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Developmental and Varietal Differences in Volatile Ester Formation and Acetyl-CoA: Alcohol Acetyl Transferase Activities in Apple (*Malus domestica* Borkh.) Fruit

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Apple (*Malus domestica* Borkh.) cultivars differ in their aroma and composition of volatile acetates in their fruit flesh and peel. Cv. Fuji flesh contains substantial levels of 2-methyl butyl acetate (fruity banana-like odor), while the flesh of cv. Granny Smith apples lacks this compound. Granny Smith apples accumulate mainly hexyl acetate (apple-pear odor) in their peel. Feeding experiments indicated that Fuji apples were able to convert hexanol and 2-methyl butanol to their respective acetate derivatives in vivo, while Granny Smith apples could only convert exogenous hexanol to hexyl acetate. Differential substrate specificities of the in vitro acetyl-CoA:alcohol acetyl transferase (AAT) activities were also detected among cultivars. In Granny Smith apples, the AAT activity was detected only in the peel, and its specificity was almost exclusively restricted to hexanol and cis-3-hexenol. In Fuji apples, the AAT activity was detected in both peel and flesh and apparently accepted a broader range of alcohols as substrates than the Granny Smith enzyme activity. Our data strongly suggest that different AAT activities are operational in apple tissues and cultivars and that these differences contribute to the variation observed in the accumulation of volatile acetates.

KEYWORDS: Aroma; acetyl-CoA dependent acetyl transferase; apple (*Malus domestica* Borkh.)

INTRODUCTION

The unique aroma of apple fruit is due to complex mixtures of volatile esters, alcohols, aldehydes, ketones, and ethers (*1*, *2*). Volatile esters such as 2-methyl butyl acetate, 2-methyl butyl acetate, hexyl acetate, ethyl butanoate, butyl acetate, and others have been found in different apple cultivars (*3*−*7*). Among these, 2-methyl butyl acetate and butyl acetate are considered as the most important volatile esters contributing to the characteristic apple aroma (*1*, *8*). Little is known about the accumulation of volatile esters during apple maturation. Among the key enzymes in the production of volatile esters are the alcohol acetyl transferases (AATs). This family of enzymes was first characterized in microorganisms and utilizes CoA esters as acyl donors. AATs transfer an acyl moiety from acetyl-CoA or other acyl-CoA derivatives into the corresponding alcohols, forming an ester and generating free CoA (*9*). AATs have been found in many plants and plant tissues including apple (*Malus domestica* Borkh.) (*8*), banana (*Musa sapientum* L.) (*9*), strawberry (*Fragaria vesca* L., Fragar) (*10*), and melon (*Cucumis melo* L.) fruits (*11*); in the flowers of *Clarkia breweri* (*12*, *13*) and roses (*Rosa x hybrida*) (*14*); and in tobacco (*Nicotiana tabacum*) and *Arabidopsis thaliana* leaves (*15*).

AAT activity has been detected in many apple varieties including cv. Fuji, cv. Royal Gala, cv. Golden Delicious, cv. Granny Smith, and cv. Pacific Rose (*3*−*7*). Fuji is considered one of the most aromatic apples while the green apple cultivar Granny Smith is considered to be one of the least aromatic apples (*7*, *16*−*18*). Despite the presence of AAT in various plant species, it is still not known whether a single AAT is responsible for the entire spectrum of volatile esters biosynthesized in apple fruits or whether several types of AATs are involved. We undertook a comparative study focusing on analyses of the content of different volatile acetates and their possible biosynthetic enzymes in cv. Fuji as compared to cv. Granny Smith. By combining gas chromatography−mass spectrometry (GC-MS) together with biochemical analyses, we demonstrate here that different apple fruit tissues and cultivars contain a different spectrum of volatile acetates, have the ability to differentially form volatile acetates from exogenous precursors, and display different substrate specificities in the AAT activities measured in cell-free extracts in vitro. Our results strongly suggest that more than one type of AAT activity is involved in the formation of volatile esters in apple fruits.

