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A Simple and Efficient System for Green Note Compound Biogenesis by Use of Certain Lipoxygenase and Hydroperoxide Lyase Sources

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Six-carbon (C_6) aldehydes and alcohols are important components of the aroma and flavor of fruits and vegetables. Soybean lipoxygenase (LOX) isozyme LOX 3 was reported not only to produce less 13-hydroperoxides, precursors of C_6 aldehydes, but also to convert them to ketodiene products. Here, we examined the effects of LOX 3 on hexenal formation from linolenic acid homogenized with watermelon 13-hydroperoxide lyase (HL)-overexpressing *Nicotiana tabacum* leaves and soybean acetone powder. Compared to the wild type, which contains LOXs 1, 2, and 3, the elimination of LOX 3 in LOX 1 + 2 facilitates greater production of hexenals. The use of LOX 2 alone yielded the highest hexenal production, while a two-step conversion was required for LOX 1 to produce hexenals at high levels due to different pH optima of the enzymes involved. These results clearly demonstrate that the soybeans lacking LOX 3 in combination with watermelon HL-overexpressing leaf tissues greatly enhance hexenal formation.

KEYWORDS: Hydroperoxide lyase; hexenal; lipoxygenase; LOX 3; linolenic acid hydroperoxide; watermelon; *Citrullus lanatus*; soybean; *Glycine max*

INTRODUCTION

Six-carbon (C_6) aldehydes and alcohols are a major group of bioactive, oxygenated lipid metabolites called oxylipins (*1*, *2*). These volatile compounds such as leaf aldehyde [(2*E*)-hexenal] and leaf alcohol [(3*Z*)-hexenol] are characteristic of the fresh, green odor of cut leaves and important components of the aroma and flavor of fruits and vegetables. Some of the C_6 aldehydes and alcohols have also been known to have antimicrobial and antifungal properties (*3*−*6*) as well as negative effects on arthropods directly and indirectly (*7*−*9*). Recently, these volatiles were also reported to induce defense-related genes in plants (*10*, *11*) as well as the emission of other volatiles (*12*, *13*).

The enzymes responsible for the production of these compounds are a lipoxygenase (LOX) and a fatty acid hydroperoxide lyase (HL). LOXs are a class of enzymes, widely distributed in both the plant and animal kingdoms, which oxidize unsaturated fatty acids containing a *cis*,*cis*-1,4-pentadiene system such as found in linoleic (18:2), linolenic (18:3), and arachidonic acids to produce hydroperoxides (*14*−*17*). From these hydroperoxides, HL forms very unstable hemiacetals generating aldehydes and aldehyde enols by spontaneous dissociation (*18*). C_6 aldehydes and alcohols, mentioned in the beginning, are produced by 13-HLs from 13-hydroperoxy C_{18} fatty acids. On the other hand, 9-hydroperoxy fatty acids are converted by 9- or 9/13-HLs to nine-carbon aldehydes and alcohols which are characteristic flavors of certain fruits such as cucumber (*19*−*21*).

Due to their "green" organoleptic characteristics, C_6 aldehydes and alcohols are often called "green notes" and are widely used as flavors in foods and beverages (*22*). Though these compounds can be synthesized chemically, a higher demand for "natural" compounds is driving research on the biocatalytic production of these chemicals. Many plant species were surveyed for their C_6 aldehyde formation ability (*22*−*24*), and several plant species were explored for large-scale production of green note compounds with or without addition of soy flour as a source of LOX (*22*, *25*−*29*). Plant cell cultures (*30*, *31*) as well as purified enzymes (*32*−*34*) were also evaluated, but low production and/or high cost deemed them uneconomical. Recombinant enzymes expressed in yeast or bacteria showed some promise (*35*−*37*). However, producing proteins/enzymes in plants has an advantage of lower production cost (*38*).

In soybean [*Glycine max* (L.) Merr.] seeds, three distinct isoforms of LOXs have been described, on the basis of differences in pH optima, substrate specificity, and their product formation (*39*). LOX type 1 (LOX 1) has a pH optimum of 9.0, producing 13(*S*)-hydroperoxylinoleic acid (HPOD) as the major product from 18:2 (*40*). LOX type 2 (LOX 2) with a pH optimum of 6.1 was said to form almost equal proportions of 9- and 13-HPODs. Finally LOX type 3 (LOX 3) with a pH optimum of 6.5 produces approximately 65% and 35% 9- and 13-HPODs, respectively (*39*), while no single stereoisomer was dominant (*41*). Though LOX 1 produces 13(*S*)-HPOD predominantly near its optimal pH, the percentage of 9-HPOD increases as the pH decreases, reaching about 20−23% around pH 7 (*42*−*44*). As for LOX 2, Christopher et al. (*45*) showed the ratio of 13-HPOD to 9-HPOD from LOX 2 reactions ranged from 30:70 to 62:38 depending on the conditions employed. Haslbeck and Grosch (*46*) argued that, at concentrations of 18:2 above

