The definitive version is available at www.springerlink.com

Functional characterization of a melon alcohol acyl-transferase gene family involved in the biosynthesis of ester volatiles. Identification of the crucial role of a threonine residue for enzyme activity★

Islam El-Sharkawy†, Daniel Manríquez†, Francisco B. Flores†, Farid Regad, Mondher Bouzayen, Alain Latché and Jean-Claude Pech*

*UMR 990 INRA/INPT-ENSAT "Génomique et Biotechnologie des Fruits", Av. de l'Agrobiopole, BP 32607, F-31326 Castanet-Tolosan Cedex, France (*author for correspondence; e-mail pech@ensat.fr); †These authors contributed equally to the work*

Key words: alcohol acyl-transferase, aromas, melon, site-directed mutagenesis, volatile esters

Abstract

Volatile esters, a major class of compounds contributing to the aroma of many fruit, are synthesized by alcohol acyl-transferases (AAT). We demonstrate here that, in Charentais melon (*Cucumis melo* var. *cantalupensis*), AAT are encoded by a gene family of at least four members with amino acid identity ranging from 84% (Cm-AAT1/Cm-AAT2) and 58% (Cm-AAT1/Cm-AAT3) to only 22% (Cm-AAT1/Cm-AAT4). All encoded proteins, except Cm-AAT2, were enzymatically active upon expression in yeast and show differential substrate preferences. Cm-AAT1 protein produces a wide range of short and long-chain acyl esters but has strong preference for the formation of *E*-2-hexenyl acetate and hexyl hexanoate. Cm-AAT3 also accepts a wide range of substrates but with very strong preference for producing benzyl acetate. Cm-AAT4 is almost exclusively devoted to the formation of acetates, with strong preference for cinnamoyl acetate. Site directed mutagenesis demonstrated that the failure of Cm-AAT2 to produce volatile esters is related to the presence of a 268-alanine residue instead of threonine as in all active AAT proteins. Mutating 268-A into 268-T of Cm-AAT2 restored enzyme activity, while mutating 268-T into 268-A abolished activity of Cm-AAT1. Activities of all three proteins measured with the prefered substrates sharply increase during fruit ripening. The expression of all Cm-AAT genes is up-regulated during ripening and inhibited in antisense ACC oxidase melons and in fruit treated with the ethylene antagonist 1-methylcyclopropene (1-MCP), indicating a positive regulation by ethylene. The data presented in this work suggest that the multiplicity of AAT genes accounts for the great diversity of esters formed in melon.

Introduction

Aroma volatiles contribute to a large extent to the overall sensory quality of fruit. Research during the last decades has been dedicated to the identification of volatile compounds present in fruit aromas, and to the elucidation of some of the biosynthetic routes by either bioconversion or by tracing of precursors (Sanz *et al.*, 1997; D'Auria *et al.*, 2002; Dudareva *et al.*, 2004). Recently, research efforts have been directed to the isolation of genes involved in the production of aroma

★ Accession numbers Cm-AAT1 (CAA94432), Cm-AAT2 (AAL77060), Cm-AAT3 (AAW51125), and Cm-AAT4 (AAW51126).

volatiles of fruit (Aharoni *et al.*, 2000; Yahyaoui *et al.*, 2002; Beekwilder *et al.*, 2004) or scent of flowers (Dudareva and Pichersky, 2000; Shalit *et al.*, 2003). Although fruit aroma is generally a complex mixture of a wide range of compounds, volatile esters often represent the major contribution to the odour of fruit such as apple and pear (Paillard, 1990), banana (Shiota, 1993) pineapple (Elss *et al.*, 2005) and strawberry (Zabetakis and Holden, 1997). In cantaloupe melons esters represent the major group of aroma volatiles emitted and they are likely to be the key contributors to the unique aroma of ripe melon (Homatidou *et al.*, 1992; Beaulieu and Grimm, 2001). Among cantaloupe melons, the Charentais type is highly aromatic and the synthesis of aroma volatiles is regulated by the plant hormone ethylene (Flores *et al.*, 2002). Extension of shelf-life either by breeding or by genetic engineering has led to strong reduction in aroma volatiles production (Bauchot *et al.*, 1998; Aubert and Bourger, 2004).

The esterification step is catalysed by alcohol acyl-transferase enzymes (AAT) that transfer an acyl-CoA to an alcohol (Harada *et al.*, 1985). These enzymes are capable of combining different alcohols and acyl-CoAs resulting in the synthesis of a wide range of esters accounting for the diversity of esters emitted by the fruit. The alcohol moiety of the esters arises from either lipids or amino acids produced by the degradation of lipids and proteins respectively (Sanz *et al.*, 1997). The availability of the substrates and/or selectivity of the enzymes upstream of AATs may account, at least in banana, for the amount and type of ester formed (Wyllie and Fellman, 2000). However, it is possible the diversity of volatile esters arises from the multiplicity of genes encoding AAT enzymes with different specific substrate selectivity.

So far, in fruit and flowers only one or two *AAT* genes have been characterized (Dudareva *et al.*, 1998; Aharoni *et al.*, 2000; D'Auria *et al.*, 2002; Shalit *et al.*, 2003; Beekwilder *et al.*, 2004). In melon, two putative *AAT* genes have been isolated (*Cm-AAT1* and *Cm-AAT2*) with strong identity (84%), but only the *Cm-AAT1*-encoded protein showed AAT activity upon expression in yeast (Yahyaoui *et al.*, 2002). A large number of acyl-transferase genes are present in plants with around 70 members encountered in *Arabidopsis* (Pichersky and Gang, 2000). Although performing the same reaction, AAT proteins from different fruit species may be highly divergent. For instance SAAT and Cm-AAT1 have only 22% identity while they have similar preference for substrates (Aharoni *et al.*, 2000; Yahyaoui *et al.*, 2002). These considerations have led us to search for other AAT members in the melon and to characterize their substrate preferences. By comparing within-species and inter-species gene sequences we have also undertaken a site-directed mutagenesis approach to determine why *Cm-AAT2*, a gene closely related to *Cm-AAT1* was encoding a protein that was unable to produce volatile esters.

Materials and methods

Plant material and post harvest treatments

Wild-type (WT) and ACC oxidase antisense (AS) Charentais cantaloupe melons (*Cucumis melo var. Cantalupensis*, *Naud* cv. *Védrantais*) were used (Ayub *et al.*, 1996; Guis *et al.*, 1997). They were grown on a trellis in a greenhouse under standard cultural practices for fertilization and pesticide treatments. Freshly opened female flowers were tagged on the day of hand-pollination to identify fruit of known age. Melons were harvested after 32, 35, 37, 39 and 42 days after pollination (DAP) and ethylene production measured immediately after harvest. Fruit were selected for homogenous ethylene production. Stages of ripening of WT fruit, and equivalent age for AS fruit, corresponded to mature green (32 DAP), onset of ripening (35 DAP), early climacteric (37 DAP), full climacteric (39 DAP) and late climacteric (42 DAP). Antisense fruits, harvested 35 DAP, were exposed to 50 $\mu l\ l^{-1}$ ethylene for 1 and 3 days. The ethylene inhibitor, 1-methylcyclopropene (1-MCP) was also applied 35 DAP to WT fruit on the vine at 1 $\mu l\ l^{-1}$ in 3-l jars for one and three days before harvesting. Both treatments were performed with periodical flushing with air and re-injection of ethylene or 1-MCP. Vegetative tissues (leaves, stems, seeds and roots) and flowers were collected from plants grown in a greenhouse. All plant material was frozen in liquid nitrogen and stored at –80 °C.

RNA isolation

Total RNA from fruit samples was extracted using the methods described by Boss *et al.* (1996). For

leaf, stem, seed, flower, and root material, total RNA was extracted according to the RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). All RNA extracts were treated with DNase I (Promega, Madison, WI, USA) and cleaned up with a phenol-chloroform extraction.

Isolation and in silico analysis of Cm-AAT *sequences*

Cm-AAT1 and *Cm-AAT2* had been isolated prior to this study (Aggelis *et al*., 1997; Yahyaoui *et al*., 2002). *Cm-AAT3* and *Cm-AAT4* have been isolated by PCR from a cDNA library of ripe melon. The SK primer (in Bluescript: 5′-CGCTCTAGAACTAGTGGATCCC-3′) was combined with the degenerated primers, Cm-AAT (F): 5′-GTGGACTTTGGATGGGGAAARSCC-3′ and Cm-AAT (R): 5′-GGSYTTTCCCCATCCAAAGTC CAC-3′, designed from a conserved region (VDFGWGK) among plants acyl-transferase (St Pierre *et al*., 1998). The isolated fragments were cloned by using Qiagen PCR Cloning *plus* Kit (Qiagen, Valencia, CA, USA), sequenced and compared with database sequences using the BLAST program (Altschul *et al*., 1997). Extension of the partial cDNA clones was carried out using the 3′- and 5′- RACE kit (Invitrogen, Paisely, UK). First strand cDNA synthesis was carried out using 10 μg of total DNase-treated RNA in a 50-μl aliquot followed by PCR with specific Cm-AAT primers using 1 μl of cDNA. A high fidelity PCR system (BMB Indianapolis, IN, USA) was used with the following PCR parameters: 3 min template denaturation at 95 °C for one cycle, followed by 5 cycles at 95 °C (30 s), 58 °C (1 min), and 72 °C (1 min 30 s), then 25 cycles at 95 °C (30 s), 58 °C (1 min), and 72 °C (2 min) with a final 10 min extension step at 72 °C to isolate the full length *Cm-AAT* sequences. Alignments of the predicted protein sequences were performed with ClustalX (Jeanmougin *et al*., 1998) and GeneDoc (Nicholas and Nicholas, 1997). The neighbour-joining tree was constructed with PAUP* 4.0b3. The tree excluded regions of the alignment where poor matching occurred. Bootstrap values from 1000 replicates were obtained. The tree was visualised with the TreeView program (Page, 1996). Finally, a cDNA clone was isolated with homology to a β-actin sequence (AY859055). It was checked by Northern blot analysis (data not shown) that the β-actin mRNA level was similar in all treatments. The gene was, thereafter, used as an internal control in gene expression studies.

