Deficiency of Linolenic Acid in *Lefad7* Mutant Tomato Changes the Volatile Profile and Sensory Perception of Disrupted Leaf and Fruit Tissue

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**Abstract.** Six-carbon aldehydes and alcohols formed by tomato (*Lycopersicon esculentum* Mill.) leaf and fruit tissue following disruption are believed to be derived from the degradation of lipids and free fatty acids. Collectively, these C-6 volatiles comprise some of the most important aroma impact compounds. If fatty acids are the primary source of tomato volatiles, then an alteration in the fatty acid composition such as that caused by a mutation in the chloroplastic omega-3 fatty acid desaturase (*ω*-3 FAD), referred to as LeFAD7, found in the mutant line of ‘Castlemart’ termed *Lefad7*, would be reflected in the volatile profile of disrupted leaf and fruit tissue. Leaves and fruit of the *Lefad7* mutant had ≈ 10% to 15% of the linolenic acid (18:3) levels and about 1.5- to 3-fold higher linoleic acid (18:2) levels found in the parent line. Production of unsaturated C-6 aldehydes Z-3-hexenal, Z-3-hexenol, and E-2-hexenal and the alcohol Z-3-hexenol derived from 18:3 was markedly reduced in disrupted leaf and fruit tissue of the *Lefad7* mutant line. Conversely, the production of the saturated C-6 aldehyde hexanal and its alcohol, hexanol, were markedly higher in the mutant line. The shift in the volatile profile brought about by the loss of chloroplastic FAD activity in the *Lefad7* line was detected by sensory panels at high significance levels (*P* < 0.0005) and detrimentally affected fruit sensory quality. The ratios and amounts of C-6 saturated and unsaturated aldehydes and alcohols produced by tomato were dependent on substrate levels, suggesting that practices that alter the content of linoleic and linolenic acids or change their ratios can influence tomato flavor.

Perception of aroma is a very complex process involving both detection and cognitive integration (Baldwin et al., 2000). Given that the aroma of most consumables is a function of many volatiles, assessing the influence of a single volatile is a challenge, and developing strategies to improve aroma is, perhaps, even more problematic.

Of the more than 400 volatiles reported for tomato fruit (Frenkel and Jen, 1989; Grierson and Kader, 1986; Tandon et al., 2000), only 30 are present at a concentration higher than 1 nL·L⁻¹. Of those, only 16 have a positive log odor unit, which is defined as the logarithm of ratio of the concentration of an aroma volatile to its odor threshold (Buttery, 1993). A log odor unit of 0 indicates that the odorant is present at its threshold level; log odor units of 1 and –1 would indicate the odorant is at 10 times and 1/10th the threshold level, respectively. Reducing the production of those volatiles with positive log odor units, increasing the production of those volatiles with negative log odor units, or changing the ratios of impact compounds have the potential to alter sensory perception of tomato leaves and/or fruit.

Lipids and fatty acids (FA) are thought to act as precursors to the aldehydes Z-3-hexenal, hexanal, and E-2-hexenal, the alcohols 1-hexanol and Z-3-hexenol, and the ketone 1-penten-3-one (Baldwin et al., 2000), which provide some of the most important flavor notes to tomato (Table 1). Our current understanding is that the formation of these compounds involves the action of a sequence of enzymes [lipase, lipoxygenase (LOX), hydroperoxide lyase (HPL), isomerase, and alcohol dehydrogenase (ADH)] on glycerolipids containing the fatty acids linoleic acid (18:2) and linolenic acid (18:3) (Bate et al., 1998; Galliard and Matthew, 1977; Riley et al., 1996). FA composition of tomato is variable

| Aroma compound | Odor descriptor¹ | Fatty acid precursor | Log odor units² |
|----------------|------------------|----------------------|-----------------|
| Z-3-Hexenal    | Tomato, citrus   | Linolenic acid (18:3)| 3.7             |
| Hexanal        | Stale, grassy, green | Linoleic acid (18:2) | 2.8             |
| E-2-Hexenal    | Stale, green, vine | Linolenic acid (18:3) | 1.2             |
| 1-Hexanol      | Glue, green      | Linoleic acid (18:2) | –1.9            |
| Z-3-Hexenol    | Green, celery    | Linolenic acid (18:3) | 0.3             |
| 1-Penten-3-one | Fresh, sweet     | Linolenic acid (18:3) | 2.7             |
| 2-Pentenal     | Stale, oil       | Linolenic acid (18:3) | –1.0            |
| 1-Penten-3-ol  | Pungent, green, fruity | Linolenic acid (18:3) | –0.6            |

¹Tandon et al., 2000.
²Buttery, 1993.
and dependent on the cultivated variety, but in general the most abundant fatty acids are 18:3 and 18:2, which account for 60% to 70% of the total FA content (Gray et al., 1999; Li et al., 2003; Wang et al., 1996).