MATERIALS AND METHODS

Plant Material. Cv. Fuji and cv. Granny Smith apples were harvested from 10 year old trees in neighboring rows of the same orchard in El-Rom, Israel. Apples were harvested according to

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commercial standards for starch content, firmness, and color parameters. The fruit size was 70−75 mm diameter for Fuji and 80−85 mm for Granny Smith. For the developmental studies, apple fruits were harvested at intervals from June to November 2002 as indicated. Six apples selected to fit a similar size and similar ripening stage were washed with tap water, and the peels were separated from the flesh with a peeler, giving special attention to minimize the inclusion of flesh tissue in the peel tissue samples. Fruit peels and flesh tissues were immediately treated as described below for GC-MS analyses. Parallel samples from the same tissues were stored at −40 °C for less than 2 months to test for enzymatic AAT activity (see below).

Feeding Experiments. Three ripe Fuji and Granny Smith intact apples harvested as described above were put in a 3 L sealed glass container containing either 2 or 10 μL of 2-methyl-1-butanol (Aldrich, St. Louis, MO) or hexanol (Treatt, United Kingdom). Volatiles were adsorbed onto a 4 cm round Whatman no. 3 paper pad attached with tape to the inner part of the container cover. As controls, apples were put in a 3 L sealed container without the addition of any exogenous volatile compound. Fruits were incubated for 24 h at ambient temperature under the above-mentioned conditions. Then, peel and flesh were separated as described above and the tissues were analyzed by headspace solid phase microextraction−gas chromatography−mass spectrometry (HS-SPME-GC-MS) (see below).

Auto-HS-SPME-GC-MS Analyses. *Sample Preparation*. Flesh volatile compounds were extracted according to the procedure of Fallik et al. (*20*). A sample (200 g) was taken randomly and homogenized in a Waring blender for 2 min with 20% (w/v) NaCl aqueous solution (200 mL). The homogenate was centrifuged twice at 5000 rpm for 30 min at 5 °C followed by the addition of 2,6-dimethyl-5-hepten-2-ol as an internal standard to the supernatant of the centrifuged material. NaCl (3 g) was added to supernatant (10 mL) (to inhibit enzymatic reactions) and poured into 20 mL headspace vials, which were sealed and kept at 4 °C until analysis. Apple peel (3 g) was homogenized with a 20% NaCl solution (10 mL). The homogenate and NaCl (3 g) were poured into 20 mL vials followed by addition of internal standard as described above.

Auto-HS-SPME. The volatiles were adsorbed for 30 min by HS-SPME with autosampler CTC PAL (CTC Analytics, Switzerland) at ambient temperature by a 75 μm PDMS/DVB fiber [poly(dimethylsiloxane)/divinylbenzene; Supelco, PA]. The fiber was inserted into the injection port of the GC-MS for 10 min (splitless), for desorption of the volatiles.

GC-MS. GC-MS analyses were carried out in an Agilent 6890N gas chromatograph with 5973N mass selective detector (CA). The instrument was equipped with a Rtx-5 SIL column (30 m length × 0.25 mm i.d., 0.25 μm film thickness, stationary phase 95% dimethyl−5% diphenyl polysiloxane, Resteck Co. (United States). The oven temperature program was initially set to 45 °C for 5 min and then increased to 200 °C at 3 °C/min. The inlet temperature was 250 °C. The carrier gas was helium with a constant flow of 0.8 mL/min and a linear flow rate of 32 cm/s. The transfer line temperature was 280 °C. A quadrupole mass detector with electron ionization at 70 eV was used to acquire the MS data in the range of 42 to 350 *m*/*z*. A mixture of straight chain alkanes (C6−C16) (Supelco, PA) was injected into the column under the above-mentioned conditions for retention indices calculation. The identification of the volatiles was assigned by comparison of their retention indices with those of authentic standards and with literature values (*19*) and by comparison of spectral data with NIST98 and QuadLib 1607 GC-MS libraries. The quantification was carried out by comparison with the internal standard using appropriate response factors for each compound. For that, a standard mixture containing 0.2 μL/L of each compound in NaCl solution was chromatographed and the response factors were calculated as a ratio of peak area/compound amount. The compound amount in each sample was calculated as (peak area × internal standard response factor) divided by (response factor × internal standard peak area).