Real time quantitative RT-PCR

DNase-treated RNA (4 μg) was reverse transcribed in a total volume of 40 μl using Omniscript Reverse Transcription Kit (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed using 100 ng of total RNA in a 20 μl reaction volume using SYBR GREEN PCR Master Mix (PE-Applied Biosystems, Foster City, CA, USA) on an ABI PRISM 7900HT sequence-detection system. PRIMER EXPRESS software (PE-Applied Biosystems) was used to design gene-specific primers (Table 1). For all the genes studied here, optimal primer concentration was 300 nM. RT-PCR conditions were as follow: 50 °C for 2 min, followed by 95 °C for 10 min, then 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All RT-PCR experiments were run in triplicate with different cDNAs synthesized from

Table 1. Real-time quantitative PCR primers.

Name	Oligonucleotide sequence
Cm-actin-344 (F)	5′-GTGATGGTGTGAGTCACACTGTTC-3′
Cm-actin-426 (R)	5′-ACGACCAGCAAGGTCCAAAC-3′
Cm-AAT1-1181 (F)	5′-CCACAGGGGCCAGAATTACA-3′
Cm-AAT1-1284 (R)	5′-TGGAGGAGGCAAGCATAGACTT-3′
Cm-AAT2-1175 (F)	5′-CTATAATTGGAGGGTGTGGAATTATC-3′
Cm-AAT2-1311 (R)	5′-AACATTTGCCCTAAATCTTTCCAT-3′
Cm-AAT3-677 (F)	5′-CGCTTGATGACATGGCACAT-3′
Cm-AAT3-744 (R)	5′-GGCCTTACGGATAGCAGAGATC-3′
Cm-AAT4-659 (F)	5′-CAGTTGTACCCCCGTCGAGTA-3′
Cm-AAT4-734 (R)	5′-AATATCGCTTCTGATCGGAACAC-3′

three biological replicates. Each sample was run in three technical replicates on 384-well plate. For each sample, a Ct (threshold constant given by the ABI PRIM 7900 Applied Biosystem 'sds'software) value was calculated from the amplification curves by selecting the optimal ΔRn (emission of reporter dye over starting background fluorescence) in the exponential portion of the amplification plot. Relative fold differences were calculated based on the comparative Ct method using the β-actin as an internal standard. To demonstrate that the efficiencies of the different gene primers were approximately equal, the absolute value of the slope of log input amount versus ΔCt was calculated for all the *Cm-AAT* and β-actin genes and was determined to be < 0.1. To determine relative fold differences for each sample in each experiment, the Ct value for *Cm-AAT* genes was normalized to the Ct value for β-actin and was calculated relative to a calibrator (flowers for *Cm-AAT1* and *Cm-AAT2*, seeds for *Cm-AAT3*, and antisense melon fruit, 39 DAP for *Cm-AAT4*) using the formula $2^{-\Delta\Delta Ct}$.

Expression of Cm-AAT *and site-directed mutagenesis*

All *Cm-AAT* cDNAs were cloned in the pYES2.1 TOPO-TA cloning vector for regulated protein expression in yeast following the instructions provided by the manufacturer (Invitrogen, Paisely, UK). Auto-ligated construct was used as negative control. All the constructs were transformed into the *Saccharomyces cerevisiae* cell line INVSc1. The strain harbouring the correct constructions were grown in selective medium (SC-U) with 2% galactose as inducer of the recombinant protein expression, at 30 °C and 250 rpm, according to Invitrogen recommendations, until the OD_{600} of the culture reached ~4 U.

All *Cm-AAT* mutants were generated using the QuikChange (Stratagene, San Diego, CA, USA) PCR-based method with *Cm-AAT* genes cloned in the pYES2.1 TOPO-TA cloning vector. After that, all mutants were transformed into *Saccharomyces cerevisiae* as described before.

Purification of recombinant AAT

Cells were collected by centrifugation (1800 *g*, 10 min at room temperature) from 50 ml of yeast cultures induced with galactose and resuspended in buffer A (50 mM sodium phosphate pH 7.5, 10% v/v glycerol, 0.3 M NaCl) containing 2 mM β-mercaptoethanol. The cells were mechanically ground in liquid nitrogen for 2 min and stored at −80 °C until needed. To extract AAT enzyme, the powder was thawed and centrifuged at 45 000 *g* for 20 min at 4 °C. The crude extract obtained was purified by an affinity column designed to purify polyhistidine-tagged proteins (BD Talon Metal Affinity, BD Biosciences), according to the manufacturer protocol. Briefly, the enzyme was fixed to the resin in the presence of buffer A, and after removing the unbound proteins with several washes with the same buffer the recombinant protein was eluted with buffer A containing 150 mM imidazole. Proteins were quantified according to Bradford (1976).

AAT *enzyme activity assay with recombinant proteins*

AAT activity was assayed in 500 μl total volume containing 70 μl of purified protein (10–15 μg), 2 mM alcohol, 250 μM acyl-CoA and adjusted to final volume with buffer B (Tris–HCl 50 mM pH 7.5, 10% v/v glycerol, 1 mM DTT). The mixture was incubated at 30 °C for 20 min. Immediately after reaction 5 μl the internal standard (methyl benzoate 0.5 $\mu l\ l^{-1}$) were added into the mixture. The volatile compounds were extracted with 250 μl of pentane, vortexed for 1.5 min and the pentanolic phase was concentrated. The quantification of these compounds was done by gas chromatography (GC); 1 μl of concentrated pentanolic phase was injected into the GC equipment with a flame ionisation detector (GC-FID). The GC-FID equipment was made up of a Hewlett–Packard gas chromatograph (model 5890) equiped with a HP-Innowax cross-linked polyethylene glycol column (30 m × 0.25 mm × 0.25 μm). Injector and detector temperatures were 250 °C. The oven temperature was programmed from 40 °C (1 min) to 60 °C (1 min) at a rate of 2 °C min^{-1} and finally to 190 °C (5 min) at a rate of 10 °C min^{-1}. Nitrogen was used as a carrier gas at 100 kPa. Compounds were identified by comparison of retention times with those of authentic standards.

AAT *activity assay of melon fruit crude proteins*

Total protein from melon pulp was extracted using the method described by Harada *et al*. (1985) with modifications. One gram of mesocarp tissue with 1 ml of extraction buffer (250 mM Tris/HCl, pH 7.5, 1 mM DTT) was ground mechanically in liquid nitrogen during 2 min and the protein crude extract was stored at −80 °C until needed. The protein extract was thawed in ice and centrifuged at 45 000 *g* for 20 min at 4 °C. The supernatant phase was desalted using Sephadex G-25 columns (Amersham Biosciences) and eluted with buffer B. Total proteins were quantified according to Bradford (1976). The AAT assay was performed as described above, the volume of soluble fraction of protein extract was 300 μl and the total incubation time was 30 min.

Results and discussion

The AAT *gene family of melon and predicted proteins*

In the present work, two additional full-length cDNAs have been isolated, *Cm-AAT3* and *Cm-AAT4*, putatively encoding alcohol acyltransferases (AAT) that differed from the previously isolated *Cm-AAT1* and *Cm-AAT2* genes (Yahyaoui *et al*., 2002). Multiple alignment of the four putative *Cm-AAT* and other characterized *AAT* genes from fruit or flowers (Figure 1) highlighted a number of conserved motifs that are common to the plant BAHD (benzylalcohol acetyl-, anthocyanin-O-hydroxy-cinnamoyl-, anthranilate-*N*-hydroxy-cinnamoyl/benzoyl-, deacetylvindoline acetyltransferase, *O*-acyltransferases) family (St Pierre and De Luca, 2000). Among them, the HXXXD motif located in the middle of the sequence (168–172 residues in Cm-AAT1) is the only conserved motif between the higher plants *AATs* and yeast, thus suggesting that this element is involved in the catalytic activity of acyl-transfer from an acyl-CoA to an alcohol (Yoshimoto *et al*., 1999). Another highly conserved motif, DFGWG, was present near the C terminus (380–385 residues in Cm-AAT1). The recent discovery of the crystal structure of a member of the BAHD family, vinorine synthase, allowed to show that the HXXXD motif region participating in catalysis was located in the centre of the reaction channel, while the DFGWG motif, which is necessary for the reaction, was located away from the active site and seems to play a structural role (Ma *et al*., 2005). A third less conserved sequence, LXXyypXaGr, is present near the N terminus (Aharoni *et al*., 2000; St-Pierre and De Luca, 2000), corresponding to residues 76–85 in Cm-AAT1 (Figure 1).

The *Cucumis melo AAT1-4* encode proteins of 462, 461, 459, and 479 amino acids, respectively, which corresponds to approximately the average size of other plant AATs (Table 2). The deduced molecular mass was of 51.5, 51.8, 51.1, and 55.0 kDa and the theorical pIs of 8.0, 8.5, 7.64, and 7.62, respectively. Amino acid sequence identities over the whole sequence of *AAT* genes from melon and other species are presented in Table 2. There is considerable divergence among AATs not only between species, but also within *Cucumis melo*. Among melon Cm-AATs, considerable

Table 2. Amino acid sequence comparison between the predicted full length *Cucumis melo*, *Clarkia breweri*, *Fragaria x ananassa*, *Fragaria vesca*, *Musa sapientum*, and *Rosa hybrida* alcohol acyl-transferase (AAT) proteins.

