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Molecular and biochemical characteristics of a gene encoding an alcohol acyl-transferase involved in the generation of aroma volatile esters during melon ripening

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Two genes (*CM-AAT1* and *CM-AAT2*) with strong sequence homology (87% identity at the protein level) putatively involved in the formation of aroma volatile esters have been isolated from Charentais melon fruit. They belong to a large and highly divergent family of multi-functional plant acyl-transferases and show at most 21% identity to the only other fruit acyl-transferase characterized so far in strawberry. RT-PCR studies indicated that both genes were specifically expressed in fruit at increasing rates in the early and mid phases of ripening. Expression was severely reduced in ethylene-suppressed antisense ACC oxidase (AS) fruit and in wild-type (WT) fruit treated with the ethylene antagonist 1-MCP. Cloning of the two genes in yeast revealed that the CM-AAT1 protein exhibited alcohol acyl-transferase activity while no such activity could be detected for CM-AAT2 despite the strong homology between the two sequences. CM-AAT1 was capable of

producing esters from a wide range of combinations of alcohols and acyl-CoAs. The higher the carbon chain of aliphatic alcohols, the higher the activity. Branched alcohols were esterified at differential rates depending on the position of the methyl group and the nature of the acyl donor. Phenyl and benzoyl alcohols were also good substrates, but activity varied with the position and size of the aromatic residue. The *cis/trans* configuration influenced activity either positively (2-hexenol) or negatively (3-hexenol). Because ripening melons evolve the whole range of esters generated by the recombinant CM-AAT1 protein, we conclude that CM-AAT1 plays a major role in aroma volatiles formation in the melon.

Keywords: alcohol acyl-transferase; aroma; Charentais melon; ethylene; fruit ripening.

Aroma volatiles are secondary metabolites that play a major role in fruit quality. Charentais cantaloupe melon (*Cucumis melo* L., var *cantalupensis* Naud.) is characterized by abundant sweetness and very good aromatic flavour. However, due to their short storage life, breeders have directed their efforts towards the extension of shelf-life and improving yield, uniformity, and pest resistance. This has resulted in a loss of flavour. It has been shown that suppression of ethylene production [1] results in a strong inhibition of aroma volatiles in Charentais-type melons [2],

suggesting that when new cultivars generated by the breeders are affected in ethylene production and/or sensitivity this may impair flavour. The characterization of some new medium shelf-life cultivars has confirmed such an assumption [3,4].

The aroma volatiles of Charentais-type cantaloupe melons, as with other cantaloupes, comprise a complex mixture of compounds including esters, saturated and unsaturated aldehydes and alcohols, and sulphur compounds [5,6]. Among these compounds, volatile esters are quantitatively the most important and therefore represent key contributors to the aroma. Although the aromatic composition of melon is well documented, little information is available on the biochemical and molecular characterization of the enzymes involved in the metabolic pathways. The last step in the production of esters is catalysed by alcohol acyl-transferases (AAT) [7] and an alcohol acetyl-transferase has been shown to be responsible for the acetylation of alcohols in the melon [8]. A gene showing AAT activity has been isolated from strawberries [9]. In the melon, a gene putatively encoding an AAT protein had been isolated from Charentais melon fruit [10], but its functional identification was lacking. We report here on the expression pattern and characteristics of two putative AAT genes (*CM-AAT1* and *CM-AAT2*) and on the functional and biochemical characterization of the AAT enzyme encoded by the *CM-AAT1* gene.

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Abbreviations: AAT, alcohol acyl-transferase; ACO, aminocyclopropane carboxylic acid oxidase; AS: melon ACO antisense; 1-MCP: 1-methylcyclopropene; SAAT, strawberry AAT; WT, wild-type; NHCBT, *N*-hydroxy-cinnamoyl/benzoyl-transferase; BEAT, benzyl alcohol transferase; DAP, days after pollination.
Enzyme: alcohol acyl-transferase (EC 2.3.1.84).

Note: the accession numbers of the proteins referred to in this manuscript are: CM-AAT1, CAA94432 and CM-AAT2, AF468022.

MATERIALS AND METHODS

Plant material

Wild-type (WT) and ACC oxidase antisense (AS) Charentais Cantaloupe melons (*Cucumis melo* var. *Cantalupensis*, Naud cv. *Védraçais*) were used [1]. They were grown on a trellis in a greenhouse under standard cultural practices for fertilization and pesticide treatments. Flower tagging on the day of hand pollination and daily measurements of internal ethylene (WT fruit only) were performed as a guideline for harvesting fruit at various stages of ripening. AS fruits were exposed to 50 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for 24 and 72 h. The ethylene inhibitor 1-MCP was also applied to fruit on the vine at 1 $\mu\text{L}\cdot\text{L}^{-1}$ to WT and AS fruits in 3-L jars for 3 days before harvesting with periodical flushing with air and re-injection of the inhibitor. Vegetative tissues (leaves, stems and roots) were collected from plantlets grown in a growth chamber and occasionally exposed to ethylene (20 $\mu\text{L}\cdot\text{L}^{-1}$ for 24 h). All plant material was frozen in liquid nitrogen and stored at -80°C .