Substrate feeding experiments using protein extracts or homogenates from fruit demonstrated that the addition of 18:2 enhances the production of hexanal and 1-hexanol, and addition of 18:3 enhances Z-3-hexenal, E-2-hexenal, and Z-3-hexenol formation (Boukobza et al., 2001; Galliard and Matthew, 1977; Riley and Thompson, 1998). Somewhat paradoxically, no strong relationship was found between LOX, HPL, and ADH activities and the amount of volatiles produced (Yilmaz et al., 2001), suggesting that improving volatile formation is a complex goal that may not be achievable by modifying a single step in the LOX pathway.

Gray et al. (1999) measured the amounts of saturated and unsaturated C-6 aldehydes produced by tomato cultivars that differ in amount of 18:2 and 18:3. They found a significantly higher hexanal to hexenal ratio for tomato fruit in which the ratio of 18:2 to 18:3 was higher. Wang et al. (1996) found similar results using a transgenic approach to alter LOX substrate levels by constitutive expression of a yeast delta-9 desaturase gene. The amounts of 16:1, 18:1, and 18:2 in the fruit were significantly increased as was the total amount of FA. The amount of 18:3 was slightly reduced relative to the wild type. The volatiles hexanal and 1-hexanol, presumed to be derived from 18:1 and 18:2, were enhanced 267% and 407%, respectively, relative to untransformed tomato. However, three of four C-6 volatiles presumed to be derived from 18:3 also increased to a similar extent in the transformed plant material, as did several non-lipid-derived volatiles, thus raising doubts as to the interpretation of the data. A clearer picture of the relationship between endogenous fatty acid content and fruit and leaf volatiles may be derived using tomato lines possessing more dramatic shifts in fatty acid content.

One such tomato line is the fatty acid mutant identified by Howe and Ryan (1999), based on its impaired ability to respond to wounding. The mutant, spr2 (suppressed in 35S::prosytemin-mRNA-responsive), was identified as being deficient in jasmonic acid (JA) biosynthesis (Li et al., 2002) due to considerably lower levels of 18:3 than wild type (Li et al., 2003). It has been recently demonstrated that the Spr2 gene of tomato encodes a chloroplastic ω-3 FA desaturase (Li et al., 2003), now called LeFAD7. The mutation of the ω-3 FAD was shown to reduce the linolenic acid content in leaves, but did not affect root FA content. Fruit FA content was not reported.

We hypothesized that an alteration in the fatty acid composition caused by the spr2 mutation (renamed Lefad7) would be reflected in the volatile profile of disrupted leaf and fruit tissue. The current work analyzes the production of C-6 volatiles by tomato leaves and fruit at different developmental stages by two lines differing in fatty acid composition, and how this change in the volatile profile is perceived by a non-trained sensory panel.

Materials and Methods

PLANT MATERIAL. ‘Castlemart’ tomato and the mutant of this cultivar, Lefad7, obtained by ethyl methanesulfonate mutagenesis (EMS) (Howe and Ryan, 1999), were grown in a greenhouse under a constant temperature of 20 °C. Young (50% expanded) and mature (fully expanded) leaves and immature green and mature red fruit were sampled for FA and volatile analysis.

FATTY ACID COMPOSITION. Total lipids were extracted from 400 mg of leaf and 500 mg of fruit tissue with two 6-mL aliquots of hexane and once with 4 mL of 2:7 (v:v) isopropanol:hexane. Esterification/transesterification of FA was performed with methanolic:H2SO4 (2.5%) to generate fatty acid methyl esters (FAMES) (Conconi et al., 1996). FAMES were analyzed by gas chromatography (GC) with a DB-23 capillary column (J&W Scientific, Folsom, Calif.) and flame ionization detector (FID) as described by Bonaventure et al. (2003).

