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Biosynthesis of *trans*-2-Hexenal in Response to Wounding in Strawberry Fruit

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Wounded strawberry fruit produces a diverse group of volatile compounds including aldehydes, alcohols, and esters derived from the lipoxygenase (LOX) and hydroperoxide lyase (HPL) pathways. Because the wound volatiles may play an important role in plant−fungal interaction, the goal of this study was to develop a greater understanding about the biosynthesis of the major wound volatile, *trans*-2-hexenal (*t*-2-H), produced by strawberry fruit upon wounding. To that end, composition and quantity of total and free fatty acids of control and wounded strawberry fruit were analyzed. In addition, activities of the key enzymes, LOX and HPL, and production of C_6 aldehydes were determined. Intact strawberry fruit did not produce detectable *t*-2-H which is derived from α-linolenic acid (18:3). However, in response to wounding by bruising, strawberry fruit emitted *t*-2-H and its precursor *cis*-3-hexenal (*c*-3-H). The level of total lipid 18:3 in the fruit increased 2-fold in response to wounding, whereas free 18:3 declined slightly (∼30%). At 10 min following wounding, fruit exhibited a 25% increase in LOX activity, which leads to the production of 13-hydroperoxyoctadecatrienoic acid (13-HPOT) from 18:3. The activity of HPL, which catalyzes formation of *cis*-3-hexenal from 13-HPOT, increased 2-fold by 10 min after wounding. Thus, during a 15 min period after wounding, free 18:3 substrate availability and the activity of two key enzymes, LOX and HPL, changed in a manner consistent with increased *c*-3-H and *t*-2-H biosynthesis.

KEYWORDS: Strawberry fruit; wounding; total fatty acids; free fatty acids; linolenic acid; lipoxygenase; hydroperoxide lyase; C_6 aldehydes; *cis*-3-hexenal; *trans*-2-hexenal

INTRODUCTION

Oxylipins are a group of biologically active compounds derived from polyunsaturated fatty acids, mainly α-linolenic acid (LNA), an 18-carbon fatty acid (*1*). Jasmonates, aldehydes, ketols, and epoxy-, hydroxy-, and divinyl-ether derivatives are among many fatty acid derivatives that plants synthesize (*1*−*3*). Oxylipins are known to be synthesized *de novo* in response to various stresses, including wound injury. Their substrates, polyunsaturated fatty acids, are liberated from membrane lipids and converted into various oxylipins via several enzymatic steps.

Fruits and vegetables are subject to a variety of types of injury during harvest, handling, and postharvest transport, and this is especially true for soft fruit like strawberry. The injuries may not be visible or may disrupt the tissue surface and allow pathogens to penetrate into the interior. A number of volatile compounds are released upon wounding, including terpenoids, phenylpropanoids, and lipoxygenase (LOX)-derived compounds. Strawberry fruit produces a diverse group of wound volatile compounds, including aldehydes, alcohols, and esters derived from the LOX and hydroperoxide lyase (HPL) pathway (*4*). One of the major oxylipin products, trans-2-hexenal (*t*-2-H), is released from macerated leaves and fruit (*5*, *6*). *t*-2-Hexenal, a member of a class of volatiles produced by virtually all green plant tissue, is a six-carbon aldehyde derived from the LOX and HPL pathway (*7*−*9*). It may be produced from LNA released from galactolipids by a lipase or a lipase-like activity (*10*). It has been demonstrated that *t*-2-H formation occurs on a membrane where LOX and HPL activities are exhibited (*11*−*14*). Through the action of LOX, a molecular oxygen is inserted between C and H at position 13 of LNA. The resulting fatty acid hydroperoxide [(13*S*)-hydroperoxy-(9Z,11E,15Z)-octadecatrienoic acid, 13-HPOT] is cleaved by HPL, resulting in the production of 12-oxo-(9*Z*)-dodecenoic acid and *cis*-3-hexenal (*c*-3-H). The *c*-3-H isomerizes spontaneously or enzymatically into more stable *t*-2-H (*7*). The C_6 aldehydes may be further reduced to alcohols by alcohol dehydrogenase. The C_{12} oxo-acid is the precursor of traumatin, previously known as the wound signal (*15*).