AAT Activity Assays. *Preparation of Cell-Free Extracts*. Cell-free soluble protein extracts were prepared from fruits using a protocol modified from Shalit et al. (*11*, *14*). Frozen tissue (2 g) was ground in a chilled mortar and pestle in the presence of ∼0.2 g of acid-washed sand and ∼0.2 g of polyvinylpolypyrrolidone (PVPP) until a uniform powder was obtained. Ice-cold extraction buffer [(50 mM bis-Tris, pH 6.9, containing 10% (v/v) glycerol, 5 mM $Na_2S_2O_5$, 10 mM dithiothretiol (DTT), and 1% (w/v) poly(vinylpyrrolidone) (PVP 40)] was added at 10:1 (v:w) buffer:tissue ratio and ground at 4 °C until reaching an homogeneous texture. The slurry was centrifuged at 5000*g* for 40 min at 4 °C. The supernatant (crude extract) was either used fresh or kept for up to 1 month at −20 °C until its assay as described below.

AAT Enzymatic Activity in Apple Fruit Cell-Free Extracts. *Radioactive Assay*. Small-scale assays were performed by mixing 20 μL of crude extract, 10 mM alcohol substrate, and 23 μM (7.8 μCi/μmol) [^{14}C]acetyl-CoA (Moravek, Biochemicals, Brea, CA) into a final volume of 100 μL of assay buffer (50 mM bis-Tris, pH 6.9, 10% v/v glycerol, 5 mM $Na_2S_2O_5$, and 10 mM DTT). The assays were incubated for 1.5 h at 30 °C. One milliliter of hexane was added to each tube that was then vigorously vortexed and spun for 60 s at 10000*g* to separate phases. The upper hexane layers (800 μL), containing the newly formed radiolabeled acetate esters, were transferred to 5 mL scintillation tubes containing 3 mL of scintillation liquid (Ultima Gold, Packard BioSience, Meriden, United States). The radioactivity was quantified using a liquid scintillation counter (Kontron model 810, Watford Herts, United Kingdom). The enzyme activity in picokatals was calculated based on the specific activity of the substrate and using appropriate correction factors for the counting efficiency of the scintillation machine (*11*). The reaction velocities were linear for all substrates tested. Total protein concentrations were determined by the Bradford assay (*21*).

GC-MS AAT Activity Assay. Enzymatic assays were performed by mixing 10 mM the appropriate alcohol substrate, 10 mM acetyl-CoA, and 200−800 μL of crude extract in a total volume of 2 mL in assay buffer and incubated in glass tubes for 8 h at 30 °C. Two milliliters of hexane was added to each tube that was then vigorously vortexed and spun for 30 s at 2000*g* to separate phases. The upper hexane layers were dried with sodium sulfate and concentrated by a Turbo Vap II (Zymark, Hopkinton, MA) to a final volume of 400 μL. One microliter was injected to the GC-MS for the identification of volatiles generated as described above with the following modifications: oven initial temperature of 50 °C for 1 min increased to 200 °C at a rate of 5 °C/min. A mass range from 41 to 350 *m*/*z* was recorded.

RESULTS AND DISCUSSION

Headspace Analysis of Volatile Compounds from Fuji and Granny Smith Fruits. Comparative analysis of the volatiles of the flesh and peel from mature Fuji and Granny Smith apples is depicted in **Table 1**. The most prominent constituents in Fuji apples were 2-methyl butanol, *cis*-3-hexenol, *trans*-2-hexenal, and 2-methyl butyl acetate. Only minor amounts of *cis*-3-hexenol and *trans*-2-hexenal were detected in Fuji flesh as compared to its peel. The significant apple flavor 2-methyl butyl acetate was detected at high levels in both peel and flesh of Fuji apples. *trans*-2-Hexenal was the major component in Granny Smith peel. In the flesh, its amount was much lower, although it was still the major component. 2-Methyl butyl acetate was not detected in Granny Smith apples, both in peel and in flesh. The presence of other volatile acetates, such as hexyl acetate, was very limited in Granny Smith apples and is restricted to the peel. The flesh contains only hexanol, the precursor molecule of hexyl acetate, strongly suggesting that the Granny Smith flesh in intact Granny Smith apples does not possess enough enzymatic capability to metabolize hexanol to its corresponding acetate.

