Acetyl-CoA: Alcohol Acetyltransferase Activity and Aroma Formation in Ripening Melon Fruits

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Melon varieties (*Cucumis melo* L.) differ in a range of physical and chemical attributes. Sweetness and aroma are two of the most important factors in fruit quality and consumer preference. Volatile acetates are major components of the headspace of ripening cv. Arava fruits, a commercially important climacteric melon. In contrast, volatile aldehydes and alcohols are most abundant in cv. Rochet fruits, a nonclimacteric melon. The formation of volatile acetates is catalyzed by alcohol acetyltransferases (AAT), which utilize acetyl-CoA to acetylate several alcohols. Cell-free extract derived from Arava ripe melons exhibited substantial levels of AAT activity with a variety of alcohol substrates, whereas similar extracts derived from Rochet ripe melons had negligible activity. The levels of AAT activity in unripe Arava melons were also low but steadily increased during ripening. In contrast, similar extracts from Rochet fruits displayed low AAT activity during all stages of maturation. In addition, the benzyl- and 2-phenylethyl-dependent AAT activity levels seem well correlated with the total soluble solid content in Arava fruits.

Keywords: *Aroma; volatile acetates; biosynthesis; alcohol acetyltransferase; melons (Cucumis melo. L)*

INTRODUCTION

Contemporary melon cultivars differ in shape, size, color, surface netting, sweetness, flavor, and storability (*1*−*3*) and can be divided into two groups, climacteric and nonclimacteric, according to their ripening patterns. The climacteric mode of ripening is characterized by a significant increase in the levels of respiration and release of ethylene, triggering profound and rapid changes in a range of attributes, such as flavor (sweetness and aroma), as well as in other physical parameters such as firmness of the fruits and a typical slip area produced around the peduncle in climacteric melons. Some of the climacteric varieties of melons are considered to be highly aromatic [such as Galia, Charentais, and Ananas (*Cucumis melo* var. *reticulatus*)], whereas the nonclimacteric varieties are normally considered to be less aromatic, that is, Casaba-type melons such as Rochet (*Cucumis melo* var. *inodorus*).

Two of the most important parameters that determine the quality and consumer preferences of the fruits are their sweetness and aroma properties. The aroma of fruits is ordinarily composed of complex mixtures of volatile compounds present in the headspace (*4*). Volatile aldehydes, alcohols, and especially the large quantities of esters present in their headspace are likely to be the key contributors to the unique aromas of melons (*3*, *5*−*11*). Although the aromas of some of the individual volatile compounds often resemble melon or fruity notes, the peculiar aroma of melons cannot be related to any single compound.

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Many of the volatile aldehydes present in the headspace of fruits are formed by degradation of fatty acids (*12*), whereas the respective alcohols are derived from aldehydes by the action of alcohol dehydrogenases or as a result of degradation of amino acids (*13*).

Volatile esters, important contributors to the aroma of many fruits, are formed by esterification of alcohols and carboxylic acids, normally utilizing a CoA ester as the acyl donor. The ability to esterify alcohols has been previously noted in many melon cultivars by incubating fruit slices with isobutyl alcohol (*14*). The nonaromatic *inodorus* types had low esterification potential, and other cucurbits such as cucumber (*Cucumis sativus* L.), squash (*Cucurbita maxima* Duch.), and watermelon (*Citrullus lanatus* Matsumu.) lacked the ability to esterify isobutyl alcohol (*14*). The mechanism of ester formation has been studied in microorganisms, where a group of enzymes termed alcohol acetyltransferases (AAT) have been identified (*15*, *16*). AAT catalyze the transfer of an acetyl moiety from acetyl-CoA into the corresponding alcohol, forming an ester and free CoA (Figure 1).