It is well-known that *t*-2-H is synthesized from LNA through LOX and HPL in response to wounding (*16*−*18*), and the *t*-2-H could be detected within as little as 20 s after tissue disruption (*10*). Most of the *t*-2-H emitted from wounded strawberry fruit was detected within 15 min following injury (*4*). However, it is not known how *t*-2-H biosynthesis is regulated in strawberry

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fruit in response to wounding. The production of free α-linolenic acid (18:3) and the activity of the enzymes responsible for *t*-2-H production following wounding have not been reported from strawberry fruit.

trans-2-Hexenal can influence the development of the fungal pathogen *Botrytis cinerea* Pers., also known as gray mold, a major pathogen of soft fruit like strawberry. The volatile oxylipin inhibited spore germination and promoted mycelial growth in vitro at low headspace levels but inhibited it at high headspace levels (*19*). Because the wound volatile may play an important role in the plant−fungal interaction, the goal of this study was to develop a greater understanding of the biosynthesis of *t*-2-H in strawberry fruit upon wounding. To that end, composition and quantity of total and free fatty acids of control and wounded strawberry fruit were analyzed. In addition, activities of the key enzymes of the LOX pathway, LOX and HPL, and production of C_6 aldehydes were determined.

MATERIALS AND METHODS

Plant Material. The strawberry (*Fragaria* x *ananassa* Duch.) cultivar 'Tribute" was used. Plants were grown in the greenhouse or outdoors in containers. Mature red fruit was selected for uniformity of color, size, and shape on the day of use. All fruit was harvested and placed at ambient laboratory temperature for 30 min before wounding treatment.

Wounding Procedure. Wounding was performed by the method of Hamilton-Kemp et al. (*4*). Four ripe fruits (30−35 g) were placed in a 475 mL glass jar, closed with a Teflon lined screw-cap lid, and shaken at 300 rpm for 30 s in a rotary shaker (model 3540, Lab-Line Instruments, Inc., Melrose Park, IL). The shaking did not cause visible disruption of the fruit surface. Control (unwounded) fruit were sampled prior to shaking (0 min), and wounded fruit were sampled at 5, 10, and 15 min after shaking. There were four replicate experiments and four fruits were analyzed per treatment and sampling time, unless stated otherwise. Following wounding treatments, the fruits were immediately used for analyses or immersed in liquid N_2 and stored at −80 °C for future analyses.

Lipid Extraction and Fatty Acid Analysis. For total lipid extraction, fruits (8 g) frozen in liquid N_2 were macerated in a homogenizer (model 5000, Omni International, Gainesville, VA) for 1 min in 40 mL of methanol, chloroform, and water (2:1:0.8), vortexed for 1 min, and centrifuged at 6 000 g for 20 min (*20*). This was repeated four times, and supernatants were combined. A suitable amount (630 μg/(g of fruit)) of triheptadecanoin was added to the homogenate as an internal standard. Combined supernatants were dried using a rotary evaporator, and the residue was redissolved in 10 mL of chloroform. Extract (1 mL) was dried under N_2.

For total fatty acid analysis, the dried lipid sample was transmethylated with 0.5 mL of 1% sodium methoxide. The sample was shaken for 45 min, and the resulting fatty acid methyl esters were extracted with hexane. The sample was partitioned against 0.9% KCl, and aliquots of the upper hexane layer were analyzed using a Hewlett-Packard 5890 (Agilent, Wilmington, DE) gas chromatograph (GC) equipped with a flame ionization detector (FID) and a FFAP column (14 m × 0.25 mm, 0.33 μm film thickness, Agilent). Samples were analyzed using a temperature gradient of 140 °C for 1 min, increased to 235 °C at a rate of 10 °C per min, and then held at this temperature for 20 min. Helium was used as the carrier gas at a flow rate of 1 mL per min.

For free fatty acid analysis, total lipid samples were prepared in 10 mL of chloroform, as described above, except that a suitable amount (5 μg/(g of fruit)) of free heptadecanoic acid (17:0) was added to the homogenate as an internal standard. Samples were separated on 250 μm silica thin-layer chromatography (TLC) plates (Whatman, Clifton, NJ). Sample-loaded plates were developed in hexane/methyl tertiary butyl ether (MTBE)/acetic acid (HOAc) (80:20:1). Authentic free linoleic acid (18:2) was used as a loading standard. The plates were sprayed with 0.01% primulin in 80% acetone, and fatty acids were visualized under UV light (300 nm). A suitable amount (1 μg/(g of fruit)) of free nonadecanoic acid (19:0) was added to the fatty acid spot on the plate. The spot was scraped from the plate and eluted through a fiber glass column (Corning Glass Works, Corning, NY) with MTBE. The sample was evaporated under N_2 and methylated with a few drops of diazomethane in ether. The ether was dried with N_2, and the sample was dissolved in hexane. The sample was partitioned against 0.9% KCl, and the hexane layer was analyzed by GC as described above.