Protein		Amino acid identity (%)			
Name	Size	Cm-AAT1	Cm-AAT2	Cm-AAT3	Cm-AAT4
Cm-AAT1	462	–			
Cm-AAT2	461	84	–		
Cm-AAT3	459	58	60	–	
Cm-AAT4	479	22	23	21	–
Cb-BEBT	456	52	52	70	22
SAAT	452	22	22	23	34
VAAT	455	23	23	23	35
Rh-AAT	457	24	24	24	32
Cb-BEAT	433	26	25	24	28
Ban-AAT	419	31	31	34	22

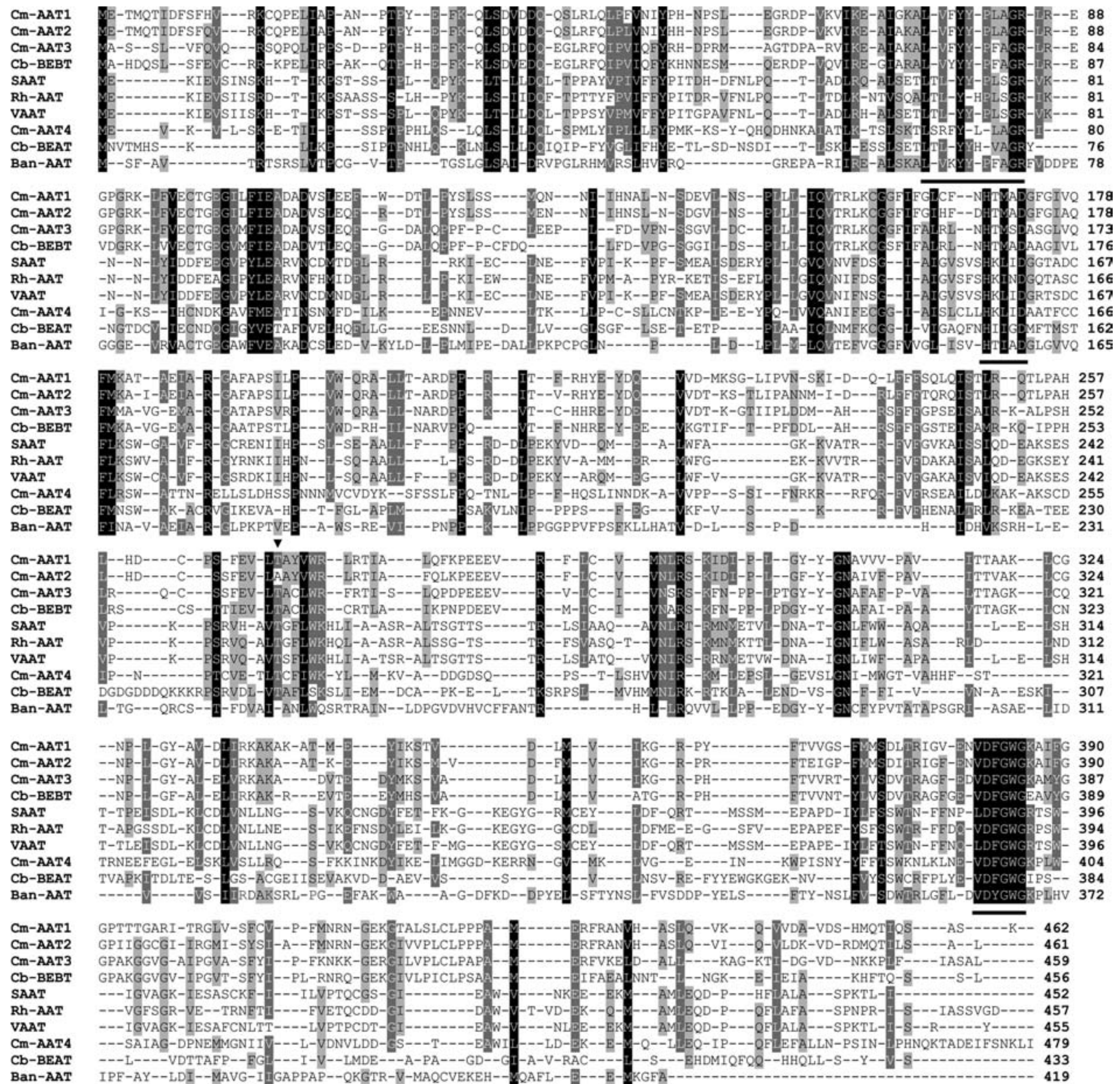

Figure 1. Amino acid sequence alignment of the *Cucumis melo* enzymes, Cm-AAT1 (CAA94432), Cm-AAT2 (AAL77060), Cm-AAT3 (AAW51125), and Cm-AAT4 (AAW51126) with closely related sequences *Clarkia breweri* Cb-BEBT (AAN09796), Cb-BEAT (AAF04787), *Musa sapientum* Ban-AAT (CAC09063), *Fragaria x ananassa* SAAT (AAG13130), *Fragaria vesca* VAAT (CAC09062), and *Rosa hybrida* Rh-AAT (AAW31948) using Clustal X program. Conserved residues are shaded in black. Dark grey shading indicates similar residues in eight out of ten of the sequences and clear grey shading indicates similar residues in six out of ten of the sequences. The underlined amino acids represent the conserved regions of plant acyl-transferase considered as playing role in the activity. The arrow represents the residue which has been studied by site-directed mutagenesis.

divergence exist between Cm-AAT1 and Cm-AAT4 with 22% identity only, although Cm-AAT1, Cm-AAT2 and Cm-AAT3 are more closely identical (84–58%). Melon Cm-AAT1-3, but not Cm-AAT4, have high identity to Cb-BEBT with 52%, 52%, and 70% identity, respectively. Within the fruit AAT characterized so far, the VAAT gene from wild strawberry, showed the highest identity (35%) with the melon Cm-AAT4 (Table 2).

A phylogenetic tree comprising ten AAT sequences from six species, *Cucumis melo*, *Fragaria*

x ananassa, *Fragaria vesca*, *Musa sapientum*, *Clarkia breweri*, and *Rosa hybrida* has been generated (Figure 2) showing three main subfamilies (I, II, and III) based on sequence conservation. Establishing a correlation between phylogenesis and functional properties of the encoded proteins is hampered by the diversity of potential substrates and by the fact that authors have not always tested the same range of precursors (combinations of alcohols and acyl-CoAs). However, published data and Table 3 allow outlining a number of functional characteristics. Cm-AAT4, which is so far the only member of subfamily I, has almost exclusive preference for producing acetate esters. However, although Cm-AAT4 is quite divergent from the other AATs, the encoded enzyme has the same strong preference for producing cinnamoyl acetate, *Z*-2-hexenyl acetate and geranyl acetate. Similarly, a member of subfamily III, Ban-AAT of banana, has also strong preference for the formation of cinnamoyl and geranyl acetate (Beekwilder *et al.*, 2004). Subfamily II comprises three members with only two of them (Rh-AAT and SAAT) having the same preference for generating geranyl acetate (Shalit *et al.*, 2003; Beekwilder *et al.*, 2004). Despite strong sequence identity, the SAAT and VAAT proteins differed in their substrate preference (Beekwilder *et al.*, 2004). Subfamily III comprises a large number of sub-branches in which very few functional similarities exist. However, the melon Cm-AAT3 and the *Clarkia* Cb-BEBT have in common a strong preference for synthesizing benzyl acetate (D'Auria *et al.*, 2002). Interestingly, this preference is also seen in Cb-BEAT which is quite divergent (Dudareva *et al.*, 1998). Another functional divergence within this subfamily that has been reported previously (Yahyaoui *et al.*, 2002) is the inability of Cm-AAT2 to generate volatile esters contrary to its closest neighbour, Cm-AAT1. A re-evaluation of the functional similarities or divergences will become possible when a wider range of acyl donors will be tested. The activity of many of the proteins mentioned in the phylogenetic tree has been tested with acetyl-CoA only as acyl donor (Cb-BEAT, Rh-AAT, VAAT, Ban-AAT).

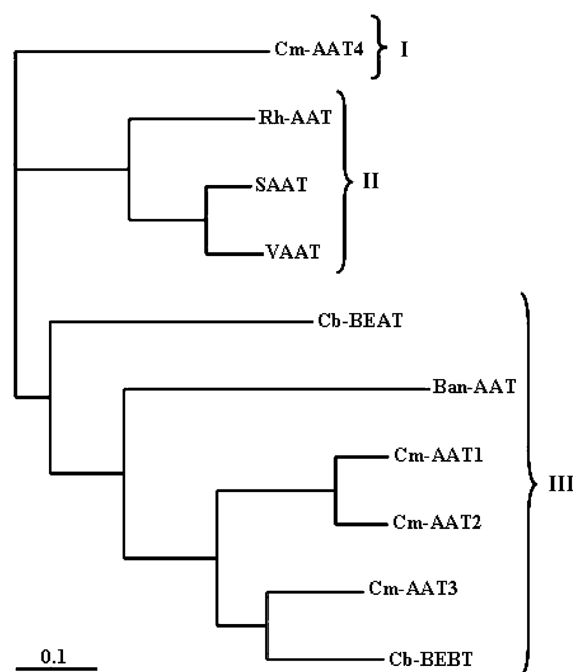

Figure 2. Phylogenetic relationships between *Cucumis melo* (Cm-AAT1, Cm-AAT2, Cm-AAT3, Cm-AAT4); *Clarkia breweri* (Cb-BEBT, Cb-BEAT); *Musa sapientum* (Ban-AAT); *Fragaria x ananassa* (SAAT); *Fragaria vesca* (VAAT); and *Rosa hybrida* (Rh-AAT) based on full-length amino acid sequence. Bootstrap confidence values from 1000 replicates are indicated I, II, and III correspond to the alcohol acyl-transferase (AAT) protein subfamilies.