The first attempt to identify AAT activity in fruits was carried out in banana slices (*17*). In this study, isoamyl acetate was synthesized from isoamyl alcohol and acetyl-CoA in partially purified cell-free protein extracts. AAT activities have been also detected in apple (*18*), strawberry (*19*, *20*), and melon fruits (*14*, *21*, *22*). In all cases, an alcohol is acetylated; the acyl donor is acetyl-CoA. The partially purified AAT from oriental sweet melons mainly esterified isobutyl alcohol to form isobutyl acetate (*21*). Other alcohols were also esterified, but benzyl alcohol, the putative precursor of benzyl

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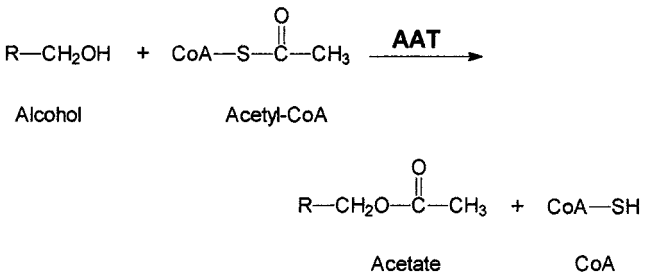

Figure 1. Formation of volatile esters by acylation of alcohols and acetyl-CoA. AATs catalyze this reaction.

acetate, and among the major components of the headspace of cv. Galia and Arava melons (*22*), was not tested.

Benzyl acetate, present in melon fruits, is also one of the most important synthetic aroma chemicals in the flavor and fragrance industry (*23*). This compound has a flowery (jasmine) odor and a bitter, pungent taste. It is the main component of jasmine and gardenia essential oils and occurs in many other essential oils as a minor component. An enzyme that catalyzes the formation of benzyl acetate in *Clarkia breweri* flowers (a native California plant, Onagraceae) has been isolated and characterized, and its gene has also been isolated (*24*). A gene coding an enzyme with AAT activity has been isolated from strawberry utilizing cDNA microarray technology (*25*).

We describe herein significant differences in the volatile acetates present in the headspace of two different melon varieties. By adapting a cell-free enzymatic assay that enabled us to measure the potential of each variety to produce benzyl and phenylethyl acetate from their respective alcohols, we attempted to biochemically rationalize the differences in the volatile profiles of the two varieties. We also monitored changes in the levels of these enzymatic activities during maturation and describe a correlation between AAT activity and total soluble solids content in ripening cv. Arava fruits.

MATERIALS AND METHODS

Plant Material. Melons were sown in April 1999 in the open field at the Newe-Ya'ar Research Center in northern Israel and grown under commercial conditions in the summer of 1999 with drip irrigation and fertilization. Fresh female flowers were tagged and hand pollinated, and fruits were harvested at different times as indicated.

Analysis of Volatiles. *Solid Phase Microextraction (SPME).* Fresh melons were peeled and cut, and the seeds were removed. The fruit flesh was then homogenized in a food processor (Braun), and 8 g of the homogenate was diluted with 2 mL of a NaCl-saturated aqueous solution. Headspace sampling was conducted immediately, utilizing a 65 μm fused-silica fiber coated with polydimethylsiloxane/divinylbenzene (PDMS/DVB) (Supelco) inserted into tightly closed 20 mL vials containing the melon homogenates. After 40 min at 65 °C, and under gentle stirring, the SPME syringe was introduced into the injector port of the GC-MS apparatus for further analysis (*22*).

GC-MS Analysis. Volatile compounds were analyzed on an HP-GCD apparatus equipped with an HP-5 (30 m × 0.25 mm) fused-silica capillary column. Helium (1 mL/min) was used as a carrier gas. The injector temperature was 250 °C, set for splitless injection. The oven was set to 50 °C for 1 min, and then the temperature was increased to 200 °C at a rate of 4 °C/min. Thermal desorption was allowed for 1.5 min. The detector temperature was 280 °C. The mass range was recorded from *m*/*z* 45 to 450, with electron energy of 70 eV. Identification of the main components was done by comparison of mass spectra and retention time data with those of authentic samples and supplemented with a Wiley GC-MS library.

