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Relationship of Ethylene Biosynthesis to Volatile Production, Related Enzymes, and Precursor Availability in Apple Peel and Flesh Tissues

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Regulation of ethylene biosynthesis or action has a major effect on volatiles production in apples. To understand the biochemical processes involved, we used Greensleeves apples from a transgenic line with a high suppression of ethylene biosynthesis. The study was focused at the level of the aroma volatile-related enzymes, including alcohol acyltransferase (AAT), alcohol dehydrogenase (ADH), and lipoxygenase (LOX) and at the level of amino acids and fatty acids as aroma volatile precursors in peel and flesh tissues. In general, volatile production, enzyme activity levels, and precursor availability were higher in the peel than the flesh and were differentially affected by ethylene regulation. AAT enzyme activity showed a clear pattern concomitant with ethylene regulation. Contrarily, ADH and LOX seem to be independent of ethylene modulation. Isoleucine, an important precursor of aroma compounds including 2-methylbutanoate esters, showed a major increase in the peel during ripening and responded significantly to ethylene regulation. Other important aroma volatiles precursors, like linoleic and linolenic acid, showed an accumulation during ripening associated with increases in aldehydes. The significance of these changes in relation to aroma volatile production is discussed.

KEYWORDS: Aroma volatiles; fatty acids; amino acids; *Malus domestica*; alcohol acyltransferase; transgenic apple fruit

INTRODUCTION

Fruit aroma is a complex trait, particularly in terms of the number of different biosynthetic pathways involved, accumulation of the final metabolites, and their regulation (*1*, *2*). In apple and other fruits, most of the studies have focused on the last step of ester formation, in which alcohol acyltransferase (AAT) enzymes have a key role (*2*−*5*). In apples, the activity of AAT appears to increase with the onset of ripening, followed by a decrease in extractable activity (*2*). Ester biosynthesis in apple is also limited by the alcohol concentration (*6*), which suggests that a critical step for ester formation may be regulated at the level of alcohol dehydrogenase (ADH) or lipoxygenase (LOX) enzymes (*7*).

Another important aspect of the aroma biosynthetic pathway is the availability of primary precursor substrates, including fatty acids and amino acids, which are highly regulated in terms of their amount and composition during fruit development (*8*, *9*). In general, fatty acids are considered to be major precursors of aroma volatiles in apples, and the biosynthetic pathway includes β-oxidation, hydroperoxyacid cleavage, and LOX action to form the respective aldehydes, ketones, acids, alcohols, and esters from lipids (*1*, *10*−*13*). Transgenic modification of fatty acid biosynthesis in plant tissues resulted in significant changes in aroma compounds (*14*). Levels of fatty acids are also highly regulated during fruit development, where they accumulate during apple ripening, especially during the climacteric peak, followed by a decline because of changes in lipid metabolism (*9*, *15*).

Amino acids are also involved in aroma biosynthesis in fruit, and their metabolism is responsible for the production of a broad number of compounds including alcohols, carbonyls, acids, and esters (*1*). The most important amino acids directly responsible for generating aroma compounds are alanine, valine, leucine, isoleucine, phenylalanine, and aspartic acid (*1*, *16*, *18*). Addition of amino acid precursors to apple tissue slices has been shown to increase the levels of total aroma volatile production (*17*). In apple, it has been shown that aspartic acid is the most abundant free amino acid followed distantly by glutamic acid, serine, and phenylalanine (*8*). Despite the importance of amino acids as potential substrates for volatile production, their levels cannot always be related to the formation of a specific aroma compound, which suggests the important role of enzymes upstream of AAT in the formation of volatiles (*19*, *20*).

Considering the importance of both aroma-related enzymes and primary substrates for aroma formation, there is little

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information about the regulation of these processes. During ripening, fatty acids accumulate in apple concomitantly with the climacteric peak, suggesting a role of ethylene as a significant regulator of fatty acid accumulation (*10*, *21*). Recently, we demonstrated the importance of ethylene in regulating overall aroma production using transgenic apple lines with a high suppression of ethylene biosynthesis (*22*, *23*). We observed that the AAT enzyme is an important point in the modulation of ester production under ethylene regulation. Additionally, changes observed in alcohol and aldehyde levels suggested that events upstream of the AAT enzyme step were also under ethylene regulation, including availability of precursors or other enzymatic steps. Therefore, the aim of the present work was to investigate some potential biochemical steps involved in the modulation of ester production under ethylene regulation, considering both aroma volatile-related enzymes and the availability of precursors responsible for their synthesis.

MATERIAL AND METHODS

Plant Material and Treatments. Transgenic Greensleeves apple fruits suppressed in ethylene biosynthesis were obtained from different lines grown in an experimental orchard in Northern California. The lines used in these experiments were transformed using binary vectors that express the cDNAs corresponding to ACC-oxidase (ACO) enzymes in an antisense orientation and have shown levels of ethylene inhibition higher than 95% (*22*, *23*). Fruits of selected Greensleeves apple lines including transgenic 68G (ACO-antisense) and nontransformed fruit (GS) were sampled at harvest and periodically during 21 days of storage at 20 °C in air (ethylene-free atmosphere). The lines were harvested when the GS fruit was in a preclimacteric stage (internal ethylene concentration lower than 0.3 μL L^{-1}). Relative humidity was maintained close to 90−95%. After 13 days of storage, fruit from the 68G line were treated with ethylene. Half of the fruit was kept at 20 °C in an ethylene-free atmosphere, and the other half was stored at 20 °C under a flow of air containing 80 μL L^{-1} ethylene during storage. Because differences in enzyme activities, fatty acids, and amino acids have been observed among fruit tissues, peel and cortical tissues were carefully separated (avoiding any contamination between tissues) and frozen in liquid N_2 and kept at −80 °C until analysis. For all biochemical analysis, three replicates of five fruit each were used.

Rates of Ethylene Production and Respiration Measurements. Within each experiment, ethylene production and respiration rates were determined on individual fruits using a static system every other day during storage. Five fruits from each replicate (three replicates per treatment) were weighed and placed in 0.5 L jars at 20 °C. The jars were sealed for 30 min before measurements. Carbon dioxide and ethylene concentrations were determined by an infrared gas analyzer (Horiba, Irvine, CA) and a Carle gas chromatograph (Hach Carle, Loveland, CO) equipped with a flame ionization detector, respectively.

