Effect of Aminocyclopropane-1-carboxylic Acid Oxidase Antisense Gene on the Formation of Volatile Esters in Cantaloupe Charentais Melon (Cv. Védrandais)

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The role of ethylene on volatile formation associated with ripening was investigated on melon hybrids transformed with an aminocyclopropane-1-carboxylic acid oxidase antisense gene. The headspace of four antisense hybrid fruits was analyzed by GC/MS and compared to that of nontransformed hybrid fruit. The major volatiles extracted from nontransformed hybrids were esters, mostly acetates. However, the most potent odorants were ethyl esters, such as ethyl butanoate, and branched-chain esters, such as ethyl 2-methylpropanoate and ethyl 2-methylbutanoate. In antisense hybrids, the total volatiles were 60−85% lower than that of the nontransformed hybrids. Volatiles with low odor values, such as ethyl, 2-methylpropyl and 2-methylbutyl acetates, were half to a fifth lower than in nontransformed hybrids, whereas potent odorants, such as ethyl 2-methylpropanoate and ethyl 2-methylbutanoate, were <3% that of nontransformed hybrids. Examination of the biosynthetic pathways of volatile esters derived from amino acids demonstrates that ethylene stimulated preferentially the synthesis of the most potent odorants.

Keywords: *Ripening; postharvest; flavor; odor value; ACC oxidase antisense gene; ethylene*

INTRODUCTION

Charentais melons (*Cucumis melo* L.) from the *Cantalupensis* group are orange-fleshed and prized for their highly aromatic flavor. A major contribution of the characteristic odor of the fruit and their overall quality perception is made by esters and to a certain extent by sulfur compounds (Kemp et al., 1972; Homatidou et al., 1992; Wyllie et al., 1996a). A significant proportion of the esters contain a branched alkyl chain and originate from valine and isoleucine (Yabumoto and Jennings, 1977; Wyllie et al., 1994, 1996b).

Unlike American cantaloupe melons belonging to the *Reticulatus* group or honeydew melons from the *Inodorus* group (Whitaker and Davis, 1962), Charentais melons have poor keeping properties and short shelf life, usually associated with a sharp climacteric phase (Hadfield et al., 1995). To improve their storage and handling characteristics, a Charentais melon line has been recently transformed with a 1-aminocyclopropane-1-carboxylic acid oxidase (ACO) antisense gene (Ayub et al., 1996). This transformation inhibited ripening via strong reduction of ethylene synthesis. Further studies showed that some ripening parameters, such as softening on the vine and rind color, were severely affected by the transformation, whereas other parameters, such as sugar content, flesh color, or softening of detached fruit, were unaffected or only slightly affected by the transformation (Guis et al., 1997).

Aroma development in melons is strongly associated with ripening (Wang et al., 1996) and represents a major characteristic in the overall quality of the fruit. Before any commercialization of the fruit, it was, therefore, important to investigate the role of the ACO antisense transformation on volatile production. Moreover, the availability of antisense fruit allowed us to investigate the extent to which volatile production depended on ethylene biosynthesis.

This study was undertaken with antisense fruit that had been crossed with lines bearing important agronomic traits. The hybrids were grown, harvested, and handled according to normal commercial practices. We were therefore able to study the aroma profiles of fruit as the consumer would find them on the supermarket shelves.