Expression of Cm-AAT *genes*

Real time PCR analysis indicated that all *Cm-AAT* genes studied here are specifically expressed in fruit. Vegetative tissues (leaves, stems, seeds and roots) and flowers exhibited no or very low expression (Figure 3) even when treated with ethylene (data not shown). Transcript levels for all four *Cm-AAT* genes were by far highest in the fruit at the peak of ethylene production (Figure 3), although transcription levels of *Cm-AAT3* was 35, 28, and 27 times lower than *Cm-AAT1*, *Cm-AAT2*, and *Cm-AAT4*, respectively. Small differences were observed in the pattern of expression before and after the peak. *Cm-AAT2* and *Cm-AAT4* expression was already detectable at 32 DAP while no expression was detected for *Cm-AAT1* and *Cm-AAT3*. In addition, transcript levels were present at significant levels before and after the peak of ethylene production for *Cm-AAT1*, *Cm-AAT2*, and *Cm-AAT4* (35, 37 and 42 DAP) while transcripts for *Cm-AAT3* were very low at the same stages (Figure 3). In AS melon fruit where ethylene was strongly suppressed by antisense *ACC oxidase* (*ACO*) mRNA

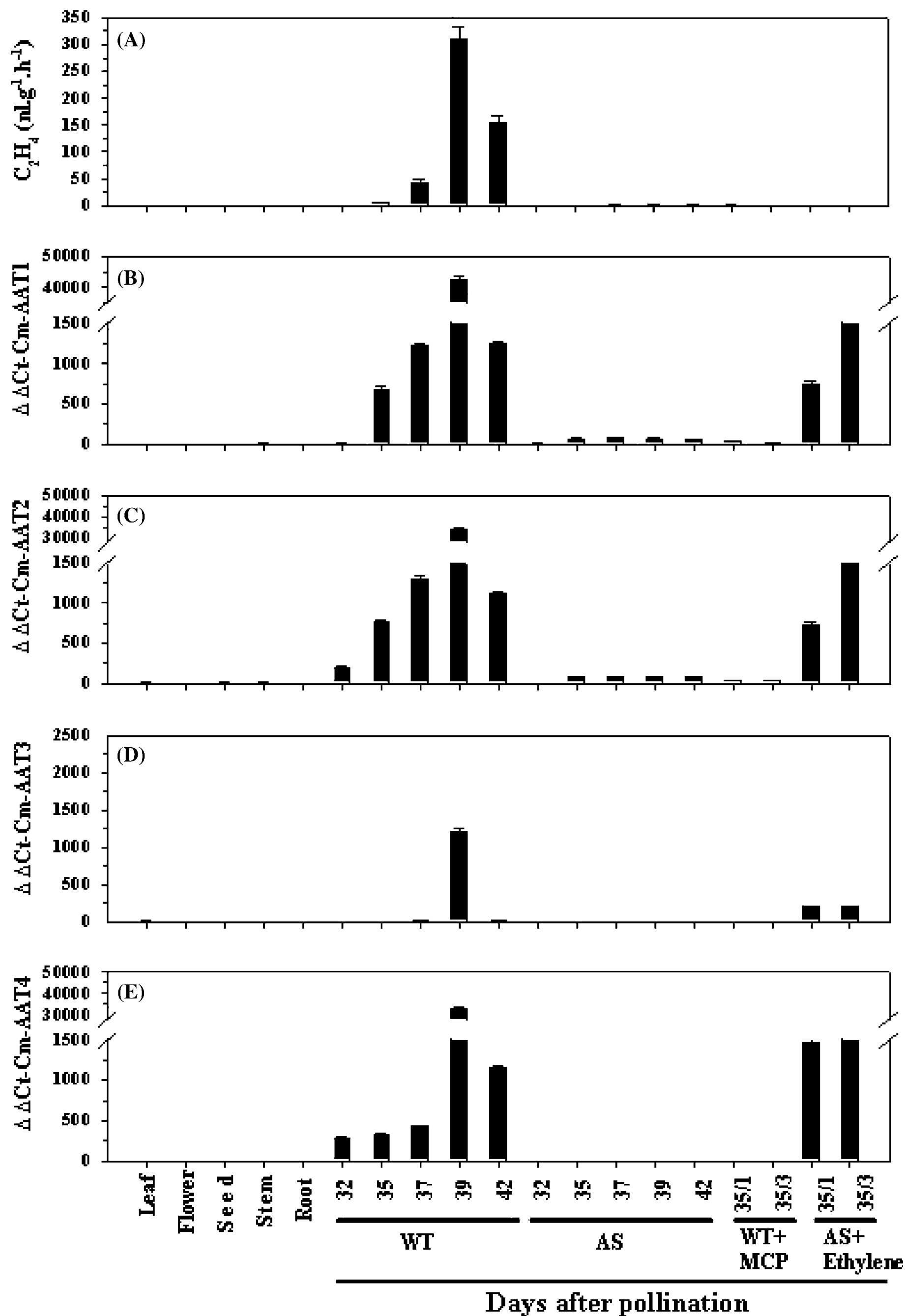

Figure 3. Ethylene production and *Cm-AATs* gene expression from ripening stages and various organs of melon. A: ethylene production; B–E: levels of *Cm-AAT1-4* transcripts assessed by real-time quantitative PCR. The experiments were carried out in triplicate. The *x*-axis represents various organs of melon (leaf, flower, seed, stem, and root); wild-type (WT) and antisense (AS) melon fruit at different days after pollination; WT (35 DAP) fruit exposed to 1 μl l^{-1} MCP for 1 and 3 days; and AS (35 DAP) fruit treated with ethylene (50 μl l^{-1}) for 1 and 3 days. $\Delta\Delta^{Ct}$ in the *y*-axis of each figure refers to the fold difference in *Cm-AAT1*, *Cm-AAT2*, *Cm-AAT3*, and *Cm-AAT4* expression relative to flowers, seeds, and antisense melon fruit (39 DAP), respectively.

Table 3. Activity of the recombinant Cm-AAT purified enzymes towards different types of alcohols and acyl-CoAs.

	Alcohol	Carbon No.	Acyl-CoA	Carbon No.	Esters produced	Ester reported in melon	Cm-AAT1	Cm-AAT3	Cm-AAT4
1	Ethanol	C2:0	Acetyl-CoA	C2:0	Ethyl acetate	+	ND	ND	ND
2	1-Butanol	C4:0	Acetyl-CoA		1-Butyl acetate	+	1 007 ± 139	542 ± 72	ND
3	1-Hexanol	C6:0	Acetyl-CoA		1-Hexyl acetate	+	1 479 ± 94	89 ± 17	117 ± 3
4	1-Heptanol	C7:0	Acetyl-CoA		1-Heptyl acetate	+	637 ± 29	ND	352 ± 20
5	1-Octanol	C8:0	Acetyl-CoA		1-Octyl acetate	+	150 ± 1	ND	345 ± 7
6	*Z*-2-hexen-1-ol	C6:1	Acetyl-CoA		*Z*-2-Hexenyl acetate	+	799 ± 33	482 ± 12	1863 ± 59
7	*E*-2-hexen-1-ol	C6:1	Acetyl-CoA		*E*-2-Hexenyl acetate	NR	**2 941 ± 42**	131 ± 1	392 ± 2
8	*Z*-3-hexen-1-ol	C6:1	Acetyl-CoA		*Z*-3-Hexenyl acetate	+	1 243 ± 12	77 ± 0	TR
9	*E*-3-hexen-1-ol	C6:1	Acetyl-CoA		*E*-3-Hexenyl acetate	+	483 ± 48	26 ± 4	122 ± 26
10	2-Methylpropanol	C4:0	Acetyl-CoA		2-Methylpropyl acetate	+	81 ± 30[a]	ND	ND
11	2-Methyl-1-butanol	C5:0	Acetyl-CoA		2-Methyl-1-butyl acetate	+	377 ± 25	104 ± 13	ND
12	3-Methyl-1-butanol	C5:0	Acetyl-CoA		3-Methyl-1-butyl acetate	+	341 ± 23	352 ± 31	ND
13	Geraniol	C10:2	Acetyl-CoA		Geranyl acetate	NR	TR	TR	1013 ± 118
14	Benzyl alcohol	C7:3	Acetyl-CoA		Benzyl acetate	+	1 544 ± 31	**16 285 ± 1231**	547 ± 48
15	2-Phenylethyl alcohol	C6:3	Acetyl-CoA		2-Phenylethyl acetate	+	961 ± 75	163 ± 4	ND
16	Cinnamyl alcohol	C9:4	Acetyl-CoA		Cinnamoyl acetate	NR	124 ± 7	TR	**3 961 ± 219**
17	Ethanol	C2:0	Propanoyl-CoA	C3: 0	Ethyl propanoate	+	ND	ND	ND
18	1-Butanol	C4:0	Propanoyl-CoA		1-Butyl propanoate	+	ND	67 ± 5[a]	ND
19	3-Methyl-1-butanol	C5:0	Propanoyl-CoA		3-Methyl-1-butyl propanoate	NR	80 ± 2	552 ± 3	ND
20	Hexanol	C6:0	Propanoyl-CoA		Hexyl propanoate	NR	576 ± 15	83 ± 4	ND
21	2-Phenylethyl alcohol	C6: 3	Propanoyl-CoA		2-Phenylethyl propanoate	+	728 ± 27	212 ± 24	ND
22	Ethanol	C2:0	Butanoyl-CoA	C4:0	Ethyl butanoate	+	96 ± 6	205 ± 10	469 ± 42
23	Ethanol	C2:0	2-methylpropanoyl-CoA		Ethyl 2-methylpropanoate	+	ND	ND	ND
24	3-Methyl-1-butanol	C5:0	2-methylbutanoyl-CoA		3-Methyl-1-butyl-2-methylbutanoate	NR	46 ± 4	1837 ± 110	38 ± 5
25	Ethanol	C2:0	Hexanoyl-CoA	C6: 0	Ethyl hexanoate	+	28 ± 3	40 ± 5	ND
26	1-Butanol	C4:0	Hexanoyl-CoA		1-Butyl hexanoate	+	190 ± 10	298 ± 8	ND
27	3-Methyl-1-butanol	C5:0	Hexanoyl-CoA		3-Methyl-1-butyl hexanoate	+	275 ± 6	950 ± 14	ND
28	Hexanol	C6:0	Hexanoyl-CoA		Hexyl hexanoate	NR	2 635 ± 91	189 ± 20	ND
29	2-Phenylethyl alcohol	C6:3	Hexanoyl-CoA		2-Phenylethyl hexanoate	+	868 ± 103	683 ± 36	ND

Activity is expressed in pkat mg^{-1} protein as the mean ± SE of three replicates. NR, not reported in the literature; ND, non detectable; TR, traces, bold indicates maximum activity for the protein.

or where ethylene action was suppressed by treating WT fruit with the ethylene antagonist 1-MCP for one and 3 days before harvest at 35 DAP the transcript levels were strongly (*Cm-AAT1/2*) or totally (*Cm-AAT3/4*) inhibited (Figure 3). Interestingly, exposing AS fruit to ethylene for one and 3 days before harvest at 35 DAP resulted in strong stimulation in the mRNA levels of all *Cm-AAT* (Figure 3).