Determination of Volatiles. Apple cortical and skin tissues were ground using liquid nitrogen and kept at −80 °C until analysis. A total of 6 g of the tissue was homogenized in a Polytron homogenizer in the presence of 12 mL of water with 2 mM NaF (to avoid enzymatic browning). The homogenized tissue was filtered through four layers of cheesecloth and centrifuged (20000*g* for 20 min) at 4 °C. A total of 10 mL of the supernatant was placed into crimp-seal 40 mL vials containing 2 g of NaCl (to facilitate the release of volatiles compounds) (*23*, *24*). Prior to sealing the vials, 600 μL of the internal standard (IS) solution (1-octanol) was added, to obtain a 500 nL L^{-1} final concentration of the IS. A poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB, 65 μm thickness) SPME fiber was used. For SPME conditions, an equilibration at 50 °C for 30 min and desorption for 4 min were used. A GC−MS system equipped with a DB-Wax column (J&W Scientific, 30 m, 0.32 mm inside diameter, 0.25 μm film thickness) was used for analysis. Conditions for chromatography were as follows: injector at 250 °C; initial oven temperature at 40 °C held for 5 min, increased to 50 °C at 2 °C min^{-1}, increased to 200 °C at 5 °C min^{-1}, and held for 5 min. Linear velocity of the carrier gas was 35 cm s^{-1} (*23*, *25*). Mass spectra were obtained by electron ionization at 70 eV, and a spectra range of 40−250 *m*/*z* was used (*26*).

Identification of compounds was confirmed by comparison of collected mass spectra with those of authenticated reference standards when available and spectra in the National Institute for Standards and Technology (NIST) mass spectra library. The following standards were purchased from Aldrich (Sigma Aldrich): hexanal, (2E)-hexenal, hexyl hexanoate, hexyl 2-methylbutanoate, and hexyl butanoate. The standards 1-butanol, 1-hexanol, and butyl butanoate were purchased from Fluka (Sigma Aldrich).

Alcohol Acyltransferase Activity. Apple cortical (flesh) and epidermis (skin) tissues (3 g) were frozen in liquid nitrogen and homogenized with mortar and pestle in 2 mL g^{-1} (tissue) of 100 mM potassium phosphate buffer (pH 7.0) and 0.33 mg g^{-1} of polyvinylpolypirrolidone (PVPP). Once the slurry melted, it was filtered through four layers of cheesecloth and centrifuged at 28000*g* for 20 min. The supernatant was recovered and used for the enzyme assay. The assay was performed using the spectrophotometric method developed by Fellman and Mattheis (*27*). Assay components in the final concentration were 670 μL of potassium phosphate buffer (10 mM at pH 7.0), 10 μL of magnesium chloride (1 M), 100 μL of 5,5′-dithiobisnitrobenzoic acid (DTNB, 10 mM) in pH 7.0 phosphate buffer, 10 μL of 1-butanol (20 mM), and 10 μL of acetyl CoA (0.49 mM). The reaction was assayed by mixing compounds in a cuvette to a total volume of 1 mL. Progress of the esterification reaction of butanol to butyl acetate was followed by monitoring solution absorbance at 412 nm, the absorbance maximum of the thiophenol product formed by the reaction of DTNB with free CoA (*27*). One activity unit (U) was defined as the increase in one unit of absorbance per minute, and results were expressed as specific activity (mU mg^{-1} of protein) (*7*).

Alcohol Dehydrogenase Activity. For each replicate, 3 g of cortical and skin tissue was obtained and homogeneized in a Polytron homogenizer in 10 mL of 100 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer (pH 6.5) containing 2 mM dithiothreitol and 1% (w/v) polyvinylpyrolidone (PVP). The homogenate was filtered through four layers of cheesecloth and centrifuged at 27000*g* for 15 min (*28*). The supernatant was recovered and used for the enzyme assay. The reduction of acetaldehyde was followed spectrophotometrically at 25 °C by measuring the change in absorbance at 340 nm for 2 min of a reaction mixture containing 800 μL of MES buffer (100 mM at pH 6.5), 50 μL of NADH (1.6 mM), 100 μL of enzyme extract, and 50 μL of acetaldehyde (80 mM). The reaction was initiated by the addition of acetaldehyde (*28*, *29*). One activity unit (U) was defined as the increase in one unit of absorbance per minute, and results were expressed as specific activity (U mg^{-1} of protein) (*7*).

Lipoxygenase Activity. For extraction, 3 g of peel or flesh tissue was homogenized in a Polytron homogenizer in 6 mL of 100 mM phosphate buffer (pH 7.5), containing 2 mM dithiothreitol, 0.1% (v/v) Triton X-100, and 1% (w/v) PVPP. The homogenate was filtered through four layers of cheesecloth and centrifuged at 25000*g* for 15 min at 4 °C. The supernatant was recovered and used for the enzyme assay. LOX activity was assayed spectrophotometrically according to Echeverría et al. (*7*), by mixing 880 μL of 0.1 M phosphate at pH 6.5 and 150 μL of substrate solution (8.6 mM linolenic acid, 0.25% (v/v) Tween-20, and 10 mM NaOH, in 0.1 M phosphate at pH 8.0) and adding 10 μL enzyme extract. The activity was followed by measuring the increase in absorbance at 234 nm over time, because of the formation of hydroperoxides from linolenic acid. One activity unit (U) was defined as the increase in one unit of absorbance per minute, and results were expressed as specific activity (U mg^{-1} of protein) (*7*).

Fatty Acid Analysis. The total fatty acid profile was determined by fatty acid methyl ester (FAME) analysis. Briefly, 0.5 g of freeze-dried and powdered tissue (peel and cortical tissues) were extracted overnight with gentle shaking in 300 μL of toluene at room temperature. After that, transesterification was done by adding 500 μL of methanolic 0.5 N sodium methoxide. Then, 50 μL (10 mg/mL in toluene) of C17:0 internal standard was added and allowed to react at room temperature for 1 h on an orbital shaker. FAME reactions were terminated by the addition of 500 μL of a 10% NaCl solution. Then, 400 μL of heptane were added, and after shaking and centrifuging the contents, an aliquot of the upper hexane phase containing the FAME was removed in an