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100 μM, the product specificity of LOX 2 from 18:2 is more similar to the results from autoxidation due to the reaction between excess 18:2 and released hydroperoxy radicals. When 3.21 μM 18:2 was used as the substrate, LOX 2 produced 13-(*S*)-HPOD predominantly at about 75% of the total HPOD (*41*).

LOX 3, on the other hand, was reported to produce more 9-HPOD than 13-HPOD (*39*). Similar results were also obtained in our laboratory (*41*). Additionally, LOX 3 preparations have been reported to transform the primary hydroperoxy products into ketodiene products, which are not substrates of HLs (*39*). We have suggested that soybean LOX 3 reduces hexanal formation by converting 13- HPOD into forms unavailable for conversion to hexanal (*47*). Takamura et al. (*48*) also showed low hexanal formation from an −L1L2 soybean mutant line which only contains LOX 3.

In this study, we showed the significant effect of LOX 3 in the reduction of hexenal formation from 18:3 homogenized with HL-rich leaf tissues and an acetone powder of LOX-null mutant soybeans. Overexpression of watermelon HL in *Nicotiana tabacum* was shown to yield a high level of its enzymatic activity. Combined together, this system has potential for producing green note compounds on a large scale.

MATERIALS AND METHODS

Plant Material. The soybean [*G. max* (L.) Merr.] LOX mutant lines −L2L3, −L1L3, and −L3 backcrossed to Century were provided by Niels Nielsen, Purdue University (*49*). The −L2L3 line is a null for LOXs 2 and 3 (but has the normal wild-type level of LOX 1), the −L1L3 line only contains LOX 2, and −L3 lacks LOX 3. Century seeds contain LOXs 1, 2, and 3.

The soybean seed acetone powder was prepared as follows: Seeds were ground in a coffee grinder to a fine flour. The flour was defatted by washing with an ample volume of cold acetone (−20 °C) followed by an ample volume of cold diethyl ether (−20 °C). The defatted flour was dried with nitrogen gas.

Transgenic *N. tabacum* L. cv. "Petite Havana" plants overexpressing a watermelon (*Citrullus lanatus*) HL cDNA (GenBank Accession No. AY703450) with an enhanced CMV 35S promoter were created using *Agrobacterium*-mediated transformation previously (*50*). Line no. 16 used in the experiments described here had 50 times more HL activity than wild-type *N. tabacum* plants. The transgenic plants were grown on a shelf by a north-facing window in a room maintained at 25 ± 3 °C and supplemented with fluorescent lighting.

LOX Enzyme Assay. The LOX activity at pH 6 was analyzed using a modified protocol from Axelrod et al. (*39*). The acetone powder (100 mg) from soybean mutant lines as well as the wild type was extracted with 1.0 mL of 50 mM sodium phosphate buffer, pH 6.8, for 20 min on ice with shaking. The slurry was filtered through a layer of Miracloth, and the filtrate was centrifuged for 1 min at 6000*g* at 4 °C. The supernatants were used for the assay. The assay was carried out with 400 μM 18:3 in 50 mM phosphate buffer, pH 6.0. The hydroperoxide formation was measured spectrophotometrically at 235 nm with an extinction coefficient of 23000 M^{-1} cm^{-1}.

LOX Product Formation. A 2 mg sample of 18:3 in 20 μL of ethanol was homogenized with 0, 2, 5, or 10 mg dry weight (dw) of soybean acetone powder (−L3) in 2.0 mL of water for 2.0 min at room temperature using a Tissumizer (Tekmar, Cincinnati, OH) at 50% output. The reaction mixture was left standing for 2.0 min and the reaction stopped by addition of 5 drops of 1 M citric acid solution. A 1 mg sample of heptadecanoic acid in 20 μL of ethanol was added as an internal standard. The reaction products were extracted with 1 mL of methyl *tert*-butyl ether (MTBE) after addition of 1 g of NaCl to reduce the water content in the organic phase. The MTBE extracts were treated with triphenylphosphine for 40 min at room temperature to reduce hydroperoxides into hydroxides. The solvent was dried by a stream of nitrogen gas, and the residues were methylated with ethereal diazomethane. Excess diazomethane and ether were dried again, and the residues were dissolved in 1.0 mL of hexane with 0.001% butylated hydroxytoluene.