Enzyme Extraction. Fruits were cut into small pieces (1−2 g) and frozen at −40 °C for up to 1 month until use. The pieces were placed in a chilled mortar and ground with a pestle in the presence of sand and 1% w/w polyvinylpolypyrollidone (PVPP) until a uniform powder was obtained. Ice-cold extraction buffer [50 mM bis Tris, pH 6.9, 10% (v/v) glycerol, 10 mM dithiothretiol (DTT), 5 mM $Na_2S_2O_5$] was added (1 volume to 1 g fresh weight of tissue), and the suspension was further extracted for an additional 30 s. The slurry was centrifuged at 20000*g* for 10 min at 4 °C. The supernatant (crude extract) was either used fresh or kept for up to 2 weeks at −40 °C until its use for enzymatic assays.

Enzyme Assays. *GC-MS Assay.* Enzymatic assays were performed by mixing 10 mM of the appropriate alcohol, 0.2 mM acetyl-CoA, and 200 μL of crude extract in a total volume of 2 mL in 100 mM phosphate buffer, pH 8.0, and incubated for 8 h at 30 °C. Two milliliters of hexane was added to each tube, which 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) to a final volume of 400 μL. One microliter was injected to the GC-MS for the identification of volatiles.

Radioactive Assay. Small-scale assays were performed by mixing 10 μL of crude extract, 10 mM benzyl (or other) alcohol, and 23 μM (7.8 μCi/μmol) [^{14}C]acetyl-CoA (Amersham) into a final volume of 50 μL of assay buffer (100 mM phosphate buffer, pH 8.0). The assays were incubated for 1 h at 30 °C. One milliliter of hexane was added to each tube, which was then vigorously vortexed and spun for 30 s at 5000*g* to separate phases. The upper hexane layers, containing the newly formed radiolabeled alcohol acetate, were transferred to 5 mL scintillation tubes containing 3 mL of scintillation liquid [2.5 phenyloxazol (PPO, 4 g/L), 2,2-*p*-phenylene-bis(5-phenyloxazol) (POPOP, 0.05 g/L), and 10% (v/v) Triton in toluene]. The radioactivity was quantified using a liquid scintillation counter (Kontron model 810). Enzyme activity in picokatals was calculated on the basis of the specific activity of the substrate and using appropriate correction factors for the counting efficiency of the scintillation machine (>0.90).

Protein Determination. The Bradford assay (*26*) utilizing the Bio-Rad protein assay reagent (Bio-Rad) was used. Absorbance at 595 nm was determined using a spectrophotometer (Uvikon 810). Bovine serum albumin (Sigma) served as a standard.

Total Soluble Solids (TSS) Determination. TSS were measured directly from freshly cut fruits utilizing a digital refractometer (PR-100, Atago) calibrated against pure water. TSS was calculated by averaging five different determinations per melon fruit and expressed as Brix degrees (*27*).

RESULTS AND DISCUSSION

Composition of the Volatiles Present in the Headspace of Cv. Arava and Rochet Melon Fruits. The SPME GC-MS analysis method was used to identify the volatiles present in the headspace of Arava and Rochet fruits during their maturation. The results are shown in Table 1. In mature Arava fruits (35−45 days after pollination) 77% of the total volatiles comprise volatile acetates, mostly aliphatic (57%), but other groups such as aromatic acetates (20%) and other esters (7%) are also prominent. In these melons, low levels of medium-chain alcohols (4.2%) and low levels of terpenes (0.9%) and lactones (0.1%) were also detected.

In contrast to Arava ripe fruits, in Arava unripe fruits the headspace composition was very different and the volatile composition included mainly medium-chain aliphatic aldehydes (74%), almost lacking in Arava ripe fruits, and lower levels of alcohols (13%).

Arava is an aromatic melon variety in contrast to Rochet, a Casaba-type nonaromatic melon, and this is