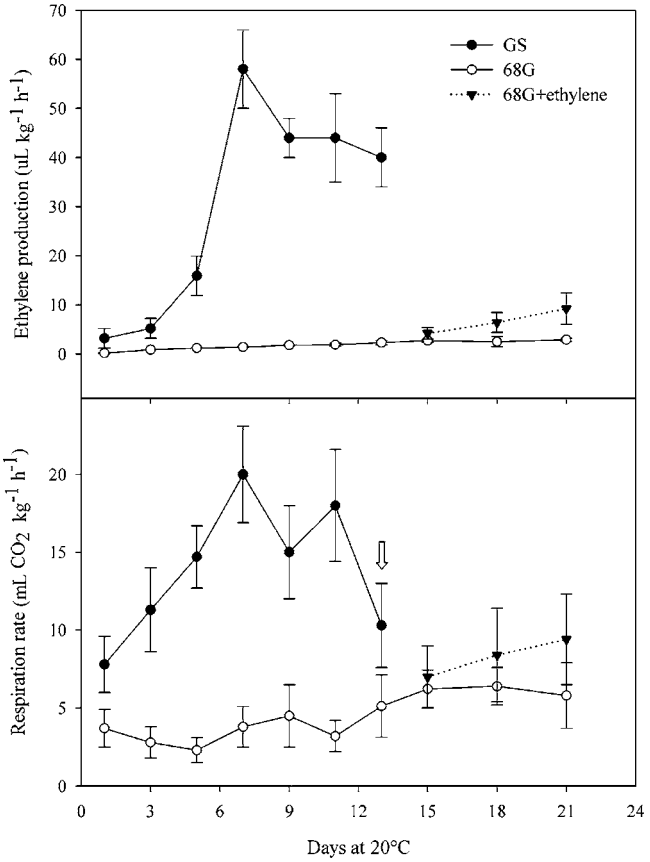

Figure 1. Ethylene production and respiration rates (means of 3 replicates ± SE) of two Greensleeves apple lines (GS, nontransformed line; 68G, ACO antisense) held at 20 °C for 21 days. The arrow indicates the beginning of application of 80 μL L^{-1} ethylene to 68G.

autosampler vial for GC−MS analysis. A GC−MS system equipped with a DB-Wax column (Agilent, 30 m, 0.25 mm inside diameter, 0.25 μm film thickness) was used for analysis. Conditions for chromatography were injector at 250 °C, initial oven temperature started at 50 °C, increased to 200 °C at 25 °C min^{-1}, increased to 230 °C at 3 °C min^{-1}, and held for 4 min, with 20 min of total running time. Mass spectra were obtained by electron ionization at 70 eV. MS Source temperature was 200 °C, and MS Quad temperature was 180 °C. Identification of compounds was confirmed by a comparison of collected mass spectra with those of authenticated reference standards.

Amino Acids Determination. For extraction, 3 g of peel or flesh tissue was homogenized in a Polytron homogenizer in 6 mL of 70% ethanol (*30*). The homogenate was filtered through four layers of cheesecloth and centrifuged at 25000*g* for 10 min at 4 °C. The supernatant was recovered and sent to the Molecular Structure Facility, UC Davis, for amino acid analysis (*31*). Briefly, samples were dissolved in Li citrate buffer containing 100 nmol mL^{-1} aminoethyl cysteinyl (AECys), which was used as the internal standard to correct for any variation in the operating conditions of the analyzer over time. Amino acid content was quantified using a Beckman 6300 (Li-based) analyzer equipped with the software Beckman System Gold that identifies any peak that falls within predetermined limits of known amino acid retention times. The standard injection volume was 50 μL. The amino acid composition was calculated and expressed as mole percent.

RESULTS AND DISCUSSION

Ethylene Biosynthesis and Respiration Rate. Ethylene production and respiration rates were strongly reduced in the transgenic line 68G, with a complete suppression or delay of the climacteric peak for up to 21 days at 20 °C (**Figure 1**). The addition of 80 μL L^{-1} of ethylene only produced a minor increase in the ethylene production, supporting the importance

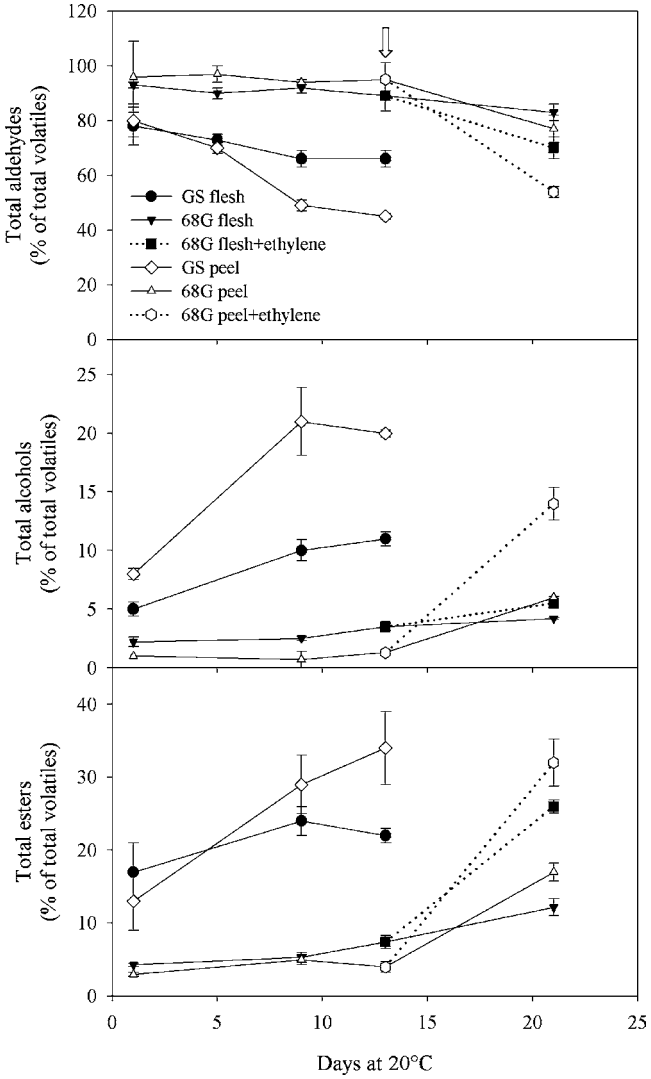

Figure 2. Relative content (means of 3 replicates ± SE) of volatile compounds of two Greensleeves apple lines (GS, nontransformed line; 68G, ACO antisense) held at 20 °C for 21 days. The arrow indicates the beginning of exposure to 80 μL L^{-1} ethylene.

of ACO in controlling ethylene biosynthesis in Greensleeves apple (*22*). The application of ethylene increased the rate of respiration after 13 days at 20 °C in the transgenic lines to nearly that of the nontransformed line (GS). This indicates that fruit from the 68G line were harvested at the appropriate maturity stage as indicated by their response to ethylene application, which is also supported by the development of other quality parameters including color and soluble solid content (data not shown).