MATERIALS AND METHODS

Plant Material. This work was carried out on Charentais melons (*Cucumis melo* var. *cantalupensis* Naud. cv. Védrandais). The R4 progeny of a line transformed with an ACO gene (Balagué et al., 1993) in antisense orientation (Ayub et al., 1996) was used as the antisense parental line (AS) to generate transformed F1 hybrids. Control melons were bred using the equivalent line without the ACO antisense gene (nT). Four other parental lines (PL1, PL2, PL3, and PL4) conferring important agronomic traits (such as resistance to pathogens or improved agronomic performances) were crossed with the nT line and with the AS line. This gave four nontransformed hybrids, PL1-nT, PL2-nT, PL3-nT, and PL4-nT, and four transformed hybrids, PL1-AS, PL2-AS, PL3-AS, and PL4-AS. Antisense fruit were assessed against nontransformed fruit from the corresponding hybrid obtained with the original nontransformed line. Plants were grown at the experimental station of Tezier Iberica, Almeria, Spain, following standard cultural practices. Nontransformed fruit were harvested when their rind turned yellow. Antisense fruit were harvested when the first leaf next to the fruit yellowed as in commercial practice. Fruit were air-freighted to the United Kingdom,

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Table 1. Flesh Firmness (FF) and Soluble Solids Content (SSC) of Melon Hybrids Sampled for Headspace Analysis[a]

	hybrid							
	PL1-nT	PL1-AS	PL2-nT	PL2-AS	PL3-nT	PL3-AS	PL4-nT	PL4-AS
FF (N)	10.0	15.0	9.1	10.3	11.3	12.0	13.9	18.7
SSC (%)	5.7	6.5	4.5	5.3	4.9	5.2	7.7	10.8

[a] Nontransformed (nT) and ACO antisense (AS) lines were crossed with parental line (PL) 1, 2, 3, or 4 to generate the hybrids.

stored for 3 days at 12 °C, and held overnight at 20 °C before analysis. The aim of this postharvest storage was to simulate transport and distribution from production sites to consumption sites.

Assessment of Flesh Firmness and Soluble Solids. The firmness of the flesh was taken at four points on a ring transversally cut at the equator at 1 cm distance of the rind with a hand-held penetrometer with an 8 mm probe (David Bishop, Heathfield, U.K.). Soluble solids were measured with a hand-held refractometer on juice from 2−4 g samples taken at a distance of 1 cm from the rind on the ring previously cut (Alavoine et al., 1988).

Aroma Extraction. Headspace concentration was carried out on Tenax TA and was followed by thermal desorption on GC/MS. Five plugs (1 cm diameter, 2 cm long, ∼10 g of tissue) were excised from the equator of each fruit, weighed, and dropped in a 250 mL flask previously filled with nitrogen and equilibrated at 37 °C. The flask was then placed in the water bath at 37 °C, and a flow of nitrogen swept the volatiles for 15 min at 100 mL/min onto a glass-lined, stainless steel trap

Table 2. Volatile Esters Identified in the Headspace of Melon Hybrids[a]

