. *Plant Cell Physiol.* **1985**, *26*, 1067−1074.

(18) Davies, C. S.; Nielsen, S. S.; Nielsen, N. C. Flavor improvement of soybean preparations by genetic removal of lipoxygense-2. *J. Am. Oil Chem. Soc.* **1987**, *64*, 1428−1432.

(19) Torres-Penaranda, A. V.; Reitmeier, C. A.; Wilson, L. A.; Fehr, W. R.; Narvel, J. M. Sensory characteristics of soymilk and tofu made from lipoxygenase-free and normal soybeans. *J. Food Sci.* **1998**, *63*, 1084−1087.

(20) Speirs, J.; Lee, E.; Holt, K.; Yong-Duk, K.; Scott, N. S.; Loveys, B.; Schuch, W. Genetic manipulation of alcohol dehydrogenase levels in ripening tomato fruits affects the balance of some flavour aldehydes and alcohols. *Plant Physiol.* **1998**, *117*, 1047−1058.

(21) Matsui, K.; Fukutomi, S.; Wilkinson, J.; Hiatt, B.; Knauf, V.; Kajiwara, T. Effect of overexpression of fatty acid 9-hydroperoxide lyase in tomatoes (*Lycopersicon esculentum* Mill.). *J. Agric. Food Chem.* **2001**, *49*, 5418−5424.

(22) Aparicio, R.; Morales, M. T.; Luna, G.; Aparicio-Ruiz, R. Biochemistry and chemistry of volatile compounds affecting consumers' attitudes toward virgin olive oil. In *Flavour and Fragrance Chemistry*; Lanzotti, V., Taglialatela-Scafati, O., Eds.; Kluwer Academic Publishers: London, U.K., 2000; pp 3−14.

(23) Anthon, G. E.; Barrett, D. M. Thermal inactivation of lipoxygenase and hydroperoxytrienoic acid lyase in tomatoes. *Food Chem*. **2003**, *81*, 275−279.
(24) León, J.; Royo, J.; Vancanney, G.; Sanz, C.; Silkowski, H.; Griffiths, G.; Sánchez-Serrano, J. J. Lipoxygense H1 gene silencing reveals a specific role in supplying fatty acid hydroperoxides for aliphatic aldehyde production. *J. Biol. Chem.* **2002**, *277*, 416−423.
(25) Vancanney, G.; Sanz, C.; Farmaki, T.; Paneque, M.; Ortego, O.; Castañera, P.; Sánchez-Serrano, J. J. Hydroperoxide lyase depletion in transgenic potato plants leads to an increase in aphid performance. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 8139−8144.
(26) Salch, Y. P.; Grove, M. J.; Takamura, H.; Gardner, H. W. Characterization of a C-5,13-cleaving enzyme of 13(*S*)-hydroperoxide of linolenic acid by soybean seed. *Plant Physiol.* **1995**, *108*, 1211−1218.
(27) Hara, A.; Radin, N. S. Lipid extraction of tissues with a low-toxicity solvent. *Anal. Biochem.* **1978**, *90*, 420−426.
(28) Vicchi, S.; Castellote, A. I.; Pizzale, L.; Conte, L. S.; Buxaderas, S.; López-Tamames, E. Analysis of virgin olive oil volatile compounds by headspace solid-phase microextraction coupled to gas chromatography with mass spectrometric and flame ionization detection. *J. Chromatogr. A* **2003**, *983*, 19−33.
(29) Morales, M. T.; Tsimidou, M. The role of volatile compounds and polyphenols in olive oil sensory quality. In *Handbook of Olive Oil: Analysis and Properties*; Harwood, J., Aparicio, R., Eds.; Aspen Publications: Gaithersburg, MD, 2000; pp 393−458.
(30) Land, D. G.; Shepherd, R. Scaling and ranking methods. In *Sensory Analysis of Foods*; Piggott, J. R., Ed.; Elsevier: London, U.K., 1988; pp 155−185.
(31) Olejnik, S. F.; Algina, J. Type I error rates and power estimates of selected parametric and nonparametric tests of scale. *J. Educ. Stat.* **1987**, *12*, 45−61.
(32) Sánchez, J.; Salas, J. J. Biogenesis of the olive oil aroma. In *Handbook of Olive Oil: Analysis and Properties*; Harwood, J., Aparicio, R., Eds.; Aspen Publications: Gaithersburg, MD, 2000; pp 79−99.
(33) Garssen, G. J.; Vliegenthart, J. F. G.; Boldingh, J. An anaerobic reaction between lipoxygenase, linoleic acid and its hydroperoxides. *Biochem. J.* **1971**, *122*, 327−332.
(34) Griffiths, G.; Leverentz, M.; Silkowski, H.; Gill, N.; Sánchez-Serrano, J. J. Lipid hydroperoxide levels in plant tissues. *J. Exp. Bot.* **2000**, *51*, 1363−1370.
(35) Bachmann, A.; Hause, B.; Maucher, H.; Garbe, E.; Voros, K.; Weichert, H.; Wasternack, C.; Feussner, I. Jasmonate-induced lipid peroxidation in barley leaves initiated by distinct 13-LOX forms of chloroplasts. *Biol. Chem.* **2002**, *383*, 1645−57.
(36) Luna Sánchez, G. Characterization of European virgin olive oils: implications of sensory analysis and consumers' attitudes. Doctorate Thesis, University of Seville, Spain, 2003.

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