Volatile Production under Ethylene Regulation. The volatile profile of Greensleeves apples was dominated by aldehydes at harvest and by esters and alcohols after 13 days at 20 °C (**Figure 2**). These changes in volatiles were more significant in peel tissue as compared to flesh tissue and with a higher presence of esters and alcohols in the peel (**Tables 1** and **2**). For aldehydes, we have consistently observed an increase between harvest and after holding for up to 21 days at 20 °C, with an accumulation of (2E)-hexenal rather than hexanal (*22*, *23*). Hexyl butanoate and hexyl 2-methyl butanoate were the most abundant esters in Greensleeves apple, followed by butyl butanoate and butyl 2-methylbutanoate, with a similar trend between the different fruit tissues examined. Under ethylene suppression conditions, fruit from the 68G line showed a major

Table 1. Volatile Composition (nL L^{-1}; Means of 3 Replicates ± SE) from Flesh Tissue of Two Greensleeves Apple Lines Evaluated at Harvest and after 21 Days at 20 °C[a]

	GS[b]			68G				
compounds	initial	9 d	13 d	initial	9 d	13 d	21 d	21 d + C_2H_4
hexanal	204 ± 21	200 ± 7	250 ± 9	264 ± 38	252 ± 3	225 ± 8	256 ± 14	253 ± 17
(2E) hexenal	80 ± 16	140 ± 10	168 ± 6	24 ± 2	31 ± 5	65 ± 15	180 ± 18	170 ± 4
butanol	0	22 ± 2	40 ± 5	ND[c]	ND	ND	8 ± 0.5	15 ± 0.4
2-methylbutanol[d]	6 ± 2	15 ± 3	24 ± 1	ND	ND	ND	3 ± 0.2	22 ± 7
hexanol	26 ± 4	106 ± 17	123 ± 7	3 ± 0.4	2 ± 0.2	4 ± 0.4	24 ± 2	65 ± 5
butyl butanoate	6 ± 2	27 ± 1	48 ± 5	ND	ND	ND	6 ± 2	32 ± 4
butyl 2-methylbutanoate[d]	3 ± 1	11 ± 1	20 ± 2	ND	ND	ND	4 ± 0.3	16 ± 2
hexyl butanoate	22 ± 9	101 ± 15	153 ± 30	4 ± 0.4	8 ± 1	5 ± 1	38 ± 7	94 ± 8
hexyl 2-methylbutanoate	10 ± 3	49 ± 10	72 ± 15	ND	ND	ND	12 ± 2	61 ± 8
hexyl propanoate[d]	0	11 ± 2	18 ± 1	ND	ND	ND	13 ± 1	19 ± 1
hexyl hexanoate	11 ± 1	13 ± 2	17 ± 1	5 ± 1	6 ± 2	8 ± 2	15 ± 1	26 ± 3

[a] Note that quantification is relative to 1-octanol (internal standard). [b] GS, nontransformed line; 68G, ACO antisense. [c] ND = not detected. [d] Tentatively identified.

Table 2. Volatile Composition (nL L^{-1}; Means of 3 Replicates ± SE) from Peel Tissue of Two Greensleeves Apple Lines Evaluated at Harvest and after 21 Days at 20 °C[a]

	GS[b]			68G				
compounds	initial	9 d	13 d	initial	9 d	13 d	21 d	21 d + C_2H_4
hexanal	670 ± 80	597 ± 30	702 ± 23	844 ± 20	843 ± 27	755 ± 35	605 ± 20	650 ± 40
(2E) hexenal	314 ± 20	562 ± 27	528 ± 23	99 ± 10	162 ± 5	290 ± 34	619 ± 15	678 ± 40
butanol	7 ± 1	30 ± 4	30 ± 1	ND	ND	ND	5 ± 1	14 ± 2
2-methylbutanol[c]	ND[d]	22 ± 4	30 ± 1	ND	ND	ND	ND	18 ± 1
hexanol	56 ± 8	124 ± 18	152 ± 10	22 ± 4	27 ± 2	41 ± 4	56 ± 1	75 ± 5
butyl butanoate	30 ± 8	67 ± 4	79 ± 8	ND	9 ± 2	20 ± 2	40 ± 4	120 ± 14
butyl 2-methylbutanoate[c]	21 ± 6	40 ± 2	45 ± 1	ND	2 ± 0.2	4 ± 1	4 ± 1	58 ± 5
hexyl butanoate	104 ± 20	150 ± 21	145 ± 5	10 ± 1	22 ± 4	40 ± 6	56 ± 6	156 ± 6
hexyl 2-methylbutanoate	55 ± 10	117 ± 14	111 ± 4	10 ± 1	11 ± 2	13 ± 2	32 ± 2	111 ± 8
hexyl propanoate[c]	30 ± 6	35 ± 1	33 ± 1	3 ± 0.5	10 ± 1	12 ± 1	25 ± 2	28 ± 5
hexyl hexanoate	17 ± 5	38 ± 4	32 ± 5	11 ± 2	4 ± 1	9 ± 1	20 ± 2	31 ± 3

[a] Note that quantification is relative to 1-octanol (internal standard). [b] GS, nontransformed line; 68G, ACO antisense. [c] Tentatively identified. [d] ND = not detected.

reduction in all groups of volatiles, especially esters and alcohols, with the levels being similar to the ones observed at harvest in the nontransformed line. Exposure of 68G fruit to ethylene for 8 days led to the recovery of the levels of alcohols and esters similar to the ones observed in the nontransformed line. These observations support the findings of earlier studies that ethylene is a modulator of volatiles responsible of aroma production (*23*, *32*, *33*). However, we also observed a small increase in volatile compounds in the 68G fruit not treated with ethylene, while the fruit was held in an ethylene-free environment over time. This indicates that there are regulatory processes other than ethylene involved in aroma production. Moreover, we did not observe any differences in aldehyde production when 68G fruit was exposed to ethylene, and therefore, we obtained the same hexanal/(2E)-hexenal ratio after 21 days at 20 °C. This suggests that some steps of aroma formation are independent of ethylene regulation.

Aroma Volatile-Related Enzymes. The levels of AAT enzyme activity were higher in the nontransformed fruit as compared to the 68G line and showed an increase between harvest and after 13 days at 20 °C (**Figure 3**). Moreover, the activity levels in peel tissue were higher than in flesh for both lines through the evaluation period. A similar observation was made for AAT levels in Fuji apples (*7*). Interestingly, 68G fruit treated with ethylene showed a sharp rise in AAT enzyme activity levels and ester accumulation. However, we have also consistently found a relatively high level of enzyme activity in 68G fruit at harvest accompanied with low levels of ester accumulation. This supports the hypothesis about the importance of other mechanisms including enzymatic activity (other than AAT), availability of substrates (alcohols and acids, for example), or initial precursors such as fatty acids or amino acids (*7*, *9*).