Preparation of LOX and HPL Crude Extracts from Strawberry Fruit. LOX and HPL crude extracts were prepared from fruits with or without freezing in liquid nitrogen over a period of 15 min after wounding, because freezing may affect the enzyme activities (*21*). The activities of frozen fruits did not differ from those of nonfrozen fruits (data not shown). This result eliminates the possibility that LOX and HPL activities were affected by freezing injury. Therefore, the activities of LOX and HPL were determined using fruit which was immediately immersed in liquid nitrogen after wounding treatment and sampling time periods. Fresh or frozen fruits (30 g) were homogenized with 4.8 g of polyvinylpolypyrrolidone (PVPP), 40 mL of 0.1 M Tris-HCl (pH 8.0), and 1 M KCl for LOX extraction (*22*). For HPL extraction, 0.1% Triton X-100 was added to the buffer used for LOX extraction. The resulting homogenates were centrifuged at 20 000 g for 20 min. The supernatants were used for enzyme assays of LOX and HPL. Protein concentration was measured with Coomassie protein assay reagent (Pierce, Rockford, IL) with bovine serum albumin as standard protein at 595 nm using a spectrophotometer (Cary 50 Bio, Varian, Walnut Creek, CA) (*23*).

LOX Assay. LOX activity was determined by monitoring the conversion of LNA to diene product at 234 nm (*24*). The crude extract prepared as described above was added to a standard assay mixture. The mixture contained 1 mL of 0.1 M sodium phosphate buffer, pH 6.0, 10 mM LNA, and an appropriate amount (5−20 μL) of LOX extract. The increase in absorbance at 234 nm was recorded for 5 min. LOX activity was determined as the formation of 13-HPOT in nmol per min per mg of protein, using an extinction coefficient of 25 000 M^{-1} cm^{-1} for 13-HPOT.

Preparation of LOX from Soybean for Synthesis of 13-HPOT. For the enzymatic reaction to synthesize 13-HPOT from LNA, LOX was extracted from the soybean genotype −LOX3, which is designated as a null for LOX3 with normal levels of LOX1 and LOX2 (*25*). Soybeans (3 g) were ground using a coffee grinder and macerated in a mortar with 5 mL of acetone stored at −20 °C. The residue in the mortar was washed twice with 5 mL of acetone and 10 mL of ethyl ether and filtered on Whatman paper with vacuum. The defatted soybean powder on the filter paper was dried under N_2 and stored at 4 °C. The soybean powder (100 mg) was extracted with 1 mL of 0.2 M sodium acetate, pH 4.5, for 1 h on a shaker and filtered through Miracloth. Ammonium sulfate (40%) was added to the filtrate, which was centrifuged at 8 000 g for 10 min. The supernatant was precipitated with ammonium sulfate (60%) and centrifuged at 8 000 g for 10 min. The pellet was dissolved in 100 μL of sodium borate buffer, pH 9.0, which was used for LOX activity. LOX activity was determined by the method described above, except that the LOX assay mixture for soybean contained 0.2 M sodium borate buffer, pH 9.0. LOX activity was defined as a 0.001 increase of absorbance at 234 nm per min.

Preparation of 13-HPOT and HPL Assay. The 13-HPOT was prepared from LNA using crude soybean LOX. 2.4 mM LNA (100 μL) and 3 750 units of crude soybean LOX were added to 15 mL of oxygenated 0.2 M sodium borate buffer, pH 9.0. The reaction was allowed to proceed on ice for 1 h under a constant flow of oxygen. The pH of the reaction mixture was adjusted to 4. The 13-HPOT produced was extracted with 20 mL of diethyl ether, and the extract was dried under N_2. The residue was dissolved in 500 μL of hexane and ethyl ether and applied to a 40 μm silica column with a 20 cm length, equilibrated with 40 mL of hexane (*26*). The column was eluted first with 50 mL of hexane/HOAc (100:1) and then with 50 mL of hexane/MTBE/HOAc (90:10:1). The 13-HPOT was collected in the next fraction eluted with 50 mL of hexane/MTBE/HOAc (75:25:1). The product was dried under N_2, dissolved in ethanol, and stored at −80 °C. The final concentration of 13-HPOT, estimated from the

Table 1. Fatty Acid Content and Composition of Strawberry Fruit[a]

	total		free	
fatty acids	μg/(g of fruit)	%	ng/(g of fruit)	%
16:0	307 ± 171	11.6 ± 1.8	1630 ± 265	27.9 ± 2.1
18:0	36 ± 16	1.5 ± 0.1	510 ± 92	8.6 ± 0.2
18:1	308 ± 108	14.0 ± 1.2	592 ± 114	10.1 ± 0.7
18:2	1020 ± 395	42.5 ± 0.4	2400 ± 559	39.7 ± 3.2
18:3	776 ± 273	30.4 ± 0.6	755 ± 85	13.7 ± 1.9
total	2250± 963	100	5890 ± 1125	100

[a] Data are means ± standard errors of four independent extractions of ripe fruit. Fruit weight represents fresh weight of fruit.

absorbance, using the extinction coefficient of 25 000 M^{-1} cm^{-1} at 234 nm, was ~0.36 mM.