The fruit-specific expression of *Cm-AATs* is in agreement with previous results reported for *Cm-AAT1* and *Cm-AAT2* (Aggelis *et al*., 1997; Yahyaoui *et al*., 2002). The expression of the *VAAT* and *SAAT* genes from wild and cultivated strawberry respectively, and banana *Ban-AAT* gene were also fruit specific and strongly induced during ripening (Medina-Suarez *et al*., 1997; Nam *et al*., 1999; Aharoni *et al*., 2000). Other *O*-acetyltransferases also show organ-specific expression as *Cb-BEBT* that expressed specifically in the stigma (D'Auria *et al*., 2002), *DAT* in the leaves (St-Pierre *et al*., 1998), *Cb-BEAT* and *Rh-AAT* in the flowers (Dudareva *et al*., 1998; Shalit *et al*., 2003).

Our data indicate that ethylene is a major regulator of all *Cm-AAT* transcript levels. Similarly, ethylene initiates *Ban-AAT* gene expression in banana (Medina-Suarez *et al*., 1997). The role of ethylene on the expression of the strawberry *SAAT* and *VAAT* has not been addressed but both genes are strongly induced during fruit ripening (Nam *et al*., 1999; Aharoni *et al*., 2000).

Alcohol acyl-transferase activity of Cm-AAT *recombinant proteins towards various substrates* in vitro

As demonstrated previously for *Cm-AAT1* (Yahyaoui *et al*., 2002), recombinant yeasts expressing *Cm-AAT3* or *Cm-AAT4* genes were capable of producing high amounts of esters upon addition of the acyl-CoA and alcohol residues, contrary to control yeast transformed with the vector only for which the amount of esters emitted was at trace levels.

The capacity of the highly purified recombinant proteins (Cm-AAT1, Cm-AAT3 and Cm-AAT4) to produce esters was tested in the presence of 29 combinations of substrates, each combination being tested individually. Table 3 shows that Cm-AAT1 and Cm-AAT3 could accept a wide range of combinations of acyl and alcohol substrates, while Cm-AAT4 was almost specific to the transfer of acetate. However, each protein showed marked preferences for the formation of some compounds. Cm-AAT1 was highly active in the formation of *E*-2-hexenyl acetate and hexyl hexanoate (almost 3000 pkat mg^{-1} protein) and to a lesser extent (between 1000 and 1500 pkat mg^{-1} protein) in the formation of four other compounds, butyl, hexyl, *Z*-3-hexenyl and benzyl acetates. These findings are in general agreement with the data reported earlier by Yahyaoui *et al*. (2002) for crude extracts of the same recombinant enzyme. Cm-AAT3 also accepted a wide range of substrates but the activity for producing benzyl acetate was very high (over 16000 pkat mg^{-1} protein) as compared to the two next prefered activities (less than 2000 pkat mg^{-1} protein for the formation of 3-methylbutyl-2-ethylbutanoate). As for Cm-AAT4, it could convert a narrowest range of substrates as compared to CmAAT1 and CmAAT3 with almost exclusive preference for the transfer of the acetyl moiety. Cm-AAT4 had highest activity for the formation of cinnamoyl acetate (almost 4000 pkat mg^{-1} protein). The next two prefered activities were for *Z*-2-hexenyl acetate (ca. 1800 pkat mg^{-1} protein) and geranyl acetate (ca. 1000 pkat mg^{-1} protein). Interestingly, the highest activity of Cm-AAT4 for the synthesis of cinnamoyl acetate is also the case for the Ban-AAT protein of banana (Beekwilder *et al*., 2004). A scheme clearly showing the range of substrates accepted and the preference of each of the three proteins is given in Figure 4.

The large majority of esters made by the three Cm-AAT enzymes (Table 3) have been reported in the aromatic volatiles produced by cantaloupe melons (Beaulieu and Grimm, 2001). However, some of the esters produced by the recombinant proteins have not been reported to be evolved by melons. This is the case for compounds which are produced at a high rate such as *E*-2-hexenyl acetate and hexyl hexanoate by CmAAT1, 3-methylbutyl 2-ethylbutanoate by Cm-AAT3 and cinnamoyl acetate and geranyl acetate by Cm-AAT4 (Table 3). Similarly, the SAAT and Ban-AAT enzymes have strong preference for generating geranyl acetate and cinnamoyl acetate, respectively, whereas these volatile esters have not been reported among volatiles strawberry and banana fruit (Shiota, 1993; Perez *et al*., 1996). This

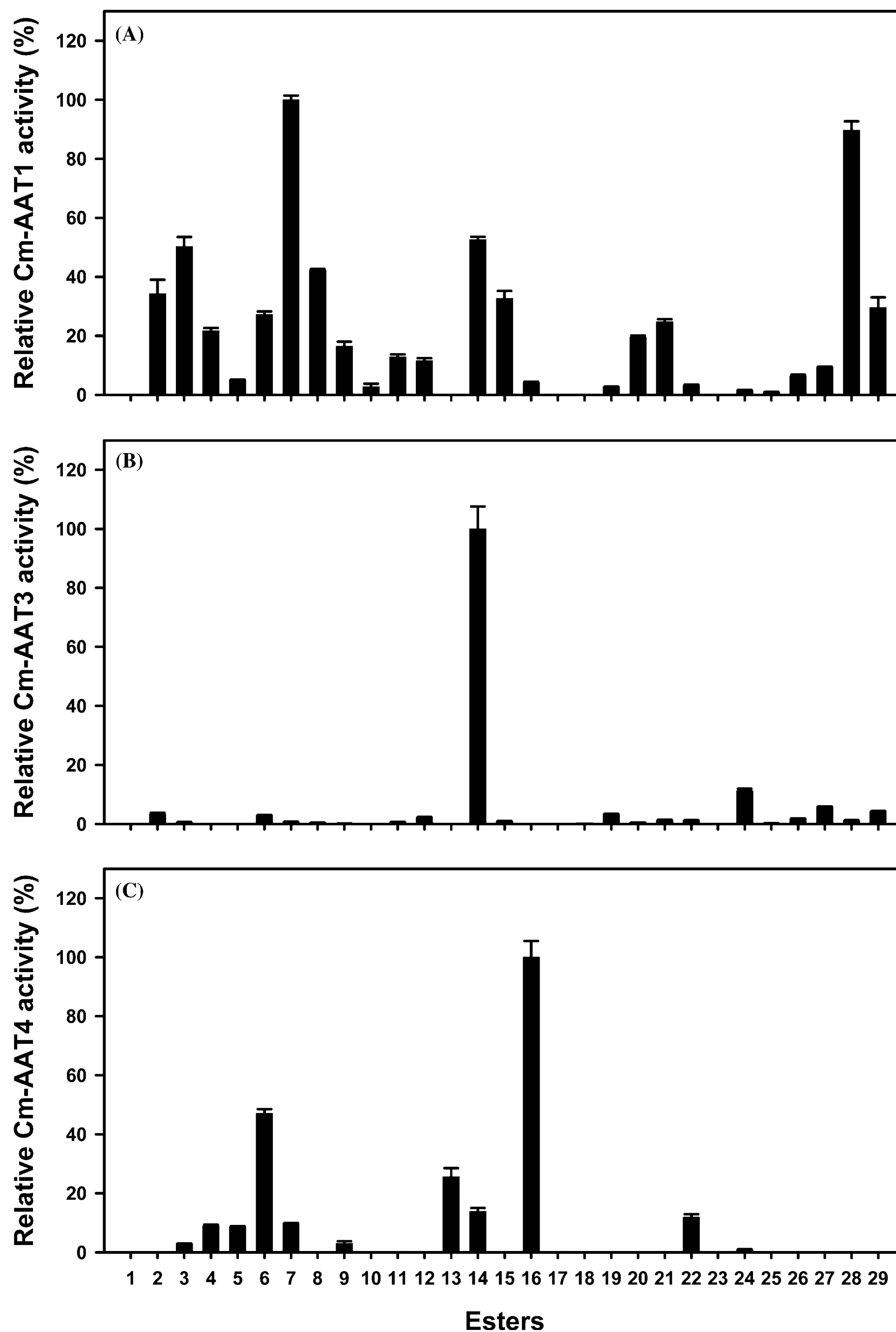

Figure 4. Substrate specificity of the recombinant Cm-AAT enzymes towards different types of alcohols and acyl-CoAs. A: Cm-AAT1; B: Cm-AAT3; C: Cm-AAT4. Activity was measured after purification of the proteins as described in Materials and methods. The number at the *x*-axis correspond to the esters listed in Tables 3 and 4. Activity is expressed as % of the most active substrate: a: 2490 pkat mg^{-1} protein; b: 16285 pkat mg^{-1} protein; c: 3961 pkat mg^{-1} protein.

indicates that, *in fructo*, the availability of the corresponding substrates may control the formation of esters. The availability of the precursors or the selectivity of enzymes upstream in the pathway has been already reported as limiting the synthesis of some esters in banana (Wyllie and Fellman,

2000). Inversely, the three recombinant Cm-AATs have low or no capacity to form ethyl esters (ethyl acetate, ethyl propanoate, and ethyl 2-methylpropanoate, Table 3) that are abundant in the volatiles evolved by melon fruit (Beaulieu and Grimm, 2001) suggesting that there are likely yet unidentified *AAT* genes within the melon genome. It has already been reported that several recombinant AATs lack capacity to form ethyl acetate, such as Cb-BEBT and Cb-BEAT (Dudareva *et al.*, 1998; D'Auria *et al.*, 2002), whereas, VAAT, Rh-AAT, SAAT, and Ban-AAT can produce only trace amounts of such esters (Shalit *et al.*, 2003; Beekwilder *et al.*, 2004).