Table 1. Main Volatile Components Identified in the Headspace of Arava and Rochet Melons by SPME

		percentage in headspace						percentage in headspace			
		Rochet		Arava				Rochet		Arava	
compound identified[a]	LRI[b]	unripe	ripe	unripe	ripe	compound identified[a]	LRI[b]	unripe	ripe	unripe	ripe
Esters											
aliphatic acetates						aromatic acetates					
hexyl acetate	1013		0.8		17.4	benzyl acetate	1164		3.2		14.1
isoamyl acetate	883		0.2		13.2						
octyl acetate	1213		0.8		11.0	phenyl propyl acetate	1370				2.1
butyl acetate	811				3.9	phenyl ethyl acetate	1257		2.1		3.5
ethyl acetate	614				2.6	(*E*) cinnamyl acetate	1455				0.1
isobutyl acetate	781				2.6	total aromatic acetates		0	5.3	0	19.8
(*Z*)-3-hexenyl acetate	1007				1.8	other esters					
heptyl acetate	1112				1.5	ethyl butyrate	806				4.4
1-octenyl 3-acetate	1199				0.8	ethyl 2-methylbutyrate	870				0.7
(*E*)-3-hexenyl acetate	998				0.6	ethyl hexanoate	1014				0.7
3-(methylthio)propyl acetate	1133				0.6	ethyl benzoate	1184				0.4
(*E*)-5-dodecenyl acetate[c]	1597				0.3	methyl cinnamate	1389				0.4
decyl acetate	1410				0.2	ethyl myristate	1726				0.1
3-nonen-1-yl acetate[c]	1140				0.2						
nonyl acetate	1325				0.1	total other esters		0	0	0	6.6
2,3-butanediol acetate[c]	1080				0.1						
ethyl 2-(methylthio)acetate	1006	0.3			0.1	total esters		0.3	7.1	0	83.3
total aliphatic acetates		0.3	1.8	0	56.9						
Aliphatic Aldehydes											
(*E*)-2-decenal	1263		0.2	0.5		2,6-nonadienal	1170			5.5	0.1
(*E*)-2-heptenal	958		0.8	2.3		decanal	1219	1.5	2.0	9.4	0.2
(*E*)-2-nonenal	1161	3.0	2.9	9.3		dodecanal	1417	0.2	0.2	1	0.1
(*E*)-2-octenal	1067		0.9	2.5		hexanal	784	0.4	1.8	3.6	
(*E*)-2-undecenal	1340		0.2	2.9		nonanal	1105	6.5	5.4	31.1	
(*E*,*E*)-2,4-decadienal	1320		0.9	2.9							
(*E*,*Z*)-2,4-decadienal	1297		0.1	0.8		total aldehydes		17.7	19.7	73.8	0.4
(*E*,*E*)-2,4-nonadienal	1205	5.9	4.3	2.1							
Alcohols											
3-nonen-1-ol	1157	16	27.8			decanol	1280				0.1
octanol	1080	0.7	0.9	0.8	2.9	3,6-nonadienol[c]	1165	17	8.8		
nonanol + 6-nonenol[c]	1176	33.5	21.2	11.8	0.9	nonadienol[c]	1280	9.2	2.6		
phenylethyl alcohol	1121				0.1						
phenyl propyl alcohol	1233				0.2	total alcohols[d]		76.6	60.3	12.6	4.3
dodecenol	1464				0.1						
Terpenes											
geranyl acetone	1463				0.4	terpinenyl acetate	1356				0.1
δ-cadinene	1530				0.1	β-caryophyllene	1427				0.05
epi-α-cadinol	1640				0.1						
β-ionone	1493				0.1	total terpenes		0	0	0	0.9
Lactones											
γ-dodecalactone	1673				0.03	γ-decalactone	1478				0.05
						total lactones		0	0	0	0.1
total volatiles		94.6	87.1	86.4	88.8						

[a] Volatile compounds from freshly cut melons were sampled using SPME and analyzed by GC-MS as described under Materials and Methods. Identification was confirmed by comparison of mass spectra and retention times with those of authentic compounds analyzed under similar conditions, as indicated. [b] LRI, linear retention index. [c] Tentative identification based on comparison of mass spectrum only.

reflected in the headspace composition of these melons (Table 1). More than 75% of the volatiles detected in Rochet unripe fruits are short- and medium-chain alcohols and ~18% are aliphatic aldehydes (mainly 2,4-nonadienal and nonanal). In general, the aroma profile of Rochet fruit changes very little with maturation up to 50 days after pollination. There is some diminution in the total level of alcohols, followed by a small increase in the levels of esters (7.1%), mainly aromatic acetates (5.3%), whereas the level of aldehydes in Rochet ripe melons remains similar to those in immature fruits.