The crude extract prepared as described above was added to a standard assay mixture which contained 1 mL of 0.1 M potassium phosphate buffer, pH 6.0, 0.1 mM 13-HPOT, 0.1 mM nicotinamide adenine dinucleotide (NADH), 50 units of alcohol dehydrogenase (one unit converts 1.0 μmol of ethanol to acetaldehyde per min at pH 8.8 at 25 °C), and an appropriate amount (5−50 μL) of HPL extract. HPL activity was determined spectrophotometrically by monitoring the oxidation of reduced NADH at 340 nm in a coupled-enzyme assay (*27*). The decrease in absorbance at 340 nm was measured for 5 min. HPL activity was expressed as the oxidation of NADH in nmol per min per mg of protein, using an extinction coefficient of 6 220 M^{-1} cm^{-1} for NADH.

Headspace Sampling and Analysis of C_6 Aldehydes. For C_6 aldehyde measurement, a 475 mL glass jar containing the fruit was sealed with a Teflon-lined plastic screw cap prior to shaking. Fruit were shaken as previously described. At 5, 10, and 15 min following the wounding treatments, vapor-phase volatile compounds were directly sampled from headspace of the jar using a gastight syringe. The volatiles collected (450 μL) were injected into a GC (Hewlett-Packard 5890 II, Agilent), equipped with a 60 m × 0.32 mm DB-5 column with a 1 μm film thickness (J & W Scientific, Folsom, CA) and an FID. The operating conditions were 50 °C for 5 min and then a temperature increase of 2 °C per min to 100 °C. Helium was used as the carrier gas at a flow rate of 30 $cm \cdot s^{-1}$. The volatile compounds were identified by both retention time and GC-MS (Hewlett-Packard GCD 1800B, Agilent) fitted with a 25 m × 0.25 mm DB-5 column (Agilent). The operating conditions were 40 °C for 5 min, and the temperature was increased by 2 °C per min to 200 °C. Spectra were matched to those in the National Institute of Standards and Technology library, and identities were confirmed by comparing the retention times of strawberry fruit compounds with those of authentic compounds. Total headspace concentration was calculated using the response factor determined by the static dilution method described by Hamilton-Kemp et al. (*28*).

Statistical Analysis. Data were subjected to analyses to determine if there were significant linear or quadratic changes over time using SigmaStat (Systat Software, Inc., Richmond, CA).

RESULTS

Fatty Acid Content and Composition of Strawberry Fruit. The total and free fatty acid content and composition of strawberry fruits are shown in **Table 1**. Palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and linolenic (18:3) acids were detected as components of the total and free fatty acids. Total fatty acids comprised >0.2% of fruit tissue, while free fatty acids comprised <0.3% of the total fatty acids. The major components of the total fatty acids in strawberry fruit were 18:2 and 18:3. The total fatty acid contents were slightly higher (~38%) than those measured by Couture et al. (*20*). The major components of the free fatty acids in strawberry fruit were 18:2 and 16:0, followed by 18:3. Free fatty acid content was roughly 3 orders of magnitude less than the total fatty acid content. Trace amounts of palmitoleic acid (16:1Δ^9 and 16:1Δ^7) from total and free fatty acids were detected (data not shown).