EVALUATION OF AROMA COMPOUNDS. For leaf volatile analysis, one leaf disk (35 mg) from each experimental unit was crushed in a 20-mL amber glass vial and sealed. After 3 min, when volatile production is essentially complete (Boukobza et al., 2001), 1 mL of 50% CaCl2 solution was added to stop further enzymatic reactions. The vial was immediately closed with a Mininert valve (Supelco, Bellefonte, Pa.), and stored at −20 °C until volatile analysis. For volatile analysis of the homogenized leaf tissue, the homogenate was thawed and the vial heated and sampled as described below. For fruit volatile analysis, 10 g of pericarp tissue were homogenized in 5 mL of distilled water using a tissue homogenizer (PT 10/35; Brinkmann Instrument Co., Westbury, N.Y.). After 3 min in a closed 50-mL tube, 6 mL of 50% CaCl2 (w/v) solution was added and cooled immediately in a −80 °C freezer and held until analysis. Before volatile analysis, the homogenate was thawed and as soon as ice was no longer present, 5 mL of the homogenate were placed in a 20-mL amber glass vial and closed with a valved septum (Mininert valve; Supelco).

Vials containing homogenate were placed in a water bath held at 37 °C for 30 min before analysis. Volatile compounds were extracted from the headspace using a solid-phase microextraction (SPME) fiber (65 μm PDMS-DVB; Supelco) similar to the method of Song et al. (1998). The fiber was held in the vial for 3 min to allow absorption of volatile compounds. A gas chromatograph (HP 6890 Series GC; Hewlett-Packard Co., Wilmington, Del.) was used for analyte separation, and a time-of-flight mass spectrometer (Pegasus II; Leco, St. Joseph, Mich.) was used for analyte detection, identification, and quantification. The GC column (SupelcoWax-10; Supelco) was 30 m long × 0.2 mm i.d. with a 0.2 μm-thick coating. The GC oven was programmed from 40 to 220 °C at a rate of 50 °C/min and held at 200 °C for 3 min. In order to quantify the headspace concentration of hexanal, Z-3-hexenol, E-2-hexenal, 1-hexanol, and Z-3-hexenol, gas standards were created using authenticated compounds according to the method of Song et al. (1998). For leaf analysis, 0.25 μg of 2-octanone (10 μL of a 25 ng·μL−1 solution in water) was added in the vial as internal standard. For fruit samples, 0.4 μg of 2-octanone was added to the vial.

SENSORY EVALUATION. A sensory test was performed to determine if the aroma of the crushed tissues for mutant line differed from the wild type line. For leaves, five leaf disks (=170 mg) were crushed in a 20-mL vial, closed with Teflon-lined caps, and held at room temperature. One milliliter of a 50% CaCl2 aqueous solution was added after 3 min to stop enzymatic reactions. Vials were kept at −20 °C until the sensory test was performed. For fruit, 30 g of pericarp were blended and 20 mL of 50% CaCl2 solution were added after 3 min. Five milliliters of the homogenate were transferred into 20-mL vials and kept at −20 °C until the sensory test.

A triangle test for aroma was performed as indicated by Meilgaard et al. (1999). Panelists were presented with three vials; two vials held the same homogenate and the third held a different homogenate. Panelists were asked to smell each vial and choose which vial scent differed from the other two. The order in which the samples, mutant or wild type, were presented to the panelists...
were arranged so that all six possible combinations were used. There were at least five replications of each sample combination presented to panelists. For leaf analysis, 32 panelists were used; for fruit analysis, there were 36 panelists.

A preference test was performed together with the triangle test for fruit (Meilgaard et al., 1999). The panel was asked if the homogenate they chose as being different from the other two was preferable or not-preferable. The preference test was designed to qualify the sensory differences detected in the triangle test. The hypothesis tested was “more than 50% of panelists prefer one aroma over the other” (Meilgaard et al., 1999).

**Statistical analysis.** All data for FA content and headspace volatile concentration were expressed as the mean ± SE. Data were analyzed using one-way analysis of variance by PROC MIXED of SAS (version 8e; SAS Institute, Cary, N.C.). Statistical significance of sensory evaluation data was determined using Table T8 in Meilgaard et al. (1999).