RNA extraction and isolation of the full-length cDNA clones

RNA was extracted according to Griffiths *et al.* [11]. The *CM-AAT1* clone, previously named *Mel2* [10] and its homologue *CM-AAT2* have been isolated by PCR from a cDNA library of ripe melon. The SK primer (in pBluescript: 5'-XTX TCTAGAAGTGTGGATC-3') was combined with the degenerated primers, AAT3'fd: 5'-GA(TC)TT(TC)GGN TGGGGNAA(AG)GC-3' and AAT3'rev: 5'-GC(CT)TTN CCCCANCC(GA)AA(GA)TC-3', designed from a conserved region (DFGWGK) among plants acyl-transferases [12].

RT-PCR

DNase-treated RNA (5 μg) was reverse transcribed in a total volume of 50 μL using an oligo dT primer and following a standard protocol. PCR was performed by mixing: 1 μL cDNA, 5 μL *Taq* buffer 10 \times (Promega), 5 μL MgCl_2 25 mM, 2 μL dNTPs (10 mM each), 0.5 μL each primer 50 μM . *CM-AAT1* was amplified by using RSB-5': 5'-CAAAGAGCACCTCATCCAGCC-3', and FSD-3': 5'-AGGAGGCAAGCATAGACTTAACG-3'; *CM-AAT2* was amplified with RSB-5' and FSA-3': 5'-GATAATT CCACACCTCCAATTA-3'; the internal standard was amplified with act.5': 5'-gcactgaagacatccgtacttc-3' and act.3': 5'-TGGGCACGGAATCTCAGC(TC)-3'. The PCR programme was one cycle of 2 min at 95°C , 50 s at 58°C , 30 s at 72°C followed by *N* cycles of 30 s at 95°C , 50 s at 58°C , 30 s at 72°C (*N* = 31 for *CM-AAT1*; 27 for *CM-AAT2* and 26 for actin). PCR products were resolved on a 1.4% agarose gel, and transferred to Nylon membranes (NEN) and prehybridized at 65°C (2–3 h) in a buffer containing, per 100 mL: 60 mL H_2O , 25 mL 20 \times NaCl/Cit, 10 mL 50 \times Denhardt's solution, 5 mL 10% SDS. Membranes were then hybridized with two probes (*CM-AAT1* and *CM-AAT2*) and actin [^{32}P]dCTP-labelled overnight and washed at 65°C successively with: 2 \times NaCl/Cit, 0.1% SDS; 1 \times NaCl/Cit, 0.1% SDS; 0.5 \times NaCl/Cit, 0.1% SDS. Membranes were finally exposed to X-ray films and developed a few hours later.

Expression of CM-AAT1 and CM-AAT2 in yeast

Both *CM-AAT1* and *CM-AAT2* cDNAs were cloned in the pYES1.2 TOPO-TA cloning vector and yeasts (strain INVSc1) were transformed following the instructions provided by the manufacturer (Invitrogen). The strain harbouring the correct construction was incubated in selecting liquid medium according to Invitrogen recommendations, until the D_{600} of the culture reached ≈ 1 U. Cells were collected by centrifugation (1800 g, 10 min) and resuspended in fresh medium with 2% galactose as inducer.

AAT activity assay with recombinant proteins

The pellet from each 50 mL of induced culture was resuspended in 2 mL buffer A (50 mM Tris/HCl pH 7.5, 1 mM dithiothreitol) and mechanically ground in liquid nitrogen for 2 min and stored at -80°C until needed. The powder was thawed, vortexed for 1 min and centrifuged at 13 000 r.p.m. for 15 min at 4°C . The total proteins were quantified according to Bradford [13]. AAT activity was assessed in a 500 μL total volume containing 25 μL protein extract (166 μg), 40 mM R-OH (alcohol), 250 μM acyl-CoAs, 20 mM MgCl_2 (for R-OH screening only) and adjusted to 500 μL with buffer A. The mixture was incubated at 30°C for 20 min. The esters formed were extracted with 250 μL pentane containing 5 $\mu\text{L}\cdot\text{L}^{-1}$ α -pinene as internal standard, vortexed for 1 min and 1 μL of the pentanic phase was injected into the GC for analysis [14].

Quantification and esters identification

Esters were identified and quantified by injecting the corresponding pure authentic product when available. Where authentic products were not available, identification was based on the enhancement of the peak between 20 and 40 min of enzymatic reaction and the quantification was based on the response curves established for esters of the same family.

RESULTS AND DISCUSSION

Sequence analysis

Both *CM-AAT1* and *CM-AAT2* encode proteins of 461 amino acids with a theoretical molecular mass of 51.5 kDa and 51.8 and a pI of 8 and 8.5, respectively, and 87% identity at the amino acid sequence level. A BLAST search of these sequences gave the highest homologies with two protein families: (a) hypersensitivity-related proteins of *Arabidopsis* and tobacco; and (b) acyl transferases such as anthranilate *N*-hydroxy-cinnamoyl/benzoyl-transferase (NHCBT)-like protein of *Arabidopsis* and *Dianthus caryophyllus*, acetyl-CoA benzyl alcohol transferase (BEAT) of *Clarkia breweri* and other AATs involved in secondary metabolism. Multiple alignment was focused on *O*-acyl-transferases and highlighted putative functional motifs (Fig. 1). These proteins are more conserved at the N-terminal region, but most importantly, they share at least two highly conserved consensus motifs around the 160–170 (H-x-x-x-DG) and 380–390 (DFGWG) positions that are present among plants *O*-acyl-transferases [12].