Previous reports (*7*, *16*, *17*) have indicated that Granny Smith apples grown in New Zealand contain significantly low levels of volatile esters as compared to Red Delicious apples, although some aliphatic esters were found. The lack of volatile esters in Granny Smith apples in our experiments could arise from the different geographical conditions between New Zealand and Israel or from the differences in Granny Smith types known to be used commercially. The overall lower levels of esters in

Table 1. Main Volatile Compounds in the Peel and Flesh of Fuji and Granny Smith Apples

		Fuji		Granny Smith	
compound	LRI[a]	peel (μg/g FW)[f]	flesh (μg/g FW)	peel (μg/g FW)	flesh (μg/g FW)
2-methyl butanol[b]	733	0.44 (0.23)	0.85 (0.38)	tr[d]	0.01 (0.00)
cis-3-hexenal[b]	802	0.30 (0.27)	0.01 (0.01)	0.12 (0.03)	0.01 (0.00)
hexanal[b]	803	0.49 (0.13)	0.08 (0.03)	0.25 (0.05)	0.08 (0.02)
butyl acetate[b]	815	0.04 (0.01)	0.05 (0.03)	tr	tr
trans-2-hexenal[b]	851	2.31 (0.85)	0.18 (0.11)	2.79 (0.38)	0.19 (0.05)
trans-2-hexenol[b]	863	0.09 (0.08)	0.02 (0.02)	0.45 (0.08)	0.05 (0.01)
hexanol[b]	869	0.10 (0.10)	0.10 (0.09)	0.08 (0.02)	0.03 (0.01)
2-methyl butyl acetate[b]	882	0.25 (0.02)	0.32 (0.06)	ND[e]	ND
trans-2-heptenal[b]	952	0.01 (0.00)	tr	0.04 (0.02)	0.02 (0.01)
3-methyl butyl propanoate[b]	974	0.02 (0.01)	0.01 (0.00)	0.01 (0.00)	ND
1-octen-3-ol[b]	978	tr	0.03 (0.03)	0.02 (0.02)	ND
6-methyl-5-hepten-2-one[c]	993	tr	tr	0.01 (0.00)	tr
octanal[b]	1007	0.01 (0.00)	tr	0.01 (0.00)	tr
hexyl acetate[b]	1015	0.04 (0.01)	0.02 (0.01)	tr	ND
trans-2-hexenyl acetate[b]	1018	0.03 (0.01)	tr	0.01 (0.00)	tr
2-ethyl hexanol[c]	1032	0.05 (0.02)	0.04 (0.02)	ND	ND
2-methyl butyl butanoate[b]	1060	tr	tr	tr	tr
2-methyl butyl 2-methyl butanoate[c]	1105	0.01 (0.01)	tr	tr	ND
nonanal[b]	1108	0.07 (0.02)	0.01 (0.00)	0.01 (0.00)	tr
hexyl 2-methyl butanoate[b]	1235	0.02 (0.01)	tr	tr	ND
trans,trans-α-farnesene[c]	1493	0.04 (0.03)	ND	ND	ND

[a] LRI, linear retention index calculated from GC-MS data. [b] Identification by comparison of LRI and mass spectrum with authentic standard (Supelco Co.). [c] Identification by comparison of LRI and mass spectrum with literature values. [d] tr, traces; value < 0.005 μg/g FW. [e] ND, not detected. [f] μg/g FW, microgram per gram fresh weight. The number in parentheses is the standard deviation.

Granny Smith apples found in earlier work were attributed either to the lack of the corresponding precursors or to the lack or malfunction of specific enzymes required for ester formation (*7*, *16*, *17*). In a separate study, we compared the volatile content of apples grown in different altitudes in Northern Israel. The volatile content in apples grown in El Rom (1000 m above sea level) was significantly higher than apples grown in the Yizre'el valley (100 m above sea level) (data not shown).