		approximate quantity[d] (ng/g)							
compound[b]	LRI[c]	PL1-nT	PL1-AS	PL2-nT	PL2-AS	PL3-nT	PL3-AS	PL4-nT	PL4-AS
acetates									
acetic acid, ethyl ester	612	185	38	322	40	458	189	664	42
acetic acid, 1-methylethyl ester	655	tr	1	1	2	tr	tr	tr	1
acetic acid, *n*-propyl ester	712	45	17	44	16	45	4	17	12
acetic acid, 2-methylpropyl ester	771	147	72	168	105	114	31	94	53
acetic acid, butyl ester	813	91	38	146	61	89	21	55	16
acetic acid, 2-methylbutyl ester	878	128	68	103	34	112	25	71	32
acetic acid, pentyl ester	906	3	2	8	2	4	1	2	tr
acetic acid, 4-pentenyl ester[e]	890					6	tr		
acetic acid, hexyl ester	1013	40	26	84	32	68	11	44	10
acetic acid, 3-hexen-1-yl ester	1002	50	8	105	18	59	7	12	1
acetic acid, heptyl ester[e]	1114	tr	tr	3	1	1	tr	1	tr
acetic acid, octyl ester	1213	4	3	25	8	8	1	5	1
acetic acid, phenylmethyl ester[e]	1184	24	10	44	33	10	9	7	4
acetic acid, 2-phenylethyl ester	1264	3	2	7	15	2	1	2	1
acetic acid, benzenepropyl ester[e]	1382	2	tr	3	1	tr	1	tr	tr
total		723	86	1065	369	978	302	974	174
propanoates and methylpropanoates									
propanoic acid, methyl ester	615	3	6	7	9	2	1	1	1
propanoic acid, ethyl ester	710	20	tr	59	4	25	23	41	tr
propanoic acid, propyl ester	809	tr	tr					tr	tr
propanoic acid, 2-methylpropyl ester	866	1	1	1	2	1	tr	tr	tr
propanoic acid, butyl ester	908	1	1	tr	tr	tr	tr		
propanoic acid, pentyl ester	1006		tr						
2-methylpropanoic acid, methyl ester	684	1	1	4	7	16	1	1	1
2-methylpropanoic acid, ethyl ester	755	4	1	36	1	30	7	25	tr
total		30	10	108	24	74	31	68	3
butanoates and methylbutanoates									
butanoic acid, methyl ester	720	5	8	26	19	11	3	5	1
butanoic acid, ethyl ester	801	67	5	108	6	104	25	96	1
butanoic acid, propyl ester	898	1	1	2	tr	1	tr		
butanoic acid, 2-methylpropyl ester	955	1	1	1	1	1	tr		
butanoic acid, butyl ester	996	tr				tr	tr	1	tr
2-methylbutanoic acid, ethyl ester[e]	843	14	1	41	1	28	6	39	tr
total		89	15	177	27	145	35	142	2
miscellaneous aliphatic esters									
pentanoic acid, methyl ester	823			tr	tr			1	tr
pentanoic acid, ethyl ester	901	1		3	tr	1	tr		
hexanoic acid, methyl ester	925	1	tr	4	1	1	tr	tr	tr
hexanoic acid, ethyl ester	999	4	tr	27	tr	12	2	23	tr
sulfur-containing compounds									
thioic acid, *S*-methyl ester[e]	702					tr	2	1	2
2-(methylthio)acetic acid, methyl ester[e]	918					tr			
2-(methylthio)acetic acid, ethyl ester[e]	990			tr	tr	1	tr	1	

[a] Nontransformed (nT) and ACO antisense (AS) lines were crossed with parental line (PL) 1, 2, 3, or 4 to generate the hybrids. [b] Identification was confirmed by comparison of mass spectrum and LRI with those of authentic compounds analyzed under similar conditions, except where indicated. [c] Linear retention index on column BPX-5. [d] Estimated quantities in headspace from ∼10 g of fruit tissue, calculated by comparison with 130 ng of 1,2-dichlorobenzene used as internal standard; average values of three or four replicates are given; compounds identified below 0.5 ng/g are reported as "tr" (trace). [e] Tentative identification based on comparison of mass sectrum with that in the NIST/EPA/NIH database.

Table 3. Estimated Odor Values of Major Volatile Compounds Identified in the Headspace of Melon Hybrids

compound	odor threshold[a] in water (ng/mL)	approximate OV (concn/odor threshold)							
		PL1-nT	PL1-AS	PL2-nT	PL2-AS	PL3-nT	PL3-AS	PL4-nT	PL4-AS
acetic acid, ethyl ester	5	37	8	64	8	92	38	13	8
acetic acid, 2-methylpropyl ester	65	2	1	3	2	2	<1	1	1
acetic acid, butyl ester	66	1	<1	2	<1	1	<1	1	<1
acetic acid, 2-methylbutyl ester	11	12	6	9	3	10	2	6	3
acetic acid, hexyl ester	2	20	13	42	16	34	6	22	5
acetic acid, octyl ester	12	<1	<1	2	<1	<1	<1	<1	<1
propanoic acid, ethyl ester	10	2	<1	6	<1	2	2	4	<1
2-methylpropanoic acid, ethyl ester	0.1	37	3	361	12	303	68	251	2
butanoic acid, ethyl ester	1	67	5	108	6	104	25	96	<1
2-methylbutanoic acid, ethyl ester	0.3	47	2	136	2	93	21	131	<1
hexanoic acid, ethyl ester	1	4	<1	27	<1	12	2	23	<1

[a] As reported in Takeoka et al. (1989, 1991, 1992).