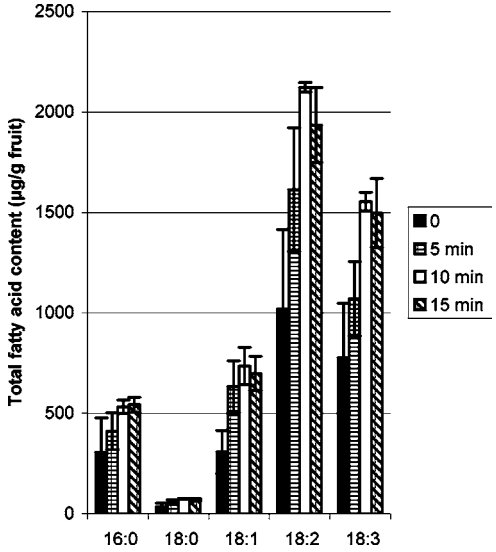

Figure 1. Changes in major components of total fatty acids in strawberry fruit in response to wounding. Bars indicate standard errors of four independent extractions of ripe fruit. Fruit weight represents fresh weight of fruit. The quadratic trends are as follows: for 16:0, $Y = 300.52 + 30.89X - 0.96X^2$, $r = 0.0.48$, $P < 0.05$; for 18:0, $Y = 35.26 + 6.7X - 0.28X^2$, $r = 0.64$, $P < 0.05$; for 18:1, $Y = 312.02 + 79.81X - 3.62X^2$, $r = 0.68$, $P < 0.05$; for 18:2, $Y = 989.4 + 182.5X - 7.8X^2$, $r = 0.66$, $P < 0.05$; and for 18:3, $Y = 739.5 + 105.8X - 3.5X^2$, $r = 0.68$, $P < 0.05$.

Changes in Total and Free Fatty Acid Content in Strawberry Fruit after Wounding. Wounding significantly increased the tissue content of each fatty acid in a quadratic pattern (**Figure 1**). The relative contribution of each fatty acid to total fatty acids remained unchanged over 15 min following wounding (data not shown). The increased pool of total fatty acids with no change in the composition suggests that the complete fatty acid biosynthetic pathway may be rapidly upregulated in wounded strawberry tissue, resulting in an increased pool of total fatty acids.

The amounts of free 16:0, 18:0, and 18:2 did not significantly change after wounding (**Figure 2**). However, free 18:1 showed a quadratic increase in concentration, with a maximum 30% greater than unwounded fruit at 10 min after wounding. In contrast, 18:3 showed a quadratic decrease with the lowest level, 30% less than unwounded levels, at 10 min. Interestingly, free 16:1 (63 ng/(g of fruit)) in unwounded fruit increased 3-fold to 183 ng/(g of fruit) at 10 min after wounding (data not shown). The accumulation of free 18:1 in wounded fruit indicates that 18:1 was hydrolyzed from membranes upon wounding, implying the presence of lipase activities. Since lipase activity is also required for the production of free 18:3 for C_6 aldehyde biosynthesis in response to wounding (*10*), the decrease of free 18:3 content at 5 min after wounding, despite increased total 18:3 content (**Figure 1**), implies that free 18:3 was metabolized into oxylipins.

Changes in LOX and HPL Activities in Strawberry Fruit after Wounding. Wounding resulted in significant quadratic changes in LOX and HPL activity over 15 min (**Figure 3**). Prior to wounding, the activities of LOX and HPL were comparable to those measured by Pérez et al. (*22*). LOX activity increased ~25% through 10 min after wounding, then decreased to the level of unwounded fruit by 15 min. In response to wounding, HPL showed the same pattern as that of LOX, about a 2-fold increase by 10 min. The quadratic decrease in free 18:3 (**Figure**

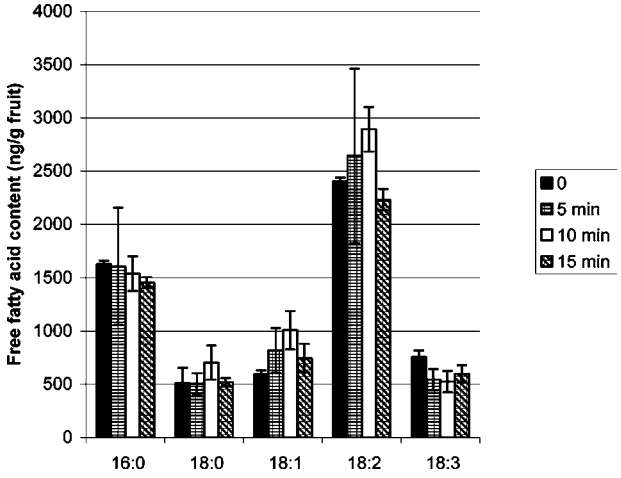

Figure 2. Changes in major components of free fatty acids in strawberry fruit in response to wounding. Bars indicate standard errors of four independent extractions of ripe fruit. Fruit weight represents fresh weight of fruit. The quadratic trends are as follows: for 18:1, $Y = 571.62 + 86.08X - 4.88X^2$, $r = 0.48$, $P < 0.05$; and for 18:3, $Y = 749.5 - 52.55X + 2.85X^2$, $r = 0.74$, $P < 0.05$. The other fatty acids did not show significant trends.