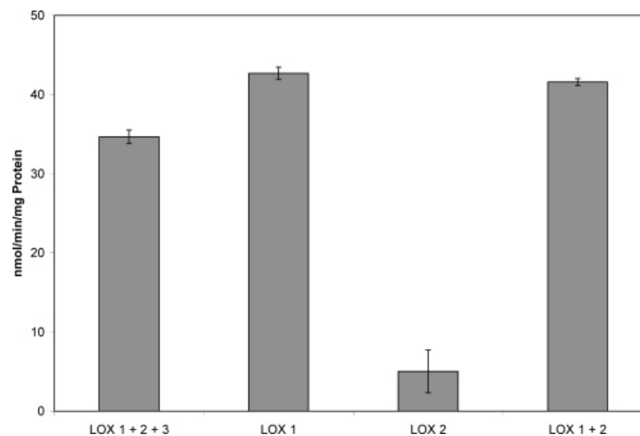

Figure 1. Lipoxygenase activity at pH 6.0 among soybean mutant lines and the wild type. LOX 1 + 2 + 3 is a Century variety which contains all three isozymes (LOXs 1, 2, and 3). LOX 1 is the –L2L3 line null for LOXs 2 and 3, LOX 2 is the –L1L3 line null for LOXs 1 and 3, and LOX 1 + 2 is the –L3 line lacking LOX 3. Each value is the average and standard error of three replications.

GC Analysis of LOX Products. The reaction products were analyzed with gas chromatography−mass spectrometry (GC−MS) using a GCD Plus (Agilent, Palo Alto, CA). The GC instrument was equipped with an HP-5 capillary column (cross-linked 5% phenylmethylsiloxane, 0.25 mm internal diameter × 30 m length), and the temperature program was as follows: 1 min at 120 °C and then increased to 275 °C at 5 °C/min. The mass spectra were acquired with a range between *m*/*z* 65 and *m*/*z* 450, and the electron impact source was operated at 70 eV.

C_6 Aldehyde Production. For the initial experiment, the 18:3 substrate was homogenized with the soybean acetone powder and watermelon HL-overexpressing leaf tissues in water. A 5 mg sample of 18:3 in 50 μL of ethanol, 25 mg of soybean acetone powder, and 50 mg fresh weight (fw) of leaf tissues were homogenized in 2.0 mL of water for 2.0 min at room temperature as described above. The reaction mixture was left standing for 2.0 min and the reaction stopped by addition of 5 drops of 1 M citric acid and 1.0 g of NaCl. A 2 μmol sample of (2*E*)-heptenal in 20 μL of ethanol was added as an internal standard, and the reaction products were extracted with 1 mL of pentane.

Since the optimal pH of watermelon HL is different from that of soybean seed LOX 1, a two-step C_6 aldehyde formation protocol was required for LOX 1. Therefore, C_6 aldehyde formation was conducted in either a two-step production for the LOX 1 or a single homogenization of all components for LOX 2 for comparison. For LOX 1, 2.0 mg of 18:3 in 20 μL of ethanol and 10 mg of soybean acetone powder were homogenized in 2.0 mL of 50 mM K_2HPO_4 (measured pH of 8.7) for 1.0 min as described above. The pH was lowered to about pH 6.4 by the addition of 0.5 mL of 840 mM KH_2PO_4. A 20 mg sample of the leaf tissues was then added and the resulting mixture further homogenized for 2.0 min. The reaction mixture was left standing for 2.0 min, and the reaction products were extracted as described above. For LOX 2, 2.0 mg of 18:3 was homogenized with 10 mg of soybean acetone powder and 20 mg of the leaf tissues in 2.5 mL of 100 mM phosphate buffer, pH 6.0, for 2.0 min. The soybean acetone powder from the wild-type Century as well as the LOX 3-null line was homogenized both sequentially like LOX 1 and simultaneously like LOX 2.

GC Analysis of Volatiles. The reaction products were analyzed with the GCD Plus. The temperature program was as follows: 5 min at 40 °C, then increased to 80 °C at 2.0 °C/min, and further increased to 260 °C at 30 °C/min. The mass spectra were acquired with a range between *m*/*z* 35 and *m*/*z* 150.