Immediate substrates, including butanol, hexanol, and 2-methyl butanol, increased similarly with ester production as ripening progressed, which may explain the low levels of ester production at harvest in both peel and flesh tissues (**Tables 1** and **2**). The enzyme ADH, responsible for the interconversion between aldehydes and alcohols, initially increased and then gradually declined in peel tissue or remained steady in flesh tissue in both lines during the holding period (**Figure 3**). ADH enzyme activity levels were higher in the fruit from the 68G line as compared to the control GS and did not change or declined slightly after the exposure to ethylene. This pattern was not associated with the changes observed in alcohol levels. This supports the idea that ADH enzyme activity may not limit alcohol supply for ester production during ripening (*7*, *34*). If ADH enzyme activity is not considered a limiting factor, the higher levels of activity observed in the peel do not explain the higher levels of alcohols observed in this tissue relative to the flesh. This indicates that upstream events involved in alcohol supply are important in this regulation, including aldehyde availability, other aroma-related enzymes, and precursor availability (fatty acids and amino acids).

The only significant change in aldehyde availability was the increase of (2E)-hexenal during ripening (**Tables 1** and **2**), which showed in general an ethylene-independent pattern. In contrast, hexanal did not show any significant change during ripening in both lines. If, as some have suggested, the lipoxygenase pathway has an important role in the generation of volatiles during

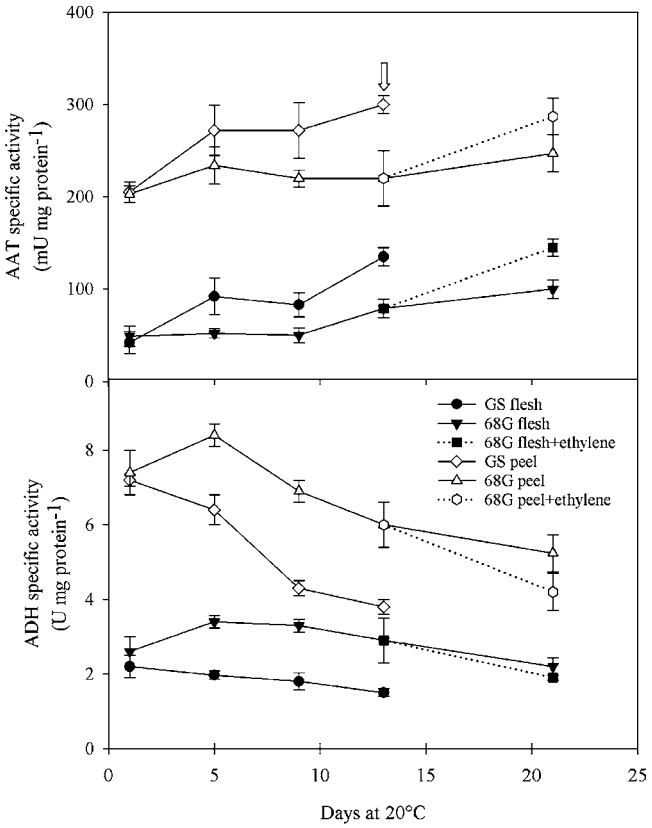

Figure 3. AAT and ADH activities (means of three replicates ± SE) of two Greensleeves apple lines (GS, nontransformed line; 68G, ACO antisense) stored at 20 °C for 21 days. The arrow indicates the beginning of application of 80 μL L^{-1} ethylene.

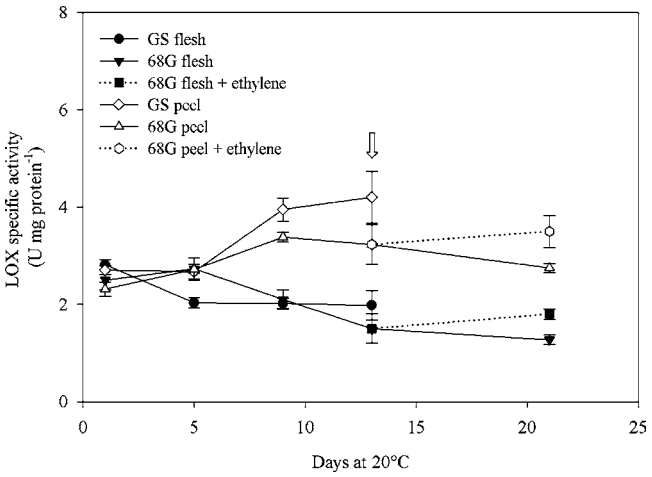

Figure 4. LOX activity (means of three replicates ± SE) of two Greensleeves apple lines (GS, nontransformed line; 68G, ACO antisense) stored at 20 °C for 21 days. The arrow indicates the beginning of application of 80 μL L^{-1} ethylene.

ripening (*1*, *10*), then the increase in (2E)-hexenal may have been due to either regulation in the enzyme cascade, including LOX, hydroperoxide lyase, or isomerase, or through the increase in the levels of the precursor linoleic acid (*1*, *35*). LOX enzyme activity levels showed a minor increase during the holding period (**Figure 4**), especially in fruit obtained from the GS line and especially in peel tissue, which was not associated with the much lower levels of aldehydes observed in fruit from the 68G line. Moreover, the exogenous application of ethylene only caused minor increases in LOX activity in fruit from the 68G line,