Feeding Exogenous Volatiles to Whole Apples in a Closed Atmosphere. One possible explanation for the observed differences in the ester content between Fuji and Granny Smith tissues could result from the lack of appropriate alcohol substrates in the fruit. Alternatively, it could be that different apple tissues have the ability to esterify different alcohols at different rates. To differentiate between these two possibilities, exogenous alcohols were supplied in a gaseous phase under controlled atmosphere conditions. As shown previously by others, intact apples readily uptake alcoholic substrates and convert them into their respective acetates derivatives (*7*, *16*, *17*). In our experimental conditions, we found that exogenous alcohol substrates in the gaseous phase were adsorbed by both the peel and the flesh (**Table 2**). Fuji apples can efficiently esterify the adsorbed 2-methyl butanol to the corresponding 2-methyl butyl acetate (up to 40−60% of the total recovered alcohols and acetates). Lower yields of esterification were observed for hexanol. In Fuji apples, hexanol was preferably utilized by the peel tissues as compared to the flesh tissues (**Table 2**). A different picture emerged from Granny Smith apples. Although 2-methyl butanol was adsorbed in significant amounts (2.6 and 3.4 μg/g FW in peel and flesh, respectively), only trace amounts of the corresponding esters were observed. The ability to convert hexanol to its corresponding acetate in Granny Smith apples was observed mainly in the peel (26%) as compared to flesh tissues (1%). Our data suggest that the different levels of volatile esters detected in Granny Smith and Fuji apples may reflect differential abilities to metabolize the appropriate alcohol precursors. This differential ability can also explain the different ester composition found in the flesh and peel of Granny Smith apples. If the limitation was solely in substrate availability, Granny Smith apples should have been able to convert exogenous 2-methyl butanol to 2-methyl butyl acetate as observed in Fuji apples.

Table 2. Conversion of Exogenous Hexanol and 2-Methyl butanol to Their Respective Acetates in Whole Apples in Closed Atmosphere

			exogenous alcohol supplied			
			2 μL		10 μL	
cultivar	tissue	exogenous alcohol	total[a] recovered (μg/g FW)	acetate[b] recovered (%)	total[a] recovered (μg/g FW)	acetate[b] recovered (%)
Fuji	peel	hexanol	0.6	20	3.4	38
	flesh		0.4	8	2.6	19
	peel	2-methyl butanol	1.7	46	5.2	58
	flesh		1.0	39	3.2	56
Granny Smith	peel	hexanol	0.5	31	1.9	26
	flesh		0.2	0	1.1	1
	peel	2-methyl butanol	2.6	3	6.5	4
	flesh		3.4	1	6.6	2

[a] Total recovered alcohols and acetates. The value defines the adsorbed alcohols and acetates and was determined by measuring the amounts of alcohol and acetates in fruits treated with alcohol precursors subtracting the amounts found in control fruits that were not treated by alcohol precursors. [b] Percent of acetates out of the total (alcohols + acetates).