RESULTS

Initially, LOX activity among mutant lines and the wild type was assayed to determine the quality of the acetone powder (**Figure 1**). There was no difference between LOX 1 and LOX 1 + 2. LOX 1 + 2 + 3 had statistically lower activity, but the

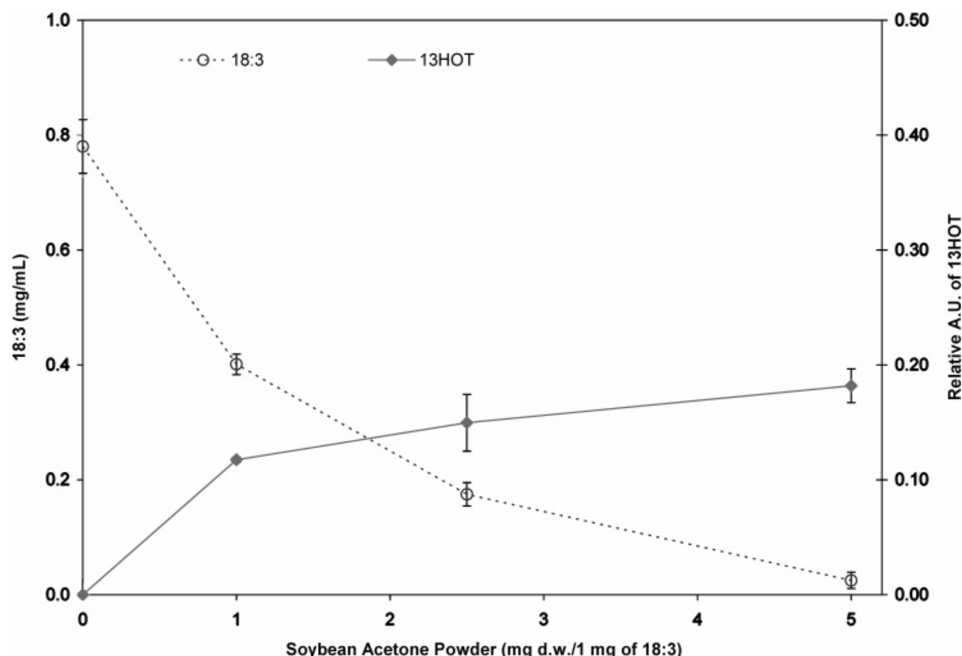

Figure 2. Conversion of linolenic acid into 13-hydroperoxylinolenic acid by various amounts of soybean acetone powder. A 0–10 mg dry weight sample of soybean acetone powder (LOX 1 + 2) was homogenized with 2.0 mg of 18:3 in 2.0 mL of H_2O and then left standing for 2 min. The 13-hydroperoxylinolenic acid formed was reduced to 13-hydroxylinolenic acid (13HOT) by triphenylphosphine. The values for 13HOT methyl ester were expressed as their area units over the area units of the internal standard heptadecanoic acid methyl ester multiplied by the concentration (mg/mL) of the latter. Each value is the average and standard error of three replications.

difference was small compared to the activity found in LOX 2. LOX 2, a rather unstable enzyme, might be inactivated during the extraction procedure we employed (*14*).

Next, the amount of soybean acetone powder required for the efficient production of HPOT was evaluated to optimize production. The analysis showed that 10 mg dw of acetone powder (LOX 1 + 2) is required to convert most of the 2 mg of 18:3 supplied in the 4 min period examined (**Figure 2**). The measurable 13-hydroxy-18:3 (13HOT) did not increase as much as the loss of 18:3. No ketodienes were detectable in the reaction products.

To evaluate the effect of soybean seed LOX isozymes on the production of hexenals by HL from 18:3, LOXs 1 and 2 as well as the wild type and a LOX 3-null line (containing both LOXs 1 and 2) were homogenized with 18:3 and watermelon HL-overexpressing leaf tissues in nonbuffered water. In all of the experiments, (3*Z*)-hexenal was the dominant product with a minor production of (2*E*)-hexenal. No hexenols could be detected possibly due to the short reaction period employed and the small amount of leaf tissues used. Therefore, the combined amount of (3*Z*)-hexenal and (2*E*)-hexenal was used as C_6 aldehyde product levels. As illustrated in **Figure 3**, the soybean line with LOX 2 only (−L1L3) and leaf tissues expressing watermelon HL produced the highest level of C_6 aldehydes from 18:3 followed by the combination including the soybean line containing LOX 1 + 2 (−L3). On the other hand, the reactions containing LOX 1 + 2 + 3 (Century) and the line with LOX 1 only (−L2L3) yielded about 4% and 10% C_6 aldehyde levels compared to that containing the LOX 2 line, respectively.