**Results**

**Fatty acid composition.** The total FA content of leaves did not differ between wild type and *Lefad7* mutant lines, averaging 29.3 μmol·g⁻¹ on a fresh weight basis. Of the eight FAs evaluated for mutant and wild type leaves, only dienoic (16:2 and 18:2) and trienoic (16:3 and 18:3) FAs differed (Fig. 1A). Mutant leaf 16:3 content was 1% of wild type, while the 18:3 content was 16% of wild type. The content of 16:2 and 18:2 in mutant leaves was 13- and 3.5-fold higher than in wild type leaves. In the wild type tomato leaf, the most abundant FA was 18:3, constituting about 44% of total FA while 18:2 constituted 18%; in *Lefad7* leaves, the most abundant was 18:2, comprising about 57% of the total (data not shown), while 18:3 represented only 7% of total FA. Octadecanoic acids accounted for ≈65% of total FA. Maturity did not affect total FA content or composition for wild type and mutant leaves (not shown).

As in leaves, the FA composition and total content in fruit was not affected by maturity stage (Fig. 1B–C) for wild type and mutant lines, averaging 2.7 μmol·g⁻¹. The content of hexadecanoic acid (16:0) was ≈20% to 30% of the total FA content and was unaffected by the mutation (data not shown). Tri- and dienoic forms of hexadecanoic acid (16:3 and 16:2) were absent or present in very low amounts in tomato fruit; whereas di- and trienoic forms of octadecanoic acids accounted for more than 60% of the total FA content (data not shown). The loss of FAD7 activity in the mutant caused the mole fraction of 18:2 to increase from ≈40% to 60%, and caused the mole fraction of 18:3 to decrease from ≈20% to 1% to 3% of the total FA content. The impact of *Lefad7* mutation on 18:3 content reduction was greater for fruit than for leaves.

**Volatile aroma compounds.** C-6 aldehydes accounted for 99% of C-6 volatiles derived from the LOX pathway for all the tissues and stages analyzed in wild type and *Lefad7* plants (data not shown). The mutation markedly altered the volatile production of the aldehydes hexanal, Z-3-hexenal, and E-2-hexenal for leaf and fruit tissue (Figs. 2–4).
In wild type leaves, the most abundant C-6 aldehyde was Z-3-hexenal (93%); in Lefad7 mutant leaves, the most abundant C-6 aldehyde was hexanal (70%) (Fig. 2A–B). In mutant leaves, Z-3-hexenal accounted for only 29% of the total C-6 aldehydes. For wild type and mutant leaves, the concentration of E-2-hexenal was low, amounting to 3% and 1% of the total, respectively. There was no difference in volatile composition and concentration for young and mature leaves.

In wild type fruit, the most abundant C-6 aldehyde was hexanal in green fruit and Z-3-hexenal in red fruit (Fig. 3). For Lefad7 fruit, Z-3-hexenal production was suppressed to extremely low levels and hexanal was the predominant C-6 aldehyde for both stages of development (Fig. 3). However, C-6 aldehyde production differed dramatically between developmental stages for wild type and mutant lines. Total C-6 aldehydes for red tomato fruit were 100-fold higher than green fruit for the wild type line and 50-fold higher for the mutant line, with no similar accompanying shift in FA content (Table 2). The greatest relative increase in the aldehydes of wild type fruit occurred for Z-3-hexenal, which increased over 200-fold between immature green and mature red stages. In mutant fruit, this aldehyde was not detected at the green immature stage, but at the red stage it was possible to detect the presence of small amount (120 nL·L⁻¹).

In terms of relative composition within the C-6 aldehydes, wild type green fruit homogenates produced more hexanal (60% of total C-6 aldehydes) than the sum of Z-3- and E-2-hexenals, while red fruit produced a greater proportion (74%) of Z-3- and E-2-hexenals (Fig. 4). Wild type leaves had a preference for the production of unsaturated aldehydes (96%), which more closely resembled the aldehyde profile of mature red fruit. Mutant leaves had a volatile profile that was somewhat depleted of unsaturated C-6 volatiles relative to wild type.

**SENSORY EVALUATION.** In the triangle test for leaves, 26 out of 32 subjects were able to correctly choose the odd sample between the three vials (Table 3). For fruit, 28 out of 36 subjects were able to differentiate the odd sample from the set of three vials. The results of both triangle tests demonstrated that the difference in volatiles of mutant and wild type leaf and fruit tissues can be readily perceived ($P < 0.0005$).