(105 mm × 3 mm i.d.) containing 85 mg of Tenax TA (Scientific Glass Engineering Ltd., Melbourne, Australia). One microliter of internal standard (130.6 μg/mL 1,2-dicholorobenzene) was added to the trap at the end of the collection, and excess solvent and any water retained on the trap were removed by purging the trap with nitrogen at 100 mL/min for 5 min.

Gas Chromatography/Mass Spectrophotometry. All analyses were performed on a Hewlett-Packard 5972 mass spectrometer, fitted with an HP5890 Series II gas chromatograph. A CHIS injection port (Scientific Glass Engineering Ltd.) was used to thermally desorb the volatiles from the Tenax trap onto the front of a BPX-5 fused silica capillary column (50 m × 0.32 mm i.d., 0.5 μm film thickness; Scientific Glass Engineering Ltd.). The Tenax traps were thermally desorbed at 250 °C in the injection port while the temperature of the oven was maintained at 0 °C for 8 min. After the desorption, the oven temperature was then raised to 50 °C over 1 min and held for 2 min before programming to 100 °C at a rate of 2.5 °C/min and then to 250 °C at a rate of 6 °C/min. Helium at 8 psi was used as the carrier gas, resulting in a flow of 1.75 mL/min at 40 °C. Mass spectra were recorded in the electron impact mode at an ionization voltage of 70 eV and a source temperature of 200 °C. A scan range of m/z 29−400 with a scan time of 0.69 s was employed. The data were controlled and stored by the HP G1034C ChemStation system.

Volatiles were identified by comparison of each mass spectrum with spectra from authentic compounds analyzed in our laboratory or with spectra in reference collections (NIST/EPA/NIH Mass Spectral database). A linear retention index (LRI) was calculated for each compound using the retention times of a homologous series of C_6−C_{22} n-alkanes analyzed under the same conditions. Wherever possible, mass spectral identifications were confirmed by comparing the LRI with those of authentic compounds analyzed under similar conditions.

Quantitation of the volatiles was based on the relation between their peak areas and that of the 1,2-dichlorobenzene internal standard, obtained from the total ion chromatograms, using a response factor of 1.

RESULTS AND DISCUSSION

Fruit Quality. Flesh firmness values of the four nontransformed hybrids were all approximately 10 N, with PL4-nT fruit being firmer (Table 1). Antisense fruit of hybrids PL1-AS and PL4-AS were ~50% firmer than corresponding nontransformed hybrid fruit, whereas antisense fruits from the hybrids PL2-AS and PL3-AS were not significantly different from their nontransformed equivalents. All fruits considered were within the limits of firmness associated with desirable texture (Mutton et al., 1981). It has been reported that flesh firmness of fruit from the pure antisense line (the transformed parental line of the hybrids used in this study) decreased steadily after harvest, even under ethylene-free conditions (Guis et al., 1997). Moreover, in a similar experiment carried out on antisense hybrid fruit grown in a glasshouse, the internal ethylene concentration at harvest was <2 ppm, but this was still physiologically significant and might induce softening during the storage period (Guis et al., 1997).

The soluble solids content (SSC) of nontransformed hybrids was <10% (Table 1), which is the minimum recommended level for good dessert quality (Mutton et al., 1981). The SSC of antisense hybrids was in general higher than that of their corresponding nontransformed fruit. Sugar accumulation in melon is not ethylene-dependent (Ayub et al., 1996), but inhibition of ethylene synthesis reduces leaf senescence, as demonstrated on ACO antisense tomato foliage (John et al., 1995). Therefore, harvest based on "first yellow leaf" allows antisense fruit to stay on the vine longer than nontranformed fruits (Ayub et al., 1996). Thus, antisense fruit can accumulate higher amounts of soluble sugars than nontransformed fruit.