AAT Activity in Ripe Arava Fruits. The major difference in the profile of volatiles examined from Arava, an aromatic melon, from those of the nonaromatic Rochet melon is the high levels of esters, mainly acetate derivatives, in Arava fruits. This suggested that AAT activities might be present in ripe Arava melons but absent in the volatile-acetate-lacking nonaromatic Rochet melons.

To validate the above hypothesis, we prepared cell-free extracts from ripe Arava fruits and analyzed their capability to acetylate benzyl alcohol from acetyl-CoA. The results are shown in Figure 2. A compound identified as benzyl acetate by its retention time on the GC and having a mass spectrum (not shown) identical to that of benzyl acetate was detected in such cell-free

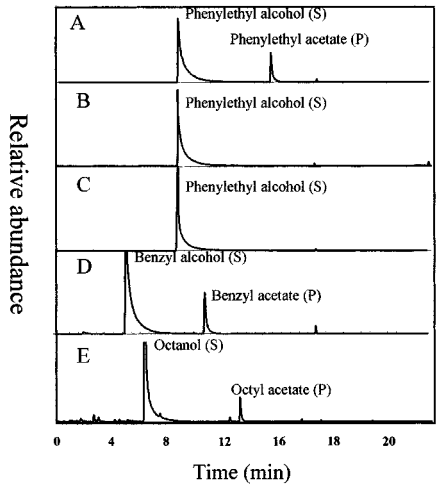

Figure 2. GC analysis of the products formed by cell-free extracts derived from Arava fruits: (A) phenylethyl alcohol and acetyl-CoA; (B) phenylethyl alcohol without acetyl-CoA; (C) phenylethyl alcohol and acetyl-CoA added to a heat-inactivated (boiled) extract; (D) benzyl alcohol and acetyl-CoA; (E) 1-octanol and acetyl-CoA. Cell-free extracts were incubated with the additions as indicated. "S" indicates substrate added and "P", the product of the enzymatic reaction.

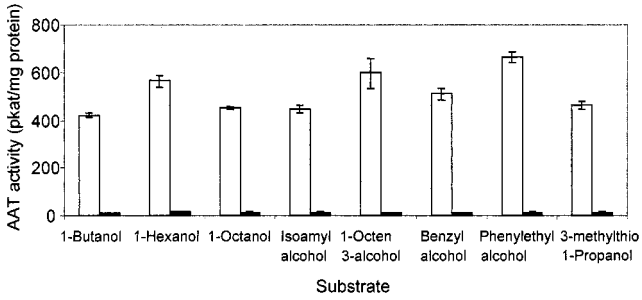

Figure 3. Substrate specificity of AAT activity in cell-free extracts of Arava and Rochet ripe fruits: (white bars) Arava; (black bars) Rochet. Cell-free extracts were incubated with [^{14}C]acetyl-CoA and 10 mM of each alcohol as substrates. Ester formation was measured utilizing the radioassay described under Materials and Methods. Averages and SE of two replicates are given.

extracts only after the addition of benzyl alcohol and acetyl-CoA (Figure 2D). When we used 2-phenylethyl alcohol as a substrate in lieu of benzyl alcohol, the product was identified as 2-phenylethyl acetate (Figure 2A). Similarly, when we used 1-octanol as substrate, the product was identified as 1-octyl acetate (Figure 2E). None of the acetates were present in the absence of acetyl-CoA or when protein extracts previously boiled for 3 min were used, as seen in Figure 2B,C.

These results suggested that cell-free extracts from Arava melons contain AAT activity, able to acetylate several alcohol substrates. The product of the reaction depends on the specific alcohol added to the reaction. No activity was detected when using cell-free extracts derived from Rochet melons under these conditions (data not shown).