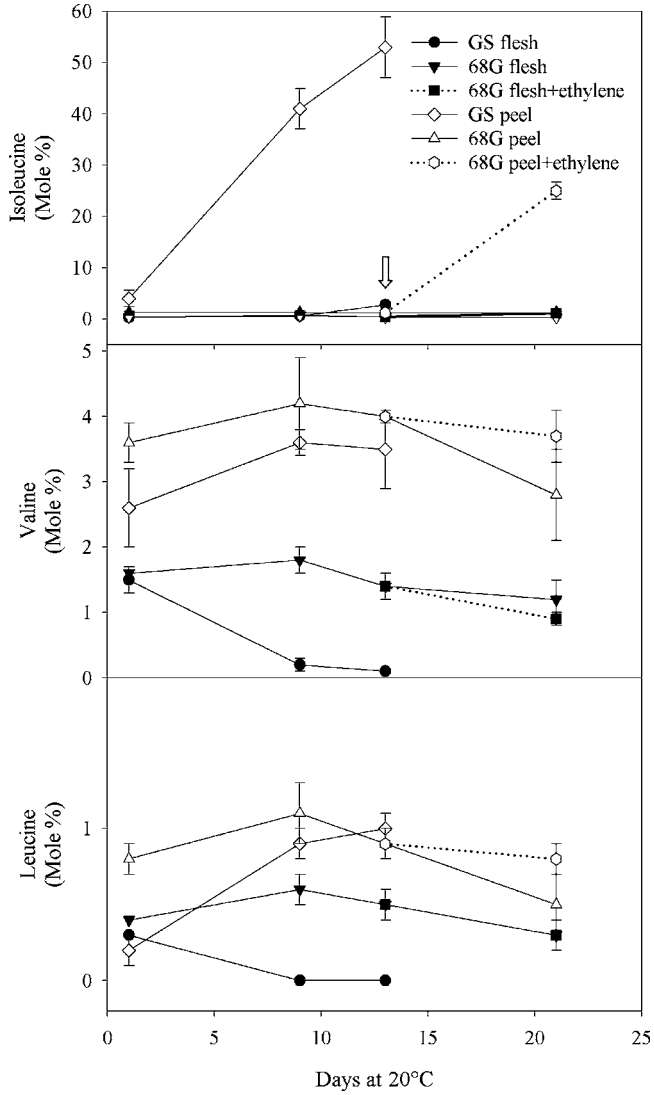

Figure 5. Content (mol %; means of 3 replicates ± SE) of isoleucine, valine, and leucine in two Greensleeves apple lines (GS, nontransformed line; 68G, ACO antisense) held at 20 °C for 21 days. The arrow indicates the beginning of exposure to 80 μL L^{-1} ethylene.

which was also not related to a significant increase in the levels of aldehydes. Because the LOX enzyme can use as a substrate both linoleic and linoleic acid, just differences in substrate specificity or substrate supply could explain the difference in aldehyde accumulation. Therefore, our data suggest that LOX enzyme activity is not a limiting step for aldehyde accumulation, as was suggested by Griffiths et al. (*35*). Other enzymes in the pathway, such as hydroperoxide lyase, that have shown substrate specificity may play a role in the generation of volatiles; however, further work should be done to elucidate their role during ripening of apple fruit.

This analysis involving the three most important enzymes involved in aroma biosynthesis allows us to suggest that, despite the importance of the AAT enzyme as a control step under ethylene regulation, aroma precursors (fatty acids and amino acids) also seem to play an important role generating aldehydes, alcohols, and acids for ester accumulation.

Amino Acids Levels. The importance of amino acids as aroma precursors has been studied and recognized in apples and other fruits (*17*, *20*, *36*). However, despite the importance of amino acids as a substrate for the production of volatiles and other secondary metabolites, only limited information about

Table 3. Mole Fraction (3 Replicates ± SE) of Amino Acids from Flesh Tissue of Two Greensleeves Apple Lines Evaluated at Harvest and after 21 Days at 20 °C

	GS[a]			68G				
amino acids	initial	9 d	13 d	initial	9 d	13 d	21 d	21 d + C_2H_4
taurine	0.6 ± 0.0	3.3 ± 1.0	1.3 ± 0.2	0.4 ± 0.1	0.6 ± 0.1	0.6 ± 0.1	0.8 ± 0.2	0.6 ± 0.0
aspartic acid	82.6 ± 0.6	125.3 ± 15.9	104.4 ± 12.8	67.5 ± 8.7	83.3 ± 9.3	79.4 ± 7.4	73.4 ± 5.8	70.4 ± 5.2
threonine	6.8 ± 0.1	4.6 ± 0.7	3.8 ± 0.1	8.6 ± 1.4	7.4 ± 0.2	6.9 ± 0.9	5.2 ± 0.7	6.0 ± 0.5
serine	17.2 ± 0.7	10.5 ± 1.6	9.3 ± 1.0	19.3 ± 1.8	18.4 ± 1.5	17.7 ± 2.3	12.5 ± 1.5	15.6 ± 1.0
asparagine	345.1 ± 5.0	329.0 ± 14.0	344.8 ± 14.6	342.6 ± 12.5	335.7 ± 16.9	352.3 ± 7.6	379.0 ± 6.7	377.4 ± 10.4
glutamic acid	13.7 ± 3.1	15.1 ± 3.7	22.3 ± 4.6	22.2 ± 1.6	24.1 ± 2.8	18.3 ± 1.7	8.0 ± 0.6	4.5 ± 0.3
glutamine	2.9 ± 0.3	1.4 ± 0.3	2.3 ± 0.3	3.3 ± 0.1	1.2 ± 0.2	1.0 ± 0.2	0.8 ± 0.1	0.6 ± 0.1
proline	3.8 ± 0.8	0.3 ± 0.0	0.5 ± 0.1	6.2 ± 0.5	5.1 ± 1.0	5.8 ± 1.6	3.8 ± 0.7	5.9 ± 2.1
glycine	1.3 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	1.3 ± 0.1	1.3 ± 0.2	0.9 ± 0.2	0.7 ± 0.2	1.1 ± 0.1
alanine	12.8 ± 0.5	4.8 ± 2.0	3.2 ± 0.8	14.9 ± 1.3	6.8 ± 1.9	8.1 ± 0.5	7.9 ± 0.1	7.8 ± 1.4
valine	1.5 ± 0.2	0.2 ± 0.1	0.1 ± 0.0	1.6 ± 0.1	1.8 ± 0.2	1.4 ± 0.2	1.2 ± 0.3	0.9 ± 0.1
methionine	1.9 ± 0.2	0.7 ± 0.1	1.0 ± 0.0	3.0 ± 0.4	2.6 ± 0.5	2.0 ± 0.3	1.5 ± 0.1	1.0 ± 0.2
isoleucine	0.3 ± 0.0	0.6 ± 0.2	2.8 ± 0.5	0.4 ± 0.0	0.7 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	1.1 ± 0.3
leucine	0.3 ± 0.0	0	0	0.4 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
phenylalanine	0.6 ± 0.1	0	0	0.6 ± 0.1	0.6 ± 0.0	0.5 ± 0.1	0.2 ± 0.1	0
alanine	1.9 ± 0.4	0	0	1.7 ± 0.2	1.7 ± 0.1	1.8 ± 0.5	0	2.1 ± 0.3
aminobutyric acid	5.5 ± 1.0	2.8 ± 0.7	2.0 ± 0.2	5.8 ± 0.8	5.3 ± 1.0	4.6 ± 0.7	3.2 ± 0.7	5.4 ± 0.8
lysine	0.4 ± 0.1	0.1 ± 0.0	0.6 ± 0.1	0.8 ± 0.2	1.0 ± 0.1	0.8 ± 0.1	0.4 ± 0.0	0.3 ± 0.0
histidine	0.8 ± 0.2	0.6 ± 0.2	0.7 ± 0.2	0.7 ± 0.1	0.6 ± 0.1	0.4 ± 0.0	0	0.2 ± 0.1
arginine	0.4 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.5 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	0.4 ± 0.1	0.2 ± 0.0
total	500 ± 131	500 ± 84	500 ± 41	502 ± 30	500 ± 36	504 ± 25	500 ± 18	502 ± 23

[a] GS, nontransformed line; 68G, ACO antisense.