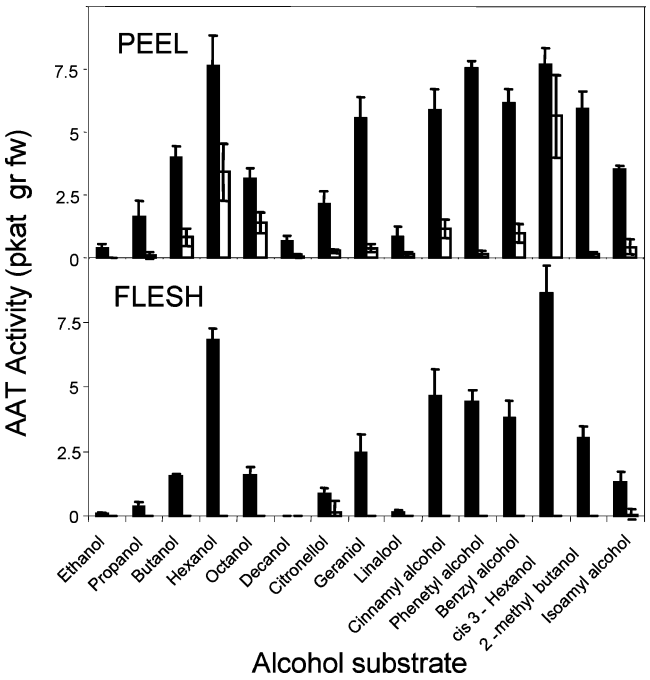

Figure 1. Substrate specificity of AAT activities in cell-free extracts of Fuji and Granny Smith apple fruits. Cell-free extracts were incubated with ^{14}C-acetyl-CoA and 10 mM each alcohol as indicated. Ester formation was measured utilizing the radioassay described in the Materials and Methods. (**Top**) Cell-free extracts derived from peel tissues. (**Bottom**) Cell-free extracts derived from flesh tissues. Black bars, Fuji; white bars, Granny Smith. Averages and SEs of two replicates are given.

Comparative Analysis of AAT Activity and Their Substrate Specificity in Mature Fuji and Granny Smith Apple Fruits. Cell-free extracts derived from Fuji apples were able to esterify many alcohols to their respective acetate esters derivatives (**Figure 1**). The efficient alcoholic substrates included short chain alcohols such as hexanol and *cis*-3-hexenol, monoterpene alcohols such as geraniol, and aromatic alcohols such as

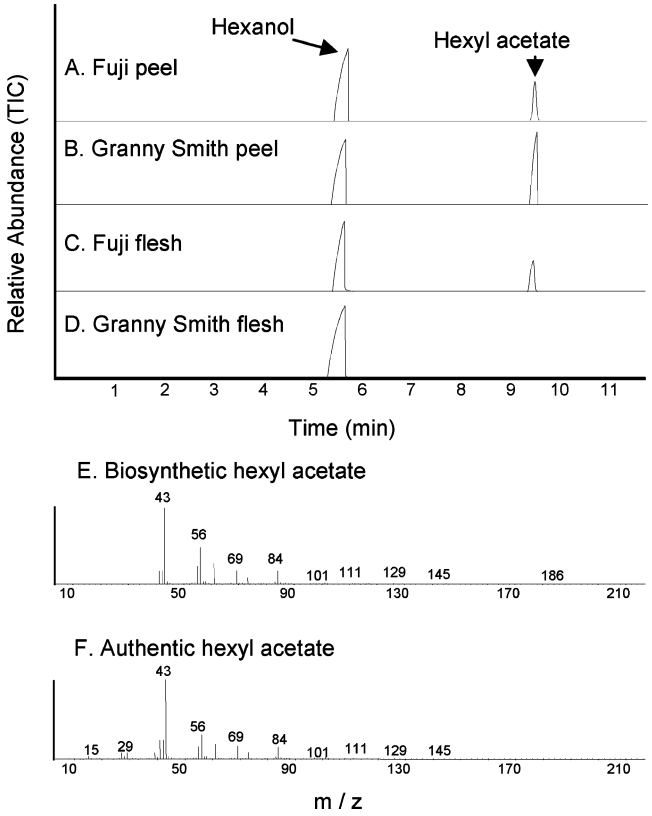

Figure 2. Identification by GC-MS of the hexyl acetate produced in vitro by apple fruit cell-free extracts. Cell-free extracts were incubated with hexanol and acetyl-CoA in assay buffer as described in the Materials and Methods. The identification of the hexyl acetate was done by matching it with the retention time and the mass spectrum of authentic hexyl acetate and by comparison with the computerized NIST library. (**A**) Cell-free extracts derived from Fuji peels. (**B**) Cell-free extracts derived from Granny Smith peels. (**C**) Cell-free extracts from Fuji flesh. (**D**) Cell-free extracts derived from Granny Smith flesh. Control assays devoid of either alcoholic substrate, acetyl CoA, or assays in which heat inactivated enzyme was used were all devoid of hexyl acetate (not shown). (**E**) Mass spectrum of biosynthetic hexyl acetate. (**F**) Mass spectrum of authentic hexyl acetate.