The fruit were not subjected to full sensory analysis, but informal assessment of the aroma of the intact fruit and the cut fruit by horticulturists and flavor chemists associated with the project clearly showed that all of the antisense fruit had less aroma than the nontransformed fruit.

Volatile Analysis. A total of >80 compounds were identified in the headspace volatiles of the melons. The most abundant compounds are listed in Table 2.

The main compounds identified in Charentais melons, using headspace analysis, were esters, with ethyl acetate, 2-methylpropyl acetate, and 2-methylbutyl acetate comprising at least 60% of the total volatiles collected from the nontranformed fruit. Willye et al. (1996a) also reported these compounds to be predominant in Makdimon melon aroma, although they performed volatile extraction by simultaneous distillation−extraction. Moreover, Wang et al. (1996) showed that ethyl acetate represented about two-thirds of the total volatile fraction of ripe Makdimon melon when analyzed by headspace. In their study, 2-methylbutyl acetate and ethyl butanoate were the two other major volatiles. Buttery et al. (1982) prepared a synthetic honeydew aroma mixture, that is ethyl acetate, 2-methylpropyl acetate, butyl acetate, 3-methylbutyl acetate, and benzyl acetate. All of these esters were identified in the Charentais melons except 3-methylbutyl acetate.

With the small samples used in this work, only traces of sulfur compounds were found in melon headspace, and some of them coeluted with much more abundant esters (Table 2). These included methyl and ethyl (2-methylthio)acetate. Some authors have reported that trace amounts of certain sulfur compounds such as ethyl