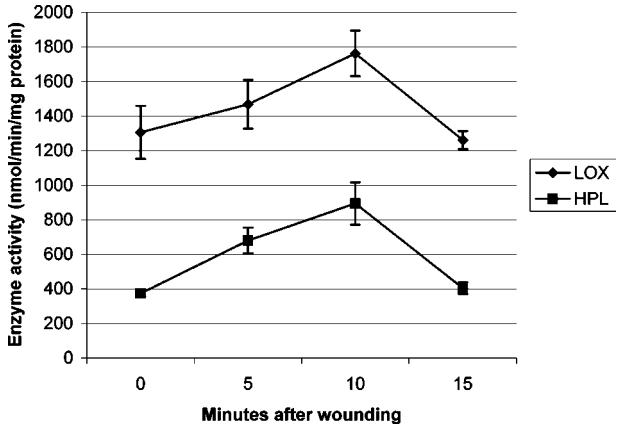

Figure 3. Changes in LOX and HPL activities in strawberry fruit in response to wounding. LOX activity was determined as the formation of 13-HPOT in nmol per min per mg of protein. HPL activity was determined as the oxidation of NADH in nmol per min per mg of protein. Bars indicate standard errors of four independent measurements of ripe fruit. The quadratic trend for LOX is $Y = 1.256 + 0.106X - 0.007X^2$, $r = 0.62$, $P < 0.05$; and for HPL, it is $Y = 0.344 + 0.125X - 0.008X^2$, $r = 0.81$, $P < 0.05$.

2) and increase in LOX and HPL activities suggest that wounding enhanced the synthesis of 13-HPOT, a product of LOX and a substrate of HPL, and that 13-HPOT was metabolized to *c*-3-H through the activity of HPL.

Changes in C_6 Aldehyde Production in Strawberry Fruit after Wounding. The vapor-phase concentrations of C_6 aldehydes measured at 5, 10, and 15 min represent the total accumulation from 0−5, 0−10, and 0−15 min, respectively. *c*-3-Hexenal was produced by unwounded fruit, while *t*-2-H was not detected (**Table 2**). Wounding induced significant changes in C_6 aldehyde production in fruit within 5 min. The vapor-phase concentration of *c*-3-H increased gradually over 15 min after wounding. In contrast, *t*-2-H was detected in the highest quantity at 5 min after wounding, maintained that level through 10 min, but declined by 15 min. The gradual emission of *c*-3-H and the rapid emission of *t*-2-H within 5 min after wounding indicate a wound-induced synthesis of C_6 aldehydes.

Table 2. Change in Vapor-Phase Concentrations of *cis*-3-Hexenal (*c*-3-H) and *trans*-2-Hexenal (*t*-2-H) in Strawberry Fruit in Response to Wounding[a]

	vapor-phase concentration (ng/(g of fruit))			
	0 min[b]	5 min[b]	10 min[b]	15 min[b]
c–3-H	2.3 ± 2.3[c]	5.2 ± 3.0	10.8 ± 0.9	12.4 ± 1.1
t–2-H	0.0 ± 0.0	13.0 ± 2.5	11.9 ± 0.2	5.9 ± 0.5

[a] Data are means ± standard errors of four independent measurements. [b] Minutes after wounding. [c] The *c*-3-H concentration in unwounded fruit was determined from two jars. Each jar contained four individual fruits. Fruit weight represents fresh weight of fruit. The quantity of *c*-3-H and *t*-2-H (ng/L in air) calculated by response factor was converted to the equivalent quantity (ng) by conversion factor (0.45) between the units. For *c*-3-H, the linear trend is $Y = 4.54 + 1.62X$, $r = 0.51$, $P < 0.05$, and the quadratic trend is $Y = 4.03 + 1.86X - 0.02X^2$, $r = 0.72$, $P < 0.05$. For *t*-2-H, the quadratic trend is $Y = 1.27 + 7.32X - 0.44X^2$, $r = 0.86$, $P < 0.05$.

DISCUSSION

It is well-known that the susceptibility of fatty acids to oxidative damage increases as fatty acid unsaturation in membranes increases. Total 18:2 and 18:3 in ripe strawberry fruit comprised >70% of the total fatty acids (**Table 1**). The high degree of fatty acid polyunsaturation of strawberry fruit may reflect sensitivity to lipid peroxidation. In contrast, 18:1, 18:2, and saturated fatty acids such as myristic acid (14:0) and 18:0 are the most abundant fatty acids in apple, apricot, mango, and tomato fruit (*6*, *29*, *30*). Total fatty acid content of strawberry fruit was within a 2-fold range of fruit of the other species.