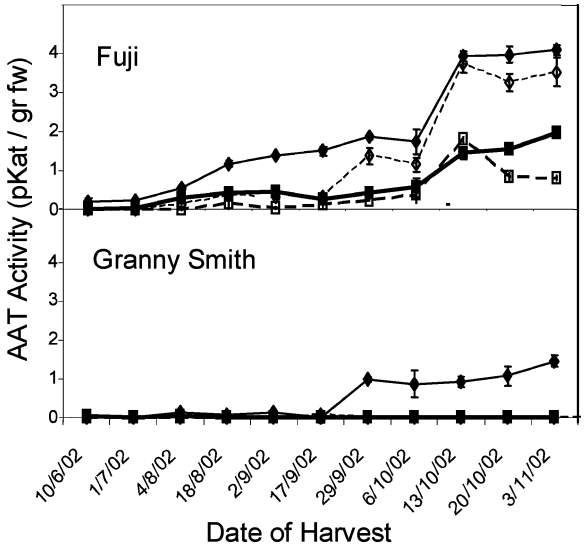

Figure 3. AAT activity in developing Fuji (**top**) and Granny Smith (**bottom**) apple fruit tissues. Cell-free extracts were prepared as described in the Materials and Methods and incubated with the substrates as indicated. Black lines and closed symbols represent values obtained from peel tissues. Broken lines and open symbols represent values from flesh tissues. Substrates used as follows: diamonds, hexanol; squares, 2-methyl butanol. Each point represents the average ± SE of three replicates, each generated from tissues pooled from two individual fruits.

cinnamyl, phenethyl, and benzyl alcohols (**Figure 1**). Interestingly, many of the acetates formed in vitro are not present in apple tissues. This phenomenon seems to occur in other plants such as melons (*11*), rose (*14*), banana (*10*), and strawberry (*10*, *20*). The ability to esterify alcohol substrates seemed relatively similar in peel and flesh tissues in Fuji (**Figure 1**). There is virtually no detectable AAT enzymatic activity in Granny Smith flesh tissues using any of the alcoholic substrates (**Figure 1**). In contrast to extracts derived from Fuji peel tissues, cell-free extracts derived from Granny Smith peels displayed much narrower substrate specificity (**Figure 1**). Extracts from peels of Granny Smith apples were apparently active only when hexanol or *cis*-3-hexenol were provided as alcoholic substrates. Butanol, octanol, and cinnamyl alcohols were also active substrates but at much lower levels. To ascertain that the products formed in the enzymatic assays were the corresponding acetates, GC-MS analyses were performed (*11*). The results for the formation of hexyl acetate from hexanol and acetyl-CoA are shown in **Figure 2**. The biosynthetically formed hexyl acetate had an identical retention time and mass spectrum as authentic hexyl acetate (**Figure 2B**). Similar confirmatory analyses were performed with 2-methyl butanol, and in this case, 2-methyl butyl acetate was detected in extracts from Fuji apples (not shown). In control enzymatic assays carried out utilizing heat-inactivated extracts or assays devoid of acetyl-CoA, no acetates were detected both in Fuji- and Granny Smith-derived extracts (not shown). In conclusion, our results indicate that the observed AAT activities in Granny Smith and Fuji tissues differ both in their substrate specificity and in their distribution among peel and flesh tissues.

AAT Activity in Developing Apple Fruit. To determine whether the observed differences in AAT activity between Granny Smith and Fuji are not solely due to differences in developmental stages between apple cultivars, we monitored the levels of AAT activity and their substrate specificities during fruit maturation. As expected from the volatile composition, during early stages of development, in which acetates were absent (not shown), no AAT activity was detected in any of the varieties, regardless of the tissue examined (**Figure 3**). AAT activity levels dramatically increased with fruit development and maturation (**Figure 3**). In essence, the ability to utilize 2-methyl butanol as a substrate was detected only in developing Fuji but not in Granny Smith fruit tissues as previously shown in mature fruits (**Figure 1** and **Table 2**). Nevertheless, the two cultivars differ in the timing of the appearance of AAT activity during development. In Fuji apples, the AAT activity is discernible already in June when a slight red color is first prominent and peaks in November, whereas AAT activity in Granny Smith apples is apparent only in September and only in the peel (**Figure 3**). Basically, the pattern of specificity to substrate and tissue remains the same during the various developmental stages of the fruit in both cultivars (**Figure 3**) and in overripe fruit (data not shown). It has been established that apples continue to produce volatile esters upon harvesting and after several months in storage (*3*). Developmental changes in AAT levels have been also associated with ripening in several fruits, including melon (*11*), strawberry (*22*), banana (*23*), and apple (*24*). AAT activity levels also varied during flower development in rose (*14*) and *Clarkia breweri* (*13*). It has been demonstrated that climacteric levels of ethylene are needed for the onset of AAT activity during ripening of climacteric melons.