To test other alcohol substrates and for the further characterization of the enzyme activity, we adapted a simple and sensitive radioassay for its determination (*24*). The results obtained are shown in Figure 3. It seems that Arava cell-free extracts can utilize many alcohols as substrates to produce their respective acetates. This includes several aliphatic, aromatic, and sulfur-containing alcohols. In any case, Rochet melons

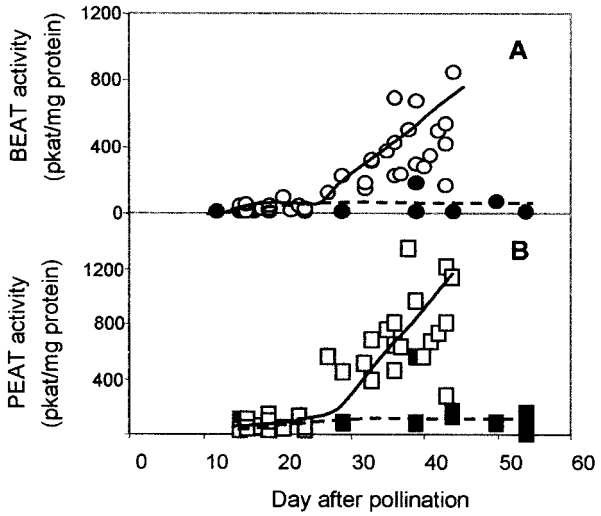

Figure 4. (A) Benzyl (BEAT) and (B) phenylethyl alcohol (PEAT) dependent acetyltransferase activities in ripening melon fruits: (solid lines, open symbols) Arava; (broken lines, solid symbols) Rochet. Each point was generated by analyzing an individual melon.

have no significant AAT activity with any of the substrates tested. This observation provides a biochemical rationale for the lack of acetates in the headspace of Rochet fruits and might explain the apparent low levels of typical melon aroma displayed by this variety.

AAT activities with an apparent broad substrate-specificity toward the alcohol acceptor substrate have been reported in banana, strawberry, and melon fruits (*21*). In oriental sweet melon (var. *makuwa* cv. Gensung), the AAT detected was mainly active toward isoamyl, butyl, and isobutyl alcohols, but not with amyl alcohol. Aromatic alcohols were not tested (*21*). The benzyl AAT activity involved in the production of the flower scent of *Clarkia breweri* primarily accepted benzyl alcohol as a substrate, whereas 2-phenylethyl alcohol and other alcohols were accepted but at lower rates (*24*). This is in contrast to the enzyme activity present in Arava fruits, which primarily accepts 2-phenylethyl alcohol as a substrate (Figure 4). The AAT activity present in strawberry fruits is more specific toward the alcohol substrate than the activity described here to be present in Arava melon fruits. Aromatic alcohols, such as benzyl and phenylethyl alcohol, were ~10-fold less efficient substrates than aliphatic alcohols for the acetyltransferase enzyme whose gene was isolated from strawberry (*25*). It is possible that Arava, an aromatic type melon, has an AAT activity with a broad substrate specificity or that Arava melon fruits possess several AAT activities with a stricter substrate specificity. It seems that in melons, more than one AAT activity is present, a conclusion based on observations reported from transgenic "delayed ripening" "Charantais" melons. In these melons the activity of 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) (a key gene in ethylene biosynthesis) is greatly repressed (*28*). Not only did this intervention affect the softening properties of the transgenic melons, but the volatiles present in the transgenic melons were greatly reduced, as compared to those in nontransformed controls (*11*). Only 20−30% of the total acetates detected in nontransformed melons were present in the headspace of the antisense-inhibited fruits (*11*), and the levels of the expression of some of the genes related to aroma formation were differentially regulated (*29*).

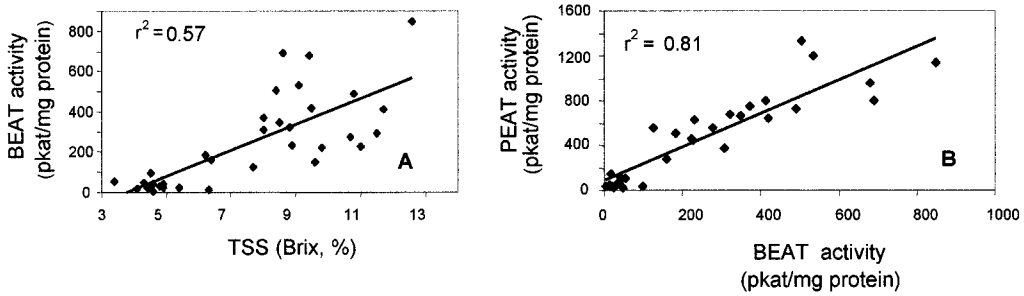

Figure 5. Correlation between benzyl alcohol acetyltransferase (BEAT) activity and TSS in ripening Arava fruits. Each value point was generated by measuring TSS and BEAT activity from one individual fruit.