Estimation of AAT *activity during melon ripening with prefered substrates of each AAT protein*

The strong preference of each Cm-AAT protein for some substrates allowed the evaluation of the corresponding activity in melon fruit. Figure 5 shows that Cm-AAT activity of all proteins was very high at 42 DAP, i.e. after the peak of ethylene when fruit was already at an advanced stage of ripening. Such a high activity occurred few days after the sharp peak of gene expression that occurred at 39 DAP (Figure 3). In all cases, activity was low at 32 DAP. It increased steadily from 35 to 42 DAP for Cm-AAT1 while a strong increase occured later around 39 DAP for Cm-AAT3 and Cm-AAT4. Ethylene-suppressed fruit (AS) showed low or strongly reduced activity for the prefered substrates of Cm-AAT1, Cm-AAT3 and Cm-AAT4, indicating strong ethylene dependence for the expression of the three proteins. This is in agreement with data on gene expression (Figure 3). Residual activity present in AS fruit may result from other yet non-identified proteins using the same substrates and showing ethylene-independent expression. This would be in agreement with previous data showing that the overall AAT activity of melon fruit was partly ethylene-independent (Flores *et al.*, 2002). However, the prefered substrates used here for determining activities are not fully specific for a given Cm-AAT, so that these data taken alone are not sufficient to conclude in the presence of other proteins.

The Charentais melon used in this study belongs to the traditional genotype, 'Védrantais' known for its fast ripening rate and their production of potent aromas. However, aroma production is very transient and limited to fully mature fruit so that consumers are often disappointed when consumption is not made at the right stage. The very sharp AAT activity just after the peak of ethylene production accounts for such a behaviour. In modern mid or long shelf-life Charentais genotypes as in American cantaloupes of the *reticulatus* type, the ripening rate is slower. Beaulieu and Grimm (2001) showed that in melons of the latter group (Sol Real genotype) there was a progressive increase of aroma production during ripening with a maximum in fully ripe fruit and a decrease in over-ripe fruit. In another genotype of cantaloupe, 'Arava', Shalit *et al.* (2001) have demonstrated that AAT activity reached its higher level in fully ripe fruit. Cell free extracts of 'Arava' were capable of acetylating a number of alcohols (1-butanol, 1-hexanol, 1-octanol, isoamyl alcohol, 1-octen-3-alcohol, benzyl alcohol, phenyl ethyl alcohol, and 3-methylthio-1-propanol), but it is probable that several AAT isoforms were present in the extract. On the other hand, Wang *et al.* (1996) had shown that in another American cantaloupe genotype (Makdimon) the highest production of esters occurred during the latest stages of ripening.

In strawberries, a non-climacteric fruit, AAT activity increased during ripening and reached its maximum at the red-dark stage with some decrease in over-ripe fruit (Perez *et al.*, 1996).

In the apple the maximum production of esters occurs at the climacteric peak, suggesting that the activity of alcohol acyl-transferases was maximum at this stage (Song and Bangerth, 1996). Ethylene plays a major role in the regulation of volatiles production in climacteric fruit. In melon and apple the production of ester volatiles is strongly depressed by ethylene suppression in transgenic lines (Bauchot *et al.*, 1998; Flores *et al.*, 2002; Defilippi *et al.*, 2004). Apples continuously require ethylene for the synthesis of volatiles (Fan *et al.*, 1998) and treatment with 1-MCP results in strong reduction in ester volatiles emission (Lurie *et al.*, 2002; Defilippi *et al.*, 2004). In the apple ethylene suppression had no effect on the production of aldehyde and alcohol precursors while it strongly reduced ester formation (Dandekar *et al.*, 2004), AAT activity and gene expression (Defilippi *et al.*, 2005). In apples treated with aminoethoxyvinylglycine (AVG), an inhibitor of ethylene biosynthesis, the production

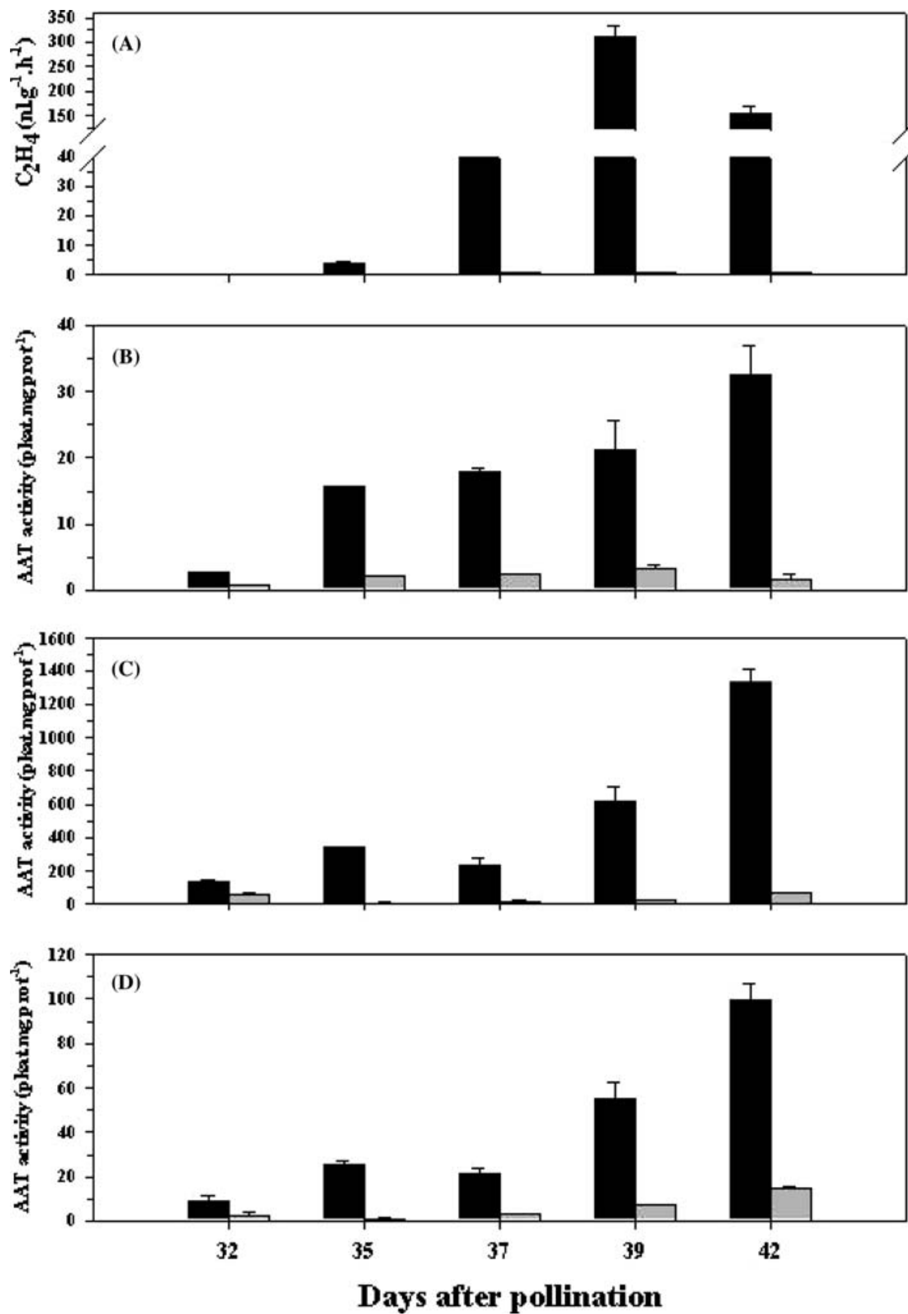

Figure 5. Ethylene production and alcohol acyl-transferases activity of crude protein extracts of WT (black bars) and AS (grey bars) melon fruit at different days after pollination (DAP). A: ethylene production. B: activity for Cm-AAT1 (measured with *E*-2-hexene-ol and acetyl-CoA), C: activity for Cm-AAT3 (measured with benzyl alcohol and acetyl-CoA) and for D: activity for Cm-AAT4 (measured with cinnamyl alcohol and acetyl-CoA). Activities were expressed in pkat mg^{-1} protein as the mean ± SE of three replicates.

of esters was reduced by 21% as compared to untreated fruit (Mir *et al*., 1999). In ethylene suppressed melon, there was an inhibition of ester formation, but it was related to both a strong inhibition of the reduction of aldehydes into alcohols by alcohol dehydrogenase and to a 50% reduction in AAT activity (Flores *et al*., 2002). In this paper, some ethylene independent

production of esters has been reported in melon fruit extracts although the three genes identified appear to be regulated by ethylene (data not shown). This suggests that other genes remain to be isolated that show ethylene-independent regulation.

Search for the failure of Cm-AAT2 *to produce volatile esters*

Yahyaoui *et al.* (2002) isolated a *Cm-AAT2* gene whose encoded protein was unable to produce volatile esters upon expression in yeast despite its strong sequence identity to Cm-AAT1 (84%, Table 2). By comparing the amino acid sequence of all active AATs isolated so far in the area between amino acids 244/263 and 255/273 (Figure 1), it appeared that alanine 268 was unique to Cm-AAT2 while other AATs had a threonine. Due to the strong difference between these two amino acids in terms of hydrophobicity, it was decided to mutate A268 into T in Cm-AAT2 and T268 into A in Cm-AAT1. After expression in yeast of the wild type and mutated versions of the two genes, it appeared that the mutated Cm-AAT2/A268T became capable of producing a wide range of volatile esters (Table 4). The six prefered products of native Cm-AAT1 were also produced at a high rate by Cm-AAT2/A268T: *E*-2-hexenyl acetate and hexyl hexanoate as well as butyl, hexyl, *Z*-3 hexenyl and benzyl acetates. There were only some differences in the activity. For instance, the capacity for benzyl acetate formation was higher in the mutant, while it was lower for *E*-2-hexenyl acetate. However, the general profile of activity and substrates preference was overall similar to Cm-AAT1 (Figure 6). Conversely mutation of T into A at amino acid 268 of

Table 4. Activities of the recombinant Cm-AAT1, Cm-AAT2 and mutated Cm-AAT1/T268A, Cm-AAT2/A268T enzymes towards different types of alcohols and acyl-CoAs.