The 2-fold increase in the unsaturated fatty acids 18:1, 18:2, and 18:3 up to 10 min after wounding of strawberry fruit (**Figure 1**) was unexpected, because previous studies showed that there was no significant change or even a decrease in the total amount of lipids and fatty acids in leaves up to 30 min after wounding (*31*, *32*). This may reflect significant differences in the wound response of different tissue types, though it should be noted that the shaking procedure used in this study to wound fruit cannot be applied to leaves. Saturated fatty acids (16:0 and 18:0) are synthesized before being incorporated into membrane lipids, while 18:3 can be synthesized from 18:1 via 18:2 by a class of desaturases on membrane lipids (*33*, *34*). The increased total fatty acids may be a consequence of cellular events in which enzymes involved in biosynthesis of fatty acids, fatty acid binding-proteins involved in transfer of fatty acids to membranes, and/or desaturases are activated to fill disrupted membrane bilayers as a wound healing process. Rapid intracellular removal of fatty acids from the cytofacial side of the plasma membrane and their subsequent esterification occurs with half-times typically <1 min in the cell (*35*). Even though a wound-induced increase of unsaturated fatty acids has not been reported previously, Spiteller (*36*) suggested that any alteration of membrane fatty acids is related to lipid peroxidation.

The fatty acids are known to be released from the plasmalemma and plastid membranes by lipases after wounding (*37*, *38*). Polyunsaturated free fatty acids liberated from membranes are oxidized by LOX or chemically metabolized to other products (*1*−*3*). In this study, the changes in the free fatty acids 18:1 and 18:3 reflect biochemical events in strawberry fruits after wounding. The continued increase in free 18:1 content through 10 min after wounding (**Figure 2**) was likely caused by buildup of fatty acids liberated from membrane lipids. The activation of lipid hydrolysis indicates the presence of a lipase and its immediate activation within 5 min in response to

wounding, even though it has not been determined whether a specific lipase is related to this early wounding response. The predominant substrate of LOX may be free 18:3, because free 18:2 did not significantly change through 10 min after wounding while free 18:3 was reduced. If LOX preferred free 18:2 to free 18:3 as a substrate, free 18:3 would have presumably increased and free 18:2 would have declined. Substrate specificity for free 18:3 by LOX has been reported (*22*). Though free 18:1 levels increased through 10 min after wounding, they decreased to the levels observed in unwounded fruit by 15 min. Because free unsaturated fatty acids are toxic to cells, it is possible that the accumulated free fatty acids were removed as part of normal cellular metabolism, detoxifying harmful unsaturated free fatty acids. A fatty acid hydroxylase can mediate the detoxification of unsaturated fatty acids as a protective response to toxic fatty acids (*39*). The free 18:1 content at 10 min after wounding may thus represent a maximal concentration in strawberry fruit that does not cause toxicity.

Free 16:0 content did not show a pattern similar to that observed for free 18:1 (**Figure 2**). The lack of a wound-induced effect on free 16:0 level is in contrast to the change in the free 16:1 levels. Even though a small amount of free 16:1 existed in fruit prior to wounding (data not shown), a notable increase in free 16:1 after wounding (63−183 ng/(g of fruit) at 10 min) argues for the existence and activation upon wounding of a 16:$0\Delta^7$ desaturase.

Free 18:3 is a precursor to the C_6 aldehydes. If a lipase is activated upon wounding in strawberry fruit, as implied by the increase in free 18:1, free 18:3 should have also increased. However, the decrease in free 18:3 levels at 5 min after wounding (**Figure 2**) implies that free 18:3 released from the plastid membrane was metabolized. The correlation between the decrease in free 18:3 level and the increase in LOX and HPL activities further supports the probability that C_6 aldehydes were produced from *de novo* synthesis of free 18:3. Thus, the data presented in this study suggest that free 18:3 generated from membrane 18:3 upon wounding is used as a source for wound-related C_6 aldehyde biosynthesis.