AAT Activity during Ripening of Melon Fruits. Because the levels of the volatile acetates in Arava melons are higher in ripe fruits than in unripe ones (Table 1), it was of interest to monitor any changes of AAT activity during the ripening process. To this end, we followed the levels of either benzyl alcohol or 2-phenylethyl alcohol dependent acetyltransferase activities in Arava and Rochet fruits. The results, as shown in Figure 4, indicate that both enzymatic activities steadily increased during the ripening of Arava melons, reaching their highest level in fully ripe fruits. In contrast, both benzyl and phenylethyl AAT-dependent activities were low during the long duration of the ripening process of Rochet melons. This correlates well with the observed increase in total volatile acetates during the ripening of Arava fruits (*22*). A significant correlation between the levels of benzyl and phenylethyl alcohol dependent acetyltransferase activities was noted (Figure 5B, $r^2 = 0.81$), but it is presently unknown if both activities reside in one or more proteins. In strawberries, it seems that one AAT enzyme is able to acetylate several alcoholic and thiolic substrates (*25*, *30*). Interestingly, in melons, the levels of the acetyltransferase activity were higher when phenylethyl alcohol was used as a substrate instead of benzyl alcohol (Figures 3 and 5B), although the levels of phenylethyl acetate in Arava headspace are lower than those of benzyl acetate in ripe Arava fruits (Table 1).

Many important physical and chemical changes take place during fruit ripening. They include color and texture changes, as well as assimilation of solids (mainly acids and sugars), and increases in the volatile constituents that compose the unique aromas. Although these processes often take place concomitantly, their biological regulation is not necessarily identical. Results of an earlier study (*31*) indicated a correlation (87%) between changes in total soluble solids and total volatiles in the melon cultivar Makdimon, which belongs to the *C. melo* var. *reticulatus* group. To further explore the possibility of a correlation between the levels of AAT activity and TSS in Arava melons, the TSS and AAT activities were measured from the same melon slices tested during maturation. The results obtained are shown in Figure 5. Although a significant ($p < 0.05$) correlation was found, a relatively low regression coefficient ($r^2 = 0.57$) may reflect that although both the TSS and the levels of volatile acetates do increase during development, these processes might not necessarily be concertedly regulated by the same factor(s). Furthermore, although sugar assimilation takes place in both Rochet and Arava cultivars during ripening (not shown), the accumulation of acetates (Table 1) and AAT activity was detected only in the aromatic Arava cultivar and only in later stages of development (Figure 4), further suggesting independent biochemical events that lead to either sugar accumulation or volatile formation processes. An indication that ethylene might be involved in the production of volatiles in melons is based on studies with ACO-inhibited transgenic melons, in which genes inferred to be related to aroma formation were also repressed (*29*). The inhibition of expression of some of these genes was reversed by exposure to exogenous ethylene. Nevertheless, parallel ripening-dependent increases in acetyltransferase activities related to aroma formation have been reported in strawberry, a nonclimacteric fruit (*20*).

In conclusion, we have reported that the profile of the volatiles in the headspace of ripe Arava melon fruits greatly differs from that obtained from unripe Arava fruits and from either ripe or unripe Rochet fruits. The major difference obtained is the high levels of volatile acetates, present almost exclusively in the aromatic Arava ripe fruits. We also followed the developmentally regulated increase in AAT activity during the ripening of Arava fruits, paralleling the accumulation of TSS.

Our results emphasize the main role of AAT in fruit aroma biogenesis. Our understanding of the enzymatic processes involved in the production of aromas will enable us to identify and manipulate genes involved in these processes to improve the aroma of melons and other fruits without compromising other agronomic properties.

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