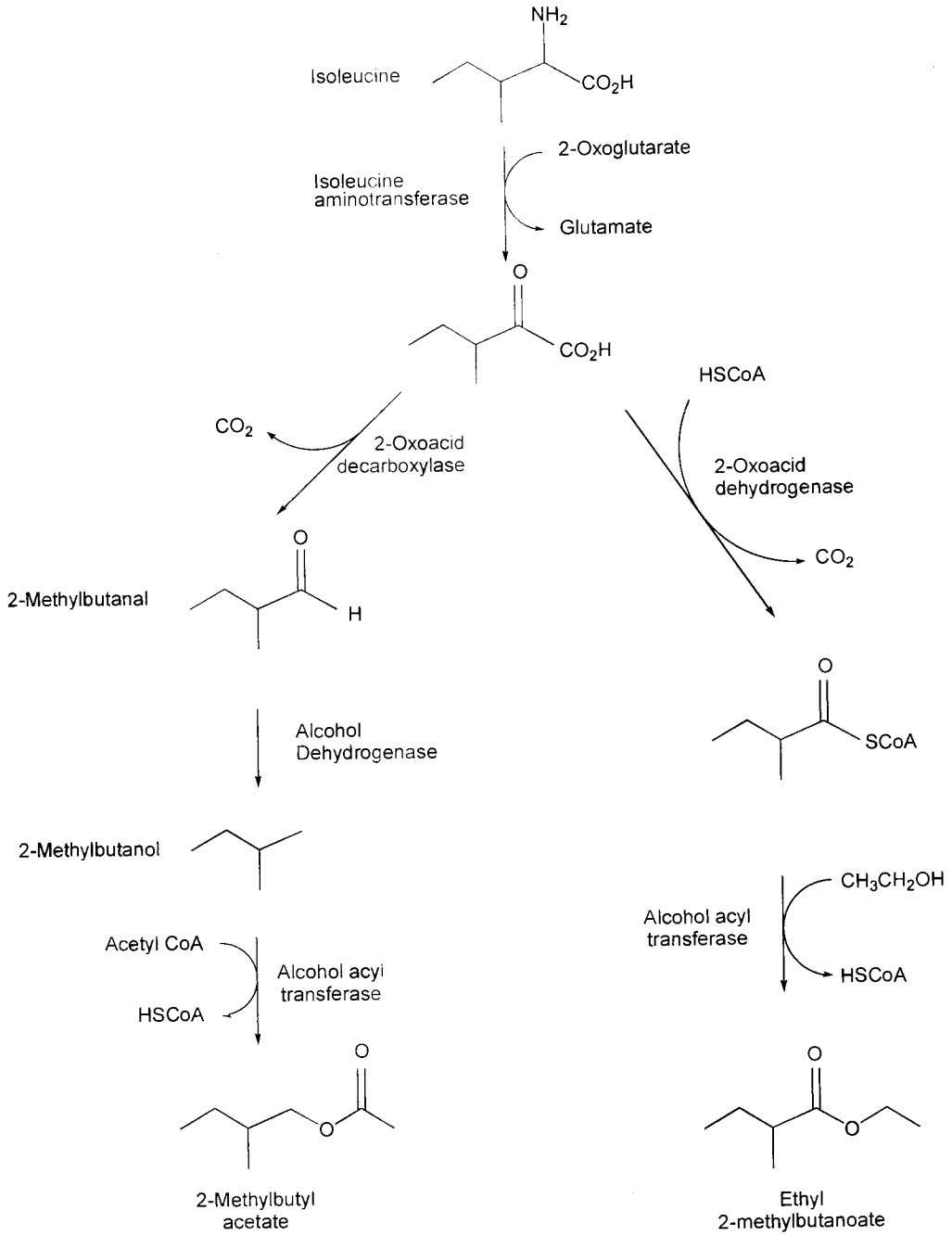

Figure 1. Biosynthetic pathways of 2-methylbutyl acetate and ethyl 2-methylbutanoate.

(2-methylthio)acetate have a major impact on the musky note of some melon aromas (Homatidou et al., 1992; Wyllie and Leach, 1992; Wyllie et al., 1994). Further work would be required, using larger samples and sulfur-specific detection methods, to evaluate the role of ethylene on the concentration of such sulfur compounds.

Only very small amounts of C_6 and C_9 aldehydes and alcohols, such as 2- and 3-hexenal, nonenal, and nonadienol, were identified in melon headspace, but their concentrations were too low to quantify and they are not included in Table 2. In contrast, Schieberle et al. (1990) found that green notes due to aldehydes were among the most potent odorants of muskmelons. However, in that work, fruit flesh had been homogenized for 90 s and then left for 5 min at 22 °C prior to extraction, thus favoring the formation of products from the degradation of fatty acids. In addition, Lester (1990) showed that lipoxygenase activity in the flesh of ripe netted muskmelon was very low. Therefore, the contribution of volatiles conferring green notes, such as aldehydes, should not be expected to be of significant importance under our extraction protocol because excised tissue was left for 15 min under an oxygen-free atmosphere during the headspace trapping and the sample was not previously blended. Our results are more in agreement with those of Wyllie and Leach (1992), who reported no discernible levels of unsaturated C_6 or C_9 aldehydes and alcohols in muskmelons.

Traces of some terpenes such as eucalyptol, geranylacetone, and limonene were also found, but the concentrations were too low to quantify and they are not included in Table 2.

Variation among Nontransformed Hybrids. Although the aroma profiles of the four nontransformed hybrids showed similar trends, major quantitative

variations were sometimes encountered. For instance, ethyl acetate content in PL1-nT hybrids was <200 ng/g and represented ~25% of the total acetates identified in their headspace. In contrast, ethyl acetate content in PL4-nT hybrids was >600 ng/g, making up nearly 70% of the acetates detected in their headspace. Other esters often showed comparable variation. Yabumoto and Jennings (1977) observed significant differences in the concentration of ethyl 2-methylbutanoate, as well as ethyl 2-methylpropanoate, among fruits showing only slight differences in maturity. Horvat and Senter (1987) and later Wang et al. (1996) showed that major changes in aroma composition occur just before abscission of the fruit from the vine. It is possible that the maturity stages of the nontransformed hybrids studied here were slightly different. However, another source of variation is due to the cultivars themselves, as several authors have already reported (Yamaguchi et al., 1977; Yabumoto et al., 1978; Wyllie and Leach, 1992). For instance, PL1-nT has been rated as a less aromatic hybrid by breeders compared to the other hybrids (A. Vermeulen and D. Lor, personal communication).