Since LOX 1 has a pH optimum of 9 (*39*) compared to the pH optimum of 6.2 for watermelon HL (*50*), a two-step hexenal formation is required for LOX 1. Therefore, to verify the effect of LOX isozyme homogenized in optimal pH conditions, hexenal formation with LOX 1 was conducted in two steps, with the first LOX reaction at pH 9 followed by the second HL reaction at pH 6, while the formation with LOX 2 was done with the combined reaction at pH 6. As illustrated in **Figure 4**, LOX 2 (−L1L3) at pH 6 produced the highest level of C_6 aldehydes, followed by LOX 1 at pH 9 and LOX 1 + 2 at pH 6 although there were no significant differences among them. On the other hand, the Century acetone powder, which contains LOX 3 along with LOXs 1 and 2, reduced the C_6 aldehyde production to about 10% of the level produced from LOX 2 or LOX 1 alone.

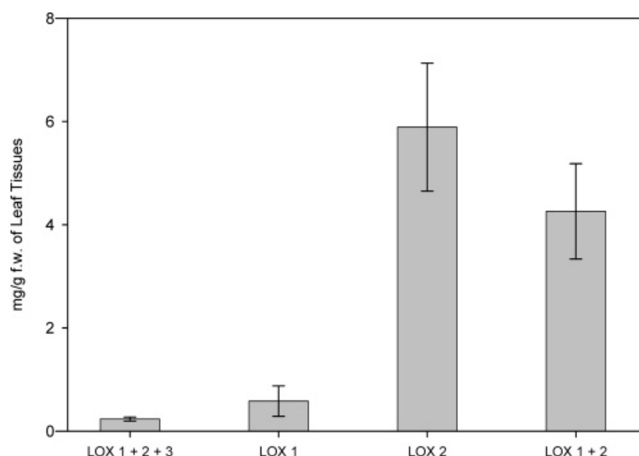

Figure 3. Comparison of the C_6 aldehyde production from linolenic acid homogenized with watermelon hydroperoxide lyase-overexpressing *Nicotiana* leaves and acetone powder from soybean lipoxygenase isozyme mutant lines in water. A 5 mg sample of 18:3, 25 mg of soybean acetone powder, and 50 mg fw of leaf tissues were homogenized in 2.0 mL of water for 2.0 min at room temperature. Each value is the average and standard error of three replications. For the explanation of mutant lines, see the caption of **Figure 1**.

As shown in **Table 1**, the conversion of 18:3 to hexenals was 50.3% when 18:3 was homogenized with LOX 2 and watermelon HL-overexpressing leaf tissues in 100 mM phosphate buffer, pH 6.0.

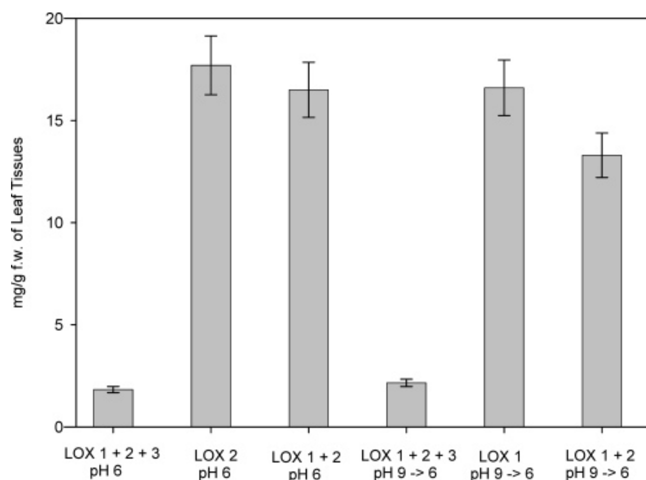

Figure 4. Comparison of C_6 aldehyde production from linolenic acid homogenized with watermelon hydroperoxide lyase-overexpressing *Nicotiana* leaves and acetone powder from soybean lipoxygenase isozyme mutant lines in buffered solutions. For the left half of the data, 2.0 mg of 18:3 was homogenized with 10 mg of soybean acetone powder and 20 mg of leaf tissues in 2.5 mL of 100 mM phosphate buffer, pH 6.0, for 2.0 min. For the right half, 18:3 and soybean acetone powder were homogenized in 2.0 mL of 50 mM K_2HPO_4 (pH 8.7) for 1.0 min, and then the pH was lowered to about 6.4 by the addition of 0.5 mL of 840 mM KH_2PO_4. The leaf tissues were promptly added, and the resulting mixture was further homogenized for 2.0 min. Each value is the average and standard error of three replications. For the explanation of mutant lines, see the caption of **Figure 1**.