No	Esters	Cm-AAT2	Cm-AAT2-A268T	Cm-AAT1	Cm-AAT1-T268A
1	Ethyl acetate	ND	ND	ND	ND
2	1-Butyl acetate	ND	898 ± 85	1 007 ± 139	ND
3	1-Hexyl acetate	ND	1 116 ± 60	1 479 ± 94	ND
4	1-Heptyl acetate	ND	254 ± 7	637 ± 29	ND
5	1-Octyl acetate	ND	60 ± 2	150 ± 1	ND
6	*Z*-2-Hexenyl acetate	ND	548 ± 15	799 ± 33	83 ± 21
7	*E*-2-Hexenyl acetate	ND	1 942 ± 215	2 941 ± 42	ND
8	*Z*-3-Hexenyl acetate	ND	1 072 ± 65	1 243 ± 12	TR
9	*E*-3-Hexenyl acetate	ND	177 ± 5	483 ± 48	40 ± 9
10	2-Methylpropyl acetate	ND	118 ± 24	81 ± 30	ND
11	2-Methyl-1-butyl acetate	ND	146 ± 8	377 ± 25	ND
12	3-Methyl-1-butyl acetate	ND	181 ± 9	341 ± 23	ND
13	Geranyl acetate	ND	TR	TR	ND
14	Benzyl acetate	ND	3 497 ± 140	1 544 ± 31	126 ± 12
15	2-Phenylethyl acetate	ND	733 ± 15	961 ± 75	43 ± 0
16	Cinnamoyl acetate	ND	154 ± 10	124 ± 7	TR
17	Ethyl propanoate	ND	ND	ND	ND
18	1-Butyl propanoate	ND	ND	ND	ND
19	3-Methyl-1-butyl propanoate	ND	109 ± 31	80 ± 2	ND
20	Hexyl propanoate	ND	341 ± 13	576 ± 15	ND
21	2-Phenylethyl propanoate	ND	1 009 ± 207	728 ± 27	TR
22	Ethyl butanoate	ND	ND	96 ± 6	ND
23	Ethyl 2-methylpropanoate	ND	ND	ND	ND
24	3-Methylbutyl 2-ethylbutanoate	ND	39 ± 0	46 ± 4	40 ± 6
25	Ethyl hexanoate	ND	25 ± 2	28 ± 3	ND
26	1-Butyl hexanoate	ND	485 ± 3	190 ± 10	ND
27	3-Methyl-1-butyl hexanoate	ND	349 ± 9	275 ± 6	ND
28	Hexyl hexanoate	ND	2 171 ± 21	2 635 ± 91	ND
29	2-Phenylethyl hexanoate	ND	861 ± 145	868 ± 103	ND

The location of the mutated amino acid is indicated by an arrow in Figure 1. Activity was measured with purified recombinant proteins. Activity is expressed in pkat mg^{-1} protein as the mean ± SE of three replicates.

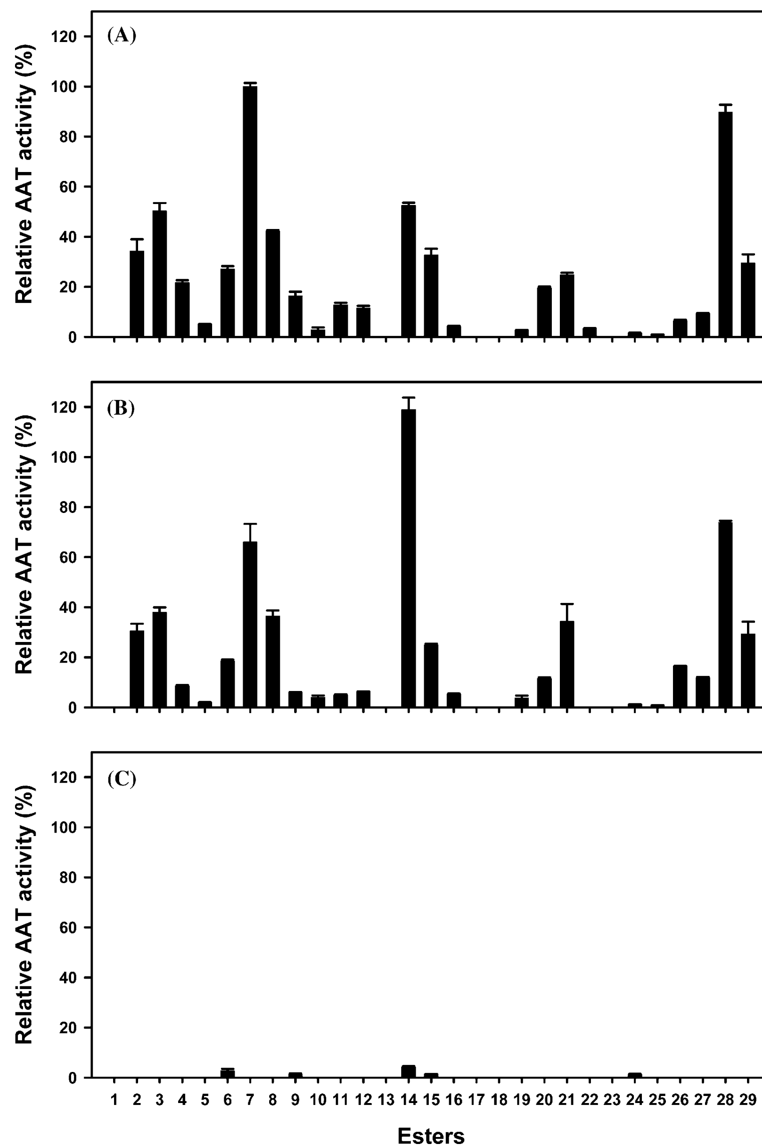

Figure 6. Effect of the Cm-AAT2/A268T and Cm-AAT1/T268A mutations on activity towards different types of alcohols and acyl-CoAs. A: wild type Cm-AAT1; B: Cm-AAT2/A268T; C: Cm-AAT1/T268A. Activity was measured after purification of the proteins as described in Materials and methods. The number at the *x*-axis correspond to the esters listed in Tables 3 and 4. Activity is expressed as % of the most active substrate of wild type Cm-AAT1: 2940 pkat mg^{-1} protein.

Cm-AAT1 resulted in a very strong reduction of activity. Only low residual and trace activity could be detected for few compounds. One single amino acid can, therefore, play a crucial role in AAT activity. It was checked that this was not due to a cloning or sequencing error by sequencing several clones from three independent PCRs and using a high fidelity DNA polymerase (Vent). Two melon sequences from oriental melon fruit (cv Gemsung) putatively encoding AAT have been deposited in data banks, GeAAT-1 (BAB78588) and GeAAT-2 (BAC58010), that also differed from all other AATs by an alanine residue at the 268 position instead of a threonine. No information is available on the activity of the encoded proteins, but the isolation of these two genes gives further arguments for the absence of sequencing errors in our *Cm-AAT2* gene. It remains, however, to determine which is the substrate preference of Cm-AAT2. Speculations on putative functions of AAT2 are

difficult due the potential wide range of substrates concerned by the very large BAHD superfamily (D'Auria *et al.*, 2002). In addition the enzymatic function has been determined in only few cases. However, this protein is probably involved in fruit ripening since the *Cm-AAT2* gene has the same pattern of expression as *Cm-AAT1*, *3* and *4*. Its expression is fruit ripening-specific and ethylene-dependent.

Conclusion

In conclusion, we demonstrate here that at least three genes encoding alcohol acyl-transferases are expressed specifically in melon fruit during ripening. Each of the encoded proteins has specific substrate preferences. Because some esters present in the melon are not produced by these three proteins, it is probable that other *Cm-AAT* genes remain to be isolated. In climacteric fruit, suppression or reduction of ethylene synthesis and action through genetics, biotechnology or inhibitors resulted in a loss or reduction of synthesis of aroma volatiles (Bauchot *et al.*, 1998; Fan *et al.*, 1998; Lurie *et al.*, 2002; Defilippi *et al.*, 2004). So far all *Cm-AAT* genes described in climacteric fruit are ethylene dependent, but the presence of ethylene independent genes is suspected that could be good markers for improving aroma production in a context where ethylene production or action has been slowed-down in order to extend shelf-life. The expression of a gene family of *AATs* specifically in the fruit as described in this work accounts for the wide range of volatiles evolved during ripening. It also probably accounts for the influence of environmental factors and storage conditions on the production and composition of aroma volatiles of fruit (Beaudry, 1999; Mattheis and Fellman, 1999).

Acknowledgements

This work was supported in part by the Midi Pyrénées Régional (Grants No. 01008920 and 03001146), the CEPIA department of INRA (ANS 2002–2003 and postdoctoral scholarship to I. E.), the National Commission for Scientific and Technological Reseach (CONICYT) from Chile (Doctoral scholarship to D.M.) and the Spanish Ministry of Education (Postdoctoral scholarship to F.B.F.).

References

Aggelis, A., John, I., Karvouny, Z. and Grierson, D. 1997. Characterization of two cDNA clones for mRNAs expressed during ripening of melon (*Cucumis melo* L.) fruits. Plant Mol. Biol. 33: 313–322.

Aharoni, A., Keizer, L.C.P., Bouwmeester, H.J., Sun, Z., Alvarez-Huerta, M., Verhoeven, H.A., Blass, J., Van Houwelingen, A.M.M.L., De Vos, R.C.H., Van der Voet, H., Jansen, R.C., Guis, M., Mol, J., Davis, R.W., Schena, M., Van Tunen, A.J. and O'Connell, A.P. 2000. Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. Plant Cell 12: 647–661.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389–3402.

Aubert, C. and Bourger, N. 2004. Investigation of volatiles in Charentais Cantaloupe melons (*Cucumis melo* var *Cantalupensis*) Characterization of aroma constituents in some cultivars. J. Agric. Food Chem. 52: 4522–4528.

Ayub, R., Guis, M., Ben Amor, M., Gillot, L., Roustan, J.P., Latché, A., Bouzayen, M. and Pech, J.C. 1996. Expression of ACC oxidase antisense gene inhibits ripening of cantaloupe melon fruits. Nat. Biotech. 14: 862–866.

Bauchot, A.-D., Mottram, D.S., Dodson, A.T. and John, P. 1998. Effect of aminocyclopropane-1-carboxylic acid oxidase antisense gene on the formation of volatile esters in Cantaloupe Charentais melon (cv. Védrantais). J. Agric. Food Chem. 46: 4787–4792.