In the preference test, the tested hypothesis was that more than 50% of the panel preferred the tomato aroma of wild type fruit. The majority of respondents expressed a preference for the aroma of the wild type tomato fruit homogenate and the hypothesis was accepted ($P < 0.0005$).

### Table 2. Ratios of C-6 volatile production and the fatty acid (FA) sources of those volatiles for red mature fruit relative to immature green fruit of wild type and Lefad7 mutant ‘Castlemart’ tomatoes. Each number represents an average of five determinations; n.d. indicates compound not detected in either red or green fruit.

| Volatile       | Wild type FA sources | Lefad7 FA sources |
|----------------|----------------------|-------------------|
| Hexanal        | 42.3                 | 49.7              |
| Z-3-Hexenal    | 234                  | n.d.              |
| E-2-Hexenal    | 50.8                 | 18.0              |
| Total C-6 aldehydes | 99.8              | 50.3              |
| 1-Hexanol      | n.d.                 | 3.0               |
| Z-3-Hexenol    | 4.0                  | n.d.              |
| Total C-6 alcohols | 4.0               | 4.0               |

*Low absolute levels of 16:3 and 18:3 reduced the accuracy of this determination.*

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ripening, which is consistent with the recent findings that an increase in LOX pathway activity accompanies fruit maturity in tomatoes. Panelist preference was determined for the 28 panels correctly identifying the odd sample for fruit.

|                          | No. of subjects |   |   |   |
|--------------------------|-----------------|---|---|---|
|                          | Correct | Total | P  |
| Triangle test, leaves    | 26      | 32    | <0.0005 |
| Triangle test, fruit     | 28      | 36    | <0.0005 |
| Preference test, fruit   | Wild type better | Total | P  |
|                          | 23      | 28    | <0.0005 |

*Meilgaard et al., 1999 (Table 8).

**Discussion**

The FA composition and content of wild type and mutant leaves in this study was similar to those previously published (Li et al., 2003). One might have expected that reduced LeFad7 expression would have reduced FA accumulation. However, Lefad7 data are consistent with observations for ω-3 FAD mutants of Arabidopsis thaliana (L.) Heynh., specifically, a sad7 mutant (Zhuang et al., 1996) and a sad3-2, sad7-2, sad8 triple mutant (Routaboul et al., 2000), for which the total FA content is not affected by the lack of ω-3 FAD activity. The lack of an impact on total FA content by a loss of ω-3 FAD activity contrasts with results of overexpression of a yeast-derived delta-9 desaturase in tomato, in which fruit FA content was increased =100% in fruit (Wang et al., 1996) and 25% in leaves (Wang et al., 2001). However, the delta-9 desaturase forms dienoic fatty acids, which act as substrates for ω-3 FAD. It is possible that FA content is controlled at a point in the pathway prior to ω-3 FAD.

The reduction of 18:3 in tomato vegetative and reproductive tissue and the accompanying dramatic decrease in the concentration of the aldehyde and alcohol volatiles derived from its peroxidation confirm that 18:3 is the primary precursor to Z-3-hexenal, E-2-hexenal, and Z-3-hexenol in tomato, as suggested by Baldwin et al. (1991) and Li et al. (2000). In mutant leaves, Z-3-hexenal, which accounted for only 29% of the total C-6 aldehydes, was most likely derived from the residual amounts of 18:3 produced by non-chloroplastic FAD (FAD3) as suggested by Li et al. (2003).

Considering that FA and C-6 volatile composition was not significantly different between young and mature leaves, the data suggest that the regulation of FA oxidation in leaves does not change developmentally as a function of the age of a plant organ. However, in fruit, the 50- to 100-fold increase in C-6 aldehyde production associated with maturation and ripening (Table 2) with no similar accompanying shift in FA content, likely reflects that an increase in LOX pathway activity accompanies fruit ripening, which is consistent with the recent findings of Chen et al. (2004) regarding TomLoxC. Changes in lipase activity could also be affecting the availability of substrate for LOX pathway. However, data for the involvement of lipase are lacking; the only lipase expression analyses during tomato fruit ripening have been done on phospholipase D (PLD) (Jandus et al., 1997; Finhoro et al., 2003; Whittaker et al., 2001). PLD activity increased during ripening process for some cultivars, but decreased or did not change in others.