Negligible levels of AAT activity were detected in nonaromatic, nonclimacteric melon varieties (*11*, *25*) and in 1-aminocyclopropane-1-carboxylate (ACC)-oxidase-antisensed melons that do not produce ethylene during ripening (*26*). In banana, the capacity to produce esters precedes the onset of ethylene biosynthesis and begins 40−50 h after the peak of ethylene emission (*23*). Observations in apple demonstrate that AAT gene expression is also regulated by ethylene (*24*). Because both Granny Smith and Fuji cultivars are climacteric (*27*, *28*), climactericity alone is not sufficient to explain the low levels of AAT activity during fruit development in Granny Smith apples. Low levels of ester production were observed in Granny Smith apples even several weeks after commercial harvest (data not shown). Moreover, in overripe fruit, the same pattern of AAT specific activity was observed, excluding the possibility that the low levels of esters observed in Granny Smith apples as compared to Fuji apples are due to harvest in inappropriate developmental stages. A differential response to ethylene has been observed among two distinct AAT genes in melon (*26*).

Varietal and tissue differences in AAT levels and substrate specificity have been demonstrated by three different experimental approaches: (i) direct comparative volatile determination using headspace analyses, (ii) examination of volatile ester content after feeding whole apples with exogenous alcohol precursors in closed atmospheres, and (iii) determination of substrate specificities in the AAT activity in cell-free extracts derived from different apple cultivars, tissues, and developmental stages. Our data support the hypothesis that the differences in ester levels and specificity found among various cultivars and tissues are not solely due to lack of production of the appropriate alcohol substrates, but it is enzymatically limited. It is presently unknown whether Fuji apples contain only one enzyme with broad substrate specificity for alcoholic acceptors or whether two or more separable enzymatic activities are involved in these biochemical conversions. Although in general AATs display broad substrate specificities toward many different alcohols, differences in the apparent substrate specificities of AAT enzymes and gene products from many sources have been noted (*29*). The strawberry AAT preferentially accepts geraniol, 1-heptanol, 1-octanol, and 1-nonanol (*10*, *22*), while the AAT from banana has the highest activity with cinnamyl alcohol (*10*). A rose AAT preferentially acetylates geraniol and citronellol (*14*). In melon, crude enzyme extracts, phenethyl alcohol was the preferred substrate, and a functionally expressed recombinant AAT gene possessed high substrate specificity toward several aliphatic and aromatic alcohols (*26*).

Our findings strongly suggest that different AATs exist in apple cultivars and tissues and their levels are differentially controlled during development. However, several other possibilities could explain the differential activity observed between Fuji and Granny Smith and between the peel and flesh of Granny Smith apples. Such explanations may include differences in enzymes that modify AAT activity (proteases, kinases, etc.) or enzymes that metabolize the end products of AAT. The purification and biochemical characterization of the enzymatic activities as well as the isolation and functional expression of their respective genes will highly contribute to our knowledge of the factors that affect the production of different volatile acetates in apple cultivars and tissues.

ABBREVIATIONS USED

AAT, alcohol acetyl transferase; GC-MS, gas chromatography−mass spectrometry; HS-SPME-GC-MS, headspace solid phase microextraction−gas chromatography−mass spectrometry; PDMS/DVB, poly(dimethylsiloxane)/divinylbenzene; PVPP, polyvinylpolypyrrolidone; DTT, dithiothretiol; ACC, 1-aminocyclopropane-1-carboxylate; FW, fresh weight; LRI, linear retention index.

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