Beaudry, R.M. 1999. Effect of O_2 and CO_2 partial pressure on selected phenomena affecting fruit and vegetable quality. Postharv. Biol. Technol. 15: 293–303.

Beaulieu, J.C. and Grimm, C. 2001. Identification of volatile compounds in cantaloupe at various developmental stages using solid phase microextraction. J. Agric. Food Chem. 49: 1345–1352.

Beekwilder, J., Alavarez-Huerta, M., Neef, E., Verstappen, F.W.A., Bouwmeester, H.J. and Aharoni, A. 2004. Functional characterization of enzymes forming volatile esters from strawberry and banana. Plant Physiol. 135: 1865–1878.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem. 72: 248–254.

Boss, P.K., Davies, C. and Robinson, S.P. 1996. Analysis of the expression os anthocyanin pathway genes in developing *Vitis vinifera* cv. Shiraz grape berries and the implications for pathway regulation. Plant Physiol. 111: 1059–1066.

Dandekar, A.M., Teo, G., Defilippi, B.G., Uratsu, S., Passey, A.J., Kader, A.A., Stow, J.R., Colgan, R.J. and Jazmes, D.J. 2004. Effect of down-regulation of ethylene biosynthesis on fruit flavor complex in apple fruit. Transgenic Res. 13: 373–384.

D'Auria, J., Chen, F. and Pichersky, E. 2002. Characterization of an acyltransferase capable of synthesizing benzylbenzoate

and other volatile esters in flowers and damaged leaves of *Clarkia breweri*. Plant Physiol. 130: 466–476.

Defilippi, B.G., Dandekar, A.M. and Kader, A.A. 2004. Impact of suppression of ethylene action or biosynthesis on flavor metabolites in apple (*Malus domestica* Borkh) fruits. J. Agr. Food Chem. 52: 5694–5701.

Defilippi, B.G., Kader, A.A. and Dandekar, A.M. 2005. Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. Plant Sci. 168: 1199–1210.

Dudareva, N., D'Auria, J.C., Nam, K.H., Raguso, R.A. and Pichersky, E. 1998. Acetyl-CoA: benzylalcohol acetyltransferase – an enzyme involved in floral scent production in *Clarkia breweri*. Plant J. 14: 297–304.

Dudareva, N. and Pichersky, E. 2000. Biochemical and molecular genetic aspects of floral scents. Plant Physiol. 122: 627–633.

Dudareva, N., Pichersky, E. and Gershenzon, J. 2004. Biochemistry of plant volatiles. Plant Physiol. 135: 1893–1902.

Elss, S., Preston, C., Hertzig, C., Heckel, F., Richling, E. and Schreier, P. 2005. Aroma profiles of pineapple fruit (*Ananas comosus* L., Merr.) and pineapple products. Lebensm-Wissu-Technol. 38: 263–274.

Fan, X., Mattheis, J. and Buchanan, D. 1998. Continuous requirement of ethylene for apple fruit volatile synthesis. J. Agric. Food Chem. 46: 1959–1963.

Flores, F., El Yahyaoui, F., De Billerbeck, G., Romojaro, F., Latché, A., Bouzayen, M., Pech, J.C. and Ambid, C. 2002. Role of ethylene in the biosynthetic pathway of aliphatic ester aroma volatiles in Charentais Cantaloupe melons. J. Exp. Bot. 53: 201–206.

Guis, M., Botondi, R., Ben Amor, M., Ayub, R., Bouzayen, M., Pech, J.C. and Latché, A 1997. Ripening-associated biochemical traits of cantaloupe charentais melons expressing an antisense ACC oxidase transgene. J. Amer. Soc. Hort. Sci. 122: 748–751.

Jeanmougin, F., Thompson, J.D., Gouy, M., Higgins, D.G. and Gibson, T.J. 1998. Multiple sequence alignment with Clustal X. Trends Biochem. Sci. 23: 403–405.

Harada, M., Ueda, Y. and Iwata, T. 1985. Purification and some properties of alcohol acyltransferase from banana fruit. Plant Cell Physiol. 26: 1067–1074.

Homatidou, V., Karvouni, S., Dourtouglou, V. and Poulos, C. 1992. Determination of total volatile components of *Cucumis melo* L. Variety Cantaloupensis. J. Agric. Food Chem. 40: 1385–1388.

Lurie, S., Pre-Aymard, C., Ravid, U., Larkov, O. and Fallik, E. 2002. Effect of 1-methylcyclopropene on volatile emission and aroma in cv Anna apples. J. Agr. Food Chem. 50: 4251–4256.

Ma, X., Koepke, J., Panjikar, S., Fritzch, G. and Stöckigt, J. 2005. Crystal structure of vinorine synthase, the first representative of the BAHD superfamily. J. Biol. Chem. 280: 13576–13583.

Mattheis, J.P. and Fellman, J.K. 1999. Pre-harvest factors influencing flavor of fresh fruit and vegetables. Postharv. Biol. Technol. 15: 227–232.

Medina-Suarez, R., Manning, K., Fletcher, J., Aked, J., Bird, C.R. and Seymour, G. 1997. Gene expression in the pulp of ripening bananas. Two-dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *in vitro* translation products and cDNA cloning of 25 different ripening-related mRNAs. Plant Physiol. 115: 453–461.

Mir, N.A., Perez, R., Schwallier, P. and Beaudry, R. 1999. Relationship between ethylene response manipulation and volatile production in Jonagold variety apples. J. Agric. Food Chem. 47: 2653–2659.

Nam, Y.W., Tichit, L., Leperlier, M., Cuerq, B., Marty, I. and Lelièvre, J.M. 1999. Isolation and characterization of mRNAs differentially expressed during ripening of wild strawberry (*Fragaria vesca* L.) fruits. Plant Mol. Biol. 39: 629–636.

Nicholas , K.B. and Nicholas H.B.J. 1997. GENEDOC: a tool for editing and annotating multiple sequence alignments, distributed by the authors.

Page, R.D.M. 1996. TREE VIEW: an application to display phylogenic trees on personal computers. Comput. Appl. Biosci. 12: 357–358.

Paillard, N.M.M. 1990. The flavour of apples, pears and quinces. In: I.D. Morton and A.J. McLeod (Eds.), Food Flavours, PartC. The Flavour of Fruit, Elsevier, Amsterdam, The Netherlands, pp. 1–41.

Perez, A.G., Sanz, C., Olías, R., Ríos, J.J. and Olías, J.M. 1996. Evolution of strawberry alcohol acyltransferase activity during fruit development and storage. J. Agric. Food Chem. 44: 3286–3290.

Pichersky, E. and Gang, D.R. 2000. Genetics and biochemistry of secondary metabolites in plants: an evolutionary perspective. Trends Plant Sci. 5: 439–445.

St Pierre, B., Laflamme, P., Alarco, A.M. and De Luca, V. 1998. The terminal *O*-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for co-enzyme A-dependent acyl transfer. Plant J. 14: 703–713.

St Pierre B. and De Luca, V. 2000. Evolution of acyltranferase genes: origin and diversification of the BAHD superfamily of acyltransferases involved in secondary metabolism. In: R.I. John, R. Romeo, L. Varin and V. De Luca (Eds.), Recent Advances in Phytochemistry. Evolution of Metabolic Pathways, vol. 34. Elsevier Science Publishing, Oxford, pp. 285–315.

Sanz, C., Olias, J.M. and Perez, A.G. 1997. Aroma biochemistry of fruit and vegetables. In: F.A. Tomas-Barberan and R.J. Robins (Eds.), Phytochemistry of Fruit and Vegetables, Oxford Science Pub, Oxford, UK, pp. 313–329.

Shalit, M., Katzir, N., Tadmor, Y., Larkov, O., Burger, Y., Schalechet, F., Lastochkin, E., Ravid, U., Amar, O., Edelstein, M., Karchi, Z. and Lewinsohn, E. 2001. Acetyl-CoA: alcohol acetyl transferase activity and aroma formation in ripening melon fruits. J. Agric. Food Chem. 49: 794–799.

Shalit, M., Guterman, I., Volpin, H., Bar, E., Tamari, T., Menda, N., Adam, Z., Zamir, D., Vainstein, A., Weiss, D., Pichersky, E. and Lewinsohn, E. 2003. Volatile ester formation in roses. Identification of an acetyl-coenzyme A geraniol/citronellol° acetyl transferase in developing rose petals. Plant Physiol. 131: 1868–1876.

Shiota, H. 1993. New esteric components in the volatiles of banana fruit (*Musa sapientum* L.). J. Agric. Food Chem. 41: 2056–2062.

Song, J. and Bangerth, F. 1996. The effect of harvest date on aroma compound production from 'Golden Delicious' apple fruit and relationship to respiration and ethylene production. Postharv. Biol. Technol. 8: 259–269.

Wang, Y., Wyllie, S.G. and Leach, D.N. 1996. Chemical changes during the development and ripening of the fruit of *Cucumis melo* (cv Makdimon). J. Agric. Food Chem. 44: 210–216l.

Wyllie, S.G. and Fellmann, J.K. 2000. Formation of volatile branched chain esters in bananas (*Musa sapientum* L.). J. Agric. Food Chem. 48: 3493–3496.

Yahyaoui, F., Wongs-Aree, C., Latché, A., Hackett, R., Grierson, D. and Pech, J.C. 2002. Molecular and biochemical characteristics of a gene encoding an alcohol acyl-transferase involved in the generation of aroma volatile esters during melon ripening. Eur. J. Biochem. 269: 2359–2366.

Yoshimoto, H., Fujiwara, D., Momma, T., Tanaka, K., Sone, H., Nagasawa, N. and Tamai, Y. 1999. Isolation and characterization of the ATF2 gene encoding alcohol acyltransferase II in the bottom fermenting yeast *Saccharomyces pastorius*. Yeast 15: 409–417.

Zabetakis, I. and Holden, M.A. 1997. Strawberry flavour: analysis and biosynthesis. J. Sci. Food Agric. 74: 421–434.