The ripening-related enhanced LOX pathway activity in fruit is most likely associated with an increase in expression of lipoxigenase genes (Griffiths et al., 1999). The gene most likely to control fruit LOX activity during ripening is TomLoxC, given that TomLoxD is not expressed in fruit (Heitz et al., 1997), and antisense-mediated silencing of TomLoxA and TomLoxB did not result in a change in volatile composition (Griffiths et al., 1999b). Further, Chen et al. (2004) demonstrated that suppression of TomLoxC, which codes for a chloroplast-targeted lipoxigenase, markedly suppresses the biosynthesis of C-6 aldehydes in tomato homogenates. The impact of TomLoxC on leaf volatiles was not reported.

Another enzyme in the pathway, HPL, is considered to undergo little change in activity during tomato fruit ripening (Riley et al., 1996). However, differences in the relative aldehyde composition (Fig. 4) between the two stages of fruit, indicate a marked increase in the production of 18:3 oxidation products (e.g., Z-3-hexenal) without a similar increase in the formation of hexanal from 18:2. This developmental change in substrate preference in the LOX pathway is most likely related to HPL, which has a higher affinity for 13-hydroperoxide of 18:3 (13-HPOT) than 13-hydroperoxide of 18:2 (13-HPOD) (Fauconnier et al., 1997; Howe et al., 2000; Suurmeijer et al., 2000). Our data therefore imply a change in HPL activity in vivo, contrary to the findings of Riley et al. (1996). The large increase in 18:3-derived C-6 volatiles relative to 18:2-derived C-6 volatiles is likely not related to LOX enzymes, which have been shown to have no specificity for 18:2 or 18:3 (Feussner and Wasternack, 2002).

The large increase in C-6 volatile production by ripening wild type tomato fruit relative to immature fruit with no change in FA content or composition suggests that an increase in volatile biosynthesis during ripening is not strictly a function of FA substrate availability. Nevertheless, FA substrate levels did impact the volatile profile since the near elimination of ω-3 FAs as a substrate for the LOX pathway did impact the capacity for synthesis of C-6 volatiles.

The perception of differences in the volatile profile of wild type and Lefad7 leaves and fruit by a sensory panel is the first demonstration that alteration in FA composition under isogenic background can alter the aroma of vegetative or reproductive tissue in tomato. The LeFad7 mutation did not appear to negatively impact LOX pathway performance, thus perceived quality changes are attributable to a single factor, the balance between dienoic and trienoic fatty acids. It is not certain, however, whether panelists were responding to a loss in 18:3-related volatiles or to an increase in 18:2-related volatiles. Likely, a trained panel would be needed to make this determination.

The data for chemical and sensory impacts of the reduction in the activity of LeFAD7 highlight an important feature of manipulating single genes in this and other metabolic pathways. Despite the relative simplicity of the LOX pathway for volatile formation, a single gene change impacts multiple compounds, suggesting that the enhancement of a single desirable compound or class of compounds, may not be achievable. While the minimal unit of change is a single gene, the minimal scope of change is likely to be numerous products of the pathway impacted. Thus, it might be difficult to develop, for instance, tomato lines in which a single volatile compound is enhanced or suppressed.

The current work demonstrated that in vivo reduction of 18:3 content in tomato tissue reduces the biosynthesis of several of the most important volatile compounds in tomato aroma, including the volatile with the highest log-odor units, Z-3-hexenal, but it does not reduce the capacity of C-6 aldehyde and alcohol production by the LOX pathway. The altered concentration of LOX pathway oxidation products, mainly aldehydes, was detected by the olfactory sense of humans, affecting the sensory
quality of tomato fruit. The current results open the possibility of improvement of tomato fruit sensory quality by changing FA composition. An increase in 18:3 content and/or the rate of oxidation of linolenic acid may result in an increase in the relative concentration of the most important tomato volatile compound, Z-3-hexenal. An increase in 18:3 content may also be attended by increases in non-target compounds such as 1-penten-3-one and other C-5 volatiles from fatty acid degradation, which may also impact fruit quality attributes and may have unintended impacts on physiological processes associated with oxylipins.

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