Table 1. Amount Needed To Produce 1 g of Hexenals[a]

linolenic acid	5.7 g
soybean acetone powder (LOX 2)	28.3 g dw
transgenic leaf tissues	56.7 g fw
yield	50.3%

[a] The numbers were calculated from the data shown in **Figure 4**.

DISCUSSION

LOX 3 has been shown to produce more 9-HPOD than 13-HPOD, while the latter is the precursor of C_6 aldehydes and alcohols (*39*). Moreover, Axelrod et al. (*39*) reported that LOX 3 transforms the primary hydroperoxy products into ketodiene products, which are not substrates of HLs. Accordingly, low hexenal formation from soybean homogenates containing LOX 3 have been reported by addition of either LOX 3 preparation to the homogenates (*47*) or the −L1L2 soybean mutant line which only contains LOX 3 (*48*). Watermelon leaves were considered to be one of the high C_6 aldehyde producers (*24*), and we were able to clone the cDNA of HL and overexpress it in *N. tabacum* leaves (*50*). These transgenic leaves showed a 50-fold increase in HL activity compared to native HL activity, reducing the influences of other hydroperoxide-metabolizing enzymes such as allene oxide synthase and peroxidase.

The experiments presented here clearly showed that inclusion of LOX 3 significantly reduced C_6 aldehyde production from 18:3 by soybean acetone powder and the watermelon HL overexpressed in *Nicotiana* leaves (**Figures 3** and **4**). Compared to the wild-type Century, which contains all three isozymes, the elimination of LOX 3 in LOX 1 + 2 facilitated about 18 or 10 times greater production of hexenals from 18:3 and watermelon HL homogenized in water, or at pH 6. LOX 2, though shown to be unstable (**Figure 1**), showed the highest potential for providing the substrate for hexenal formation by HL in water or a buffer at pH 6 when all three components are homogenized together (**Figures 3** and **4**). For LOX 1 to produce C_6 aldehydes at levels similar to those of LOX 2, an additional step of hydroperoxide synthesis prior to the homogenization with HL was required due to the high pH needed for the optimal production of 13-HPOT with LOX 1 (**Figure 4**). This additional step requires monitoring and adjusting of the pH during the production, increasing the cost of production.

Guava fruits and/or watermelon leaves are used for the industrial process of natural green note production (*26*, *51*). From guava fruit homogenates, 9.57 mg of (3*Z*)-hexenol or 3.36 mg of (2*E*)-hexenal per gram of fresh tissues can be achieved (*26*), which is the highest production rate per HL source reported so far using plant materials. As shown in **Figure 4**, we could achieve 17.7 mg of (3*Z*)-hexenal + (2*E*)-hexenal/g fresh weight using watermelon HL-overexpressing *N. tabacum* leaves and defatted seeds of soybean LOX mutant −L1L3. Whitehead et al. (*35*) listed three disadvantages of using guava fruits for commercial green note production: (1) the process has to be operated in a country where the fruits are available cheaply and freely and (2) a low specific activity per biomass requires a large-volume process and (3) also results in the disposal of large wastes. Though we have used *N. tabacum* plants as the host for transformation due to the fact that they are one of the high leaf biomass producers and are cultivated in the area around our university, different leaf crops can be transformed for the locations of choice. Also, with the higher activity per biomass [12−20 (μmol/min)/mg of protein] (*50*) than that of crude guava fruit extracts [9.3 (μmol/min)/mg of protein] (*52*), disadvantages 2 and 3 should be lessened. Finally, expressing enzymes/proteins is considered less expensive than expression in bacteria and/or yeast (*38*).

In conclusion, our results show that the simple homogenization/blending of the substrate 18:3 with soybeans lacking the LOX 3 isozyme and watermelon HL-overexpressing leaf tissues greatly improves the efficient production of natural green note compounds.

ABBREVIATIONS USED

18:2, linoleic acid; 18:3, linolenic acid; HL, hydroperoxide lyase; HPOD, hydroperoxylinoleic acid; HPOT, hydroperoxylinolenic acid; HOT, hydroxylinolenic acid; LOX, lipoxygenase; MTBE, methyl *tert*-butyl ether.

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