Table 4. Mole Fraction (Means of 3 Replicates ± SE) of Amino Acids from Peel Tissue of Two Greensleeves Apple Lines Evaluated at Harvest and after 21 Days at 20 °C

	GS[a]			68G				
amino acids	initial	9 d	13 d	initial	9 d	13 d	21 d	21 d + C_2H_4
taurine	0.7 ± 0.4	1.9 ± 0.2	1.2 ± 0.3	1.0 ± 0.1	1.2 ± 0.1	1.1 ± 0.0	1.2 ± 0.1	1.3 ± 0.2
aspartic acid	92.1 ± 6.7	72.2 ± 1.6	64.8 ± 8.1	103.4 ± 5.8	151.4 ± 8.1	164.9 ± 5.6	124.7 ± 19.7	92.4 ± 13.9
threonine	12.9 ± 1.8	6.5 ± 0.3	6.2 ± 1.2	21.7 ± 3.3	18.8 ± 2.3	18.3 ± 4.2	12.9 ± 3.1	12.9 ± 2.5
serine	30.2 ± 6.0	15.2 ± 0.9	16.0 ± 3.8	58.0 ± 9.0	56.2 ± 9.3	59.1 ± 9.8	45.1 ± 10.3	39.1 ± 5.4
asparagine	446.7 ± 90.0	265.2 ± 10.2	213.1 ± 40.8	592.7 ± 50.5	724.6 ± 50.9	601.5 ± 36.31	771.8 ± 90.7	576.2 ± 95.8
glutamic acid	47.7 ± 7.1	45.2 ± 2.9	43.9 ± 1.3	70.6 ± 3.9	73.7 ± 3.2	59.3 ± 5.2	40.0 ± 4.3	33.2 ± 1.6
glutamine	2.5 ± 0.2	3.7 ± 0.5	3.5 ± 0.6	4.1 ± 0.6	4.2 ± 0.4	3.5 ± 0.4	1.8 ± 0.9	1.6 ± 0.3
proline	0	0	0	2.7 ± 0.3	2.5 ± 0.1	1.9 ± 0.6	0.5 ± 0.1	0
glycine	1.7 ± 0.3	1.3 ± 0.1	1.3 ± 0.1	3.0 ± 0.5	3.1 ± 0.5	3.5 ± 0.2	2.7 ± 0.7	2.0 ± 0.2
alanine	19.9 ± 3.1	9.9 ± 0.6	6.9 ± 0.1	35.9 ± 4.3	28.9 ± 5.1	28.8 ± 6.3	21.3 ± 4.1	13.7 ± 2.8
valine	2.6 ± 0.6	3.6 ± 0.2	4.0 ± 0.6	3.6 ± 0.3	4.2 ± 0.7	4.0 ± 0.1	2.8 ± 0.7	3.7 ± 0.4
methionine	1.4 ± 0.2	0.2 ± 0.1	0.4 ± 0.5	4.7 ± 0.5	5.9 ± 1.4	4.5 ± 0.7	3.1 ± 1.0	1.5 ± 0.5
isoleucine	4.0 ± 1.6	41.0 ± 3.9	52.9 ± 5.9	1.3 ± 0.2	1.3 ± 0.3	1.2 ± 0.1	1.3 ± 0.2	24.8 ± 1.7
leucine	0.2 ± 0.1	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.1	1.1 ± 0.2	0.9 ± 0.1	0.5 ± 0.2	0.8 ± 0.1
phenylalanine	0	0	0	1.0 ± 0.1	1.0 ± 0.2	0.2 ± 0.0	0	0
alanine	0	0	0	0	0	0	0	0
aminobutyric acid	4.1 ± 0.0	3.4 ± 0.7	3.3 ± 0.4	7.5 ± 0.4	5.3 ± 2.0	6.2 ± 0.1	4.4 ± 0.8	5.2 ± 1.7
lysine	0	0	0.2 ± 0.0	1.7 ± 0.5	1.8 ± 0.5	1.6 ± 0.3	0.8 ± 0.3	0.1 ± 0.0
histidine	0	0	0	0	0	0	0	0
arginine	0.3 ± 0.1	0	0	0.8 ± 0.3	1.8 ± 0.4	1.8 ± 0.2	1.6 ± 0.3	0
total	670 ± 107	470 ± 21	419 ± 60	913 ± 80	1087 ± 110	962 ± 71	1035 ± 120	808 ± 110

[a] GS, nontransformed line; 68G, ACO antisense.

the changes in amino acid levels during fruit ripening is available for apple (*19*). Because our results suggested that amino acids may play an important role in the changes observed in aroma levels, we studied the amino acid composition in peel and flesh tissues during storage at 20 °C (**Tables 3** and **4**). The most abundant amino acid was asparagine followed distantly by aspartic acid, glutamic acid, serine, and alanine, as was also observed by Ackermann et al. (*8*). In general, these levels were slightly higher in peel tissue than flesh, and only minor differences in composition were observed between the two tissues. In the nontransformed apples, there were only slight changes in total amino acid levels in the flesh during ripening, but we observed a 30% drop in amino acid levels of the peel during this period (**Tables 3** and **4**). In the transgenic lines, the total levels of amino acids did not show significant changes, and only through the application of ethylene was there a minor reduction. However, individual amino acids displayed different compositional patterns during 21 days at 20 °C.