Effect of ACO Antisense Gene on Aroma Profile. Despite the variations seen among cultivars, whether due to heterogeneity of sampling or cultivar variations, the effect of ACO antisense gene was dramatic. Only ~20−30% of the total quantity of acetates obtained in nontranformed fruit headspace were present in the antisense fruit headspace (Table 2). The effect of the ACO antisense gene was even greater on propanoates and butanoates, although it was more pronounced on the PL2-AS and PL4-AS fruits compared with PL1-nT and PL3-nT fruits. Ethyl acetate was affected more by the ACO antisense than the other acetates.. The significant reduction of ethyl acetate in ACO antisense hybrids shows that the availability of either or both moieties of the ester is reduced or that their condensation, catalyzed by an alcohol acetyltransferase (Ueda et al., 1997), is regulated by ethylene.

Odor value (OV) provides a means of indicating which compounds are main contributors to an aroma. They are obtained by dividing the concentration of the compound by its known odor threshold value in water. Thus, compounds with OVs >1 are likely to contribute to the overall aroma of the product. The method does have some limitations (Mistry et al., 1997), but it can be a guide to the relative importance of the compounds in the aroma. On the basis of data available in the literature, OVs of the main volatile esters present in melon headspace have been estimated (Table 3).

Generally, acetates have higher odor threshold values than most other simple esters, such as ethyl butanoates, ethyl 2-methylpropanoate, ethyl 2-methyl butanoate, or ethyl hexanoate. Therefore, the contribution of acetates to the overall aroma may be less important than that of some other esters. Under our conditions, acetates with low OVs were generally the least depleted by the ACO antisense gene, whereas those ethyl esters showing high OVs were the most affected by the transformation.

Ethyl 2-methylpropanoate, ethyl 2-methylbutanoate, and ethyl butanoate appeared to be major contributors to the aroma in three of four nontransformed hybrids. Valine and isoleucine are likely precursors of these esters (Schreier, 1984). These ethyl esters will have the same precursors as 2-methylpropyl acetate, 2-methylbutyl acetate, and butyl acetate, respectively. Thus, the effect of the ACO antisense transformation is greater on ethyl esters compared with acetates. It shows that the pathway leading to the synthesis of isomeric esters is more affected when the ethyl provides the alcohol moiety of the ester (i.e., an ethyl ester) than when the ethyl comprises the acid moiety (i.e., an acetate). The synthesis of ethyl 2-methylbutanoate or 2-methylbutyl acetate from isoleucine readily illustrates this observation (Figure 1). Some, if not all, steps leading from isoleucine to ethyl 2-methylbutanoate via the formation of 2-methylbutyl-CoA are more affected by the ACO antisense transformation than the steps leading to 2-methylbutyl acetate via the formation of 2-methylbutanol. A similar regulation occurs in the pathway from valine to ethyl 2-methylpropanoate and to 2-methylpropyl acetate.

Conclusions. The volatiles of all the melons were dominated by straight-chain esters together with branched-chain esters derived from valine and isoleucine. When melons were transformed with an antisense ACC oxidase gene, the effect of delayed ripening on aroma profiles was very pronounced. It resulted in a considerable reduction in total volatile composition, with compounds previously described as potent odorant or with high odor value being the most affected by the transformation. It has been shown that pathways leading from amino acids to ethyl esters with branched-chain acyl groups are more strongly regulated by ethylene than the formation of acetates with branched-chain alcohol moieties.

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