Increased production of both *c*-3-H and *t*-2-H occurred along with greater activities of LOX and HPL after wounding (**Figure 3**, **Table 2**). The results are in agreement with the work by Hong et al. (*40*), who showed that an elevated level of *c*-3-H was a consequence of an increase in endogenous 13-LOX activity. In addition, *c*-3-H production requires the activity of HPL (*41*). LOX gene expression, enzymatic activity, and C_6 aldehyde synthesis have shown similar trends in other species, suggesting that the synthesis of C_6 aldehydes is a consequence of increased LOX and HPL activities (*42*, *43*). Since the increase in LOX and HPL activities occurred within 10 min in response to wounding, they may be related to posttranslational mechanisms, such as enzyme stability, presence of inhibitors, and enzyme modification. However, the possibility cannot be excluded that gene expression increased or that inactive proenzyme forms of LOX and HPL were present in sufficient amounts in cells and were rapidly translated to the enzymes upon wounding, resulting in accumulation responsible for the rapid synthesis of *t*-2-H. Even though LOX and HPL mRNA and proenzymes are known to preexist in the intact tissues of other species (*39*, *44*, *45*), it remains to be elucidated whether the quantity of LOX is actually enhanced within 10 min upon wounding in strawberry fruit.

Our data contradict the results reported by Yilmaz et al. (*46*), who indicated that enzyme activities of LOX and HPL were not a good indicator of C_6 volatile production by tomato fruit. Howe et al. (*17*) also suggested that HPL plays a minor role in the production of C_6 volatile compounds during tomato fruit ripening, since a paucity of HPL mRNA accumulation in mature green and red fruit was found. Matsui et al. (*47*) indicated that 9-LOX, the major LOX form in tomato fruit, might not contribute to the formation of flavor volatiles but that a very low level of 13-LOX could be sufficient for C_6 production. Similarly, tomato had low C_6 aldehyde-forming activity (*48*). Strawberry and tomato fruit may exhibit different metabolic flows through the pathway of LOX and HPL for the biosynthesis of *c*-3-H and *t*-2-H. Though *t*-2-H was detected in unwounded tomato fruit (*45*), *t*-2-H was detected in strawberry fruit only when the fruit was subjected to wounding in this study. Others have observed *t*-2-H from samples of ripe strawberry fruit, though their techniques of maceration or homogenization could have yielded wound-induced production of C_6 aldehydes (*22*, *49*). The differing profiles of *t*-2-H emission between tomato and strawberry fruit may indicate species differences in the regulation of LOX and HPL activities. It is possible that synthesis of C_6 aldehydes may not be affected upon wounding in plant species that favor 9-HPOT as a product. Therefore, the diverse regulatory processes involved in C_6 aldehyde production may provide an understanding of biochemical mechanisms of wound responses.

From the results obtained in this study, a model for regulation of wound-induced biosynthesis of C_6 aldehydes by strawberry fruit can be proposed. At 5 min after wounding, free 18:3 released from galactolipids was converted to *c*-3-H by LOX and HPL, and the *c*-3-H was rapidly isomerized to *t*-2-H. At 10 min after wounding, a continuing supply of free 18:3 from plastid 18:3 continued to increase activities of LOX and HPL and increased *c*-3-H production, which isomerized to maintain levels of *t*-2-H. At 15 min after wounding, free 18:3 availability was lower, leading to lower activities of LOX and HPL and a decreased overall production of the C_6 aldehydes. Such stimulation of LOX and HPL activities by free 18:3 has been reported (*45*). However, it is difficult to associate the apparent continuing emission of *c*-3-H with the decrease in free 18:3 availability and the decrease in LOX and HPL activities at 15 min after wounding, though levels of free 18:3 may be sufficient for this. It is also possible that an isomerization factor was activated upon wounding and *c*-3-H was rapidly converted to *t*-2-H, but the factor returned to a deactivated state by 15 min after wounding, resulting in the accumulation of *c*-3-H. However, it is still unclear whether the conversion of *c*-3-H to *t*-2-H in vivo can be accelerated by an enzyme in strawberry fruit, as *c*-3-H may isomerize spontaneously into the more-stable *t*-2-H (*7*). Nevertheless, the proposed model emphasizes the involvement of substrate availability and increased LOX and HPL activities with the initial burst of *t*-2-H synthesis. Vancanneyt et al. (*45*) suggested that *c*-3-H production is determined by substrate availability to HPL in potato leaves rather than by the abundance of HPL activity.

In conclusion, this is the first study to reveal aspects of regulation of *t*-2-H biosynthesis in strawberry fruit in response to wounding. Our data demonstrate that LOX and HPL activities rapidly increased upon wounding. The patterns were consistent with increased C_6 aldehyde biosynthesis. With the presence of lipase activity indicated by the increased pool of free 18:1, the decreased free 18:3 level and increased *t*-2-H production from wounded fruit implies that *de novo* synthesis of free 18:3 was required for *t*-2-H biosynthesis in response to wounding in strawberry fruit.

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