The most important amino acids involved in the biosynthesis of aroma compounds in apples are the branched chain amino acids isoleucine, leucine, and valine (*17*, *19*), and their levels during storage for 21 days at 20 °C are shown in **Figure 5**. The most significant change was observed for isoleucine levels, which showed a major and sharp rise in the peel tissue during ripening, with more than a 10-fold increase between harvest and after 21 days at 20 °C. In contrast, the levels in the flesh

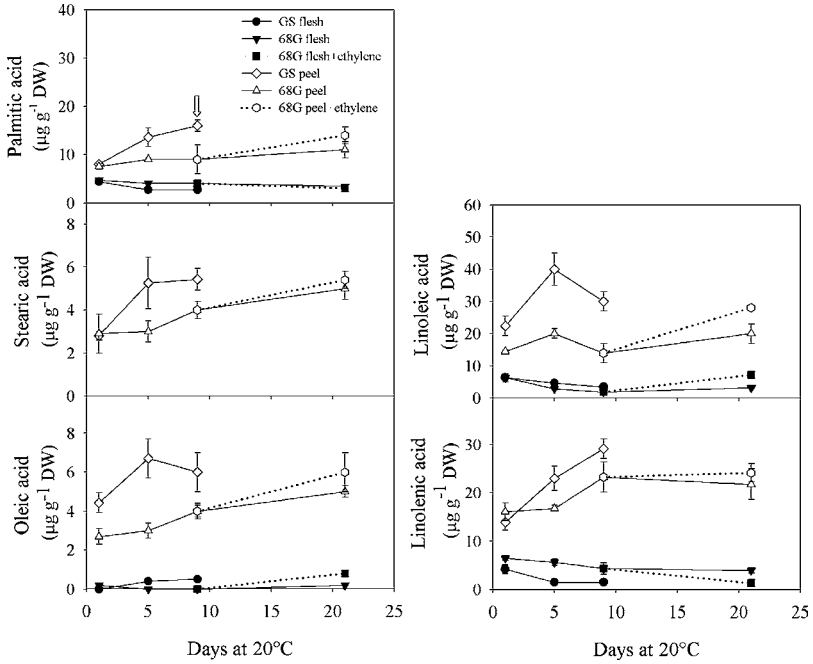

Figure 6. Concentrations of fatty acids (means of 3 replicates ± SE) in peel and flesh tissues of two Greensleeves apple lines (GS, nontransformed line; 68G, ACO antisense) held at 20 °C for 21 days. The arrow indicates the beginning of exposure to 80 μL L^{-1} ethylene.

were much lower and did not change with storage. This specific pattern of accumulation of isoleucine was previously reported for peel and flesh tissues (*37*). In fruit from the 68G lines, isoleucine levels stayed low and constant during the holding period and only showed a major increase after the application of ethylene. No major differences were observed in valine and leucine levels, with only slightly higher levels in the peel. Among aroma compounds, esters derived from isoleucine include 2-methylbutyl and 2-methylbutanoate esters (*17*, *36*). In Greensleeves apple, butyl 2-methylbutanoate and hexyl 2-methybutanoate are among the most important esters identified during ripening (*23*). As shown in **Table 2**, there was an accumulation of these compounds, especially during the first 10 days at 20 °C, when we also observed the highest rates of accumulation of isoleucine in the peel. Moreover, we only observed an increase of these esters in the transgenic lines after the application of ethylene, which was concomitant with the accumulation of isoleucine. If isoleucine is metabolized to aroma compounds, we would expect a reduction in its levels as previously observed (*38*); however, in apple peel, there is a continuous accumulation of isoleucine until late ripening (**Figure 5**). These observations seem to indicate an important role of isoleucine accumulation for ester formation during ripening. However, when we analyze ester accumulation patterns in the flesh, we observed that this accumulation is not connected with the steady levels of isoleucine and it is not clear if the low levels of isoleucine measured could explain the formation of the equivalent esters in this tissue (**Table 1**). Therefore, it seems that the complexity of biogenesis and/or regulation of ester formation differs between peel and flesh.

Fatty Acids as an Aroma Precursor. We have consistently observed in field data collected over the last 3 years that the composition of individual aldehydes behaves differently (*22*). Only (2E)-hexenal accumulates during ripening, keeping the levels of hexanal quite stable (**Tables 1** and **2**). In addition, in the transgenic line, the accumulation of (2E)-hexenal was delayed by ethylene suppression but did not show a clear response under exogenous ethylene application, which suggests an ethylene-independent pattern for its accumulation. Because LOX enzyme activity did not show significant changes explaining these differences, fatty acids were studied as potential primary precursors for aroma compounds. To analyze the changes in composition of total fatty acids in our lines, we focused the study on the ones that have previously shown a change during ripening, i.e., palmitic, stearic, oleic, linoleic, and linolenic acid (*9*, *21*). Because we have also observed important differences in the aroma biosynthetic pathway between peel and flesh tissues, we tested whether fatty acid composition may also have a different pattern among tissues, as has been reported earlier (*15*, *39*). As shown in **Figure 6**, fatty acids content was higher in the peel than in the flesh, with significant differences in the levels of linoleic and linolenic acid, which are involved in the production of hexanal and (2E)-hexenal, respectively (*1*). In peel tissue of the nontransformed fruit, most of the fatty acids accumulated during ripening. In the 68G fruit, fatty acid levels were lower and fatty acid accumulation was delayed. In general, the exogenous application of ethylene caused an increase in fatty acid levels, but this was only significant for linoleic acid. Contrary to the observed pattern for peel tissue, we did not observe any change in the levels of fatty acid in the flesh in either line during the evaluation period. The similar pattern of accumulation for linoleic and linolenic acid during ripening could explain the increase in total aldehydes, but not the consistent differences observed between hexanal and (2E)-hexenal accumulation. This supports the possibility that enzymes involved in the metabolism of fatty acids, through β-oxidation and/or lipoxygenase pathways, play an important role in determining aldehyde levels (*2*). This is also supported by the observation that in flesh tissue we observed changes in the hexanal/(2E)-hexenal ratio, without a significant change in fatty acid availability. Similar to the observed changes in isoleucine levels in the flesh, it would be interesting to demonstrate if the low levels of linoleic and linolenic acids relate to the amount of volatiles produced by this tissue.

In conclusion, the biogenesis of aroma compounds involves a series of changes during fruit ripening, and it has been demonstrated here that an important modulator of these changes is ethylene. In terms of ester biosynthesis, we have consistently

found that the AAT enzyme is under ethylene regulation and seems to play a role in determining ester formation. Other aroma-related enzymes showed mainly an ethylene-independent pattern. We also found that the availability of fatty acids and amino acids showed important changes associated with ester production under ethylene regulation, especially the amino acid isoleucine. The significant differences observed between peel and flesh in the levels of volatiles, precursors, and aroma-related enzymes indicate that the mechanism of regulation may also differ among tissues. It seems that, in flesh tissue, the AAT enzyme is a more important biochemical step than in the peel, in which the supply and metabolism of amino acids and fatty acids seems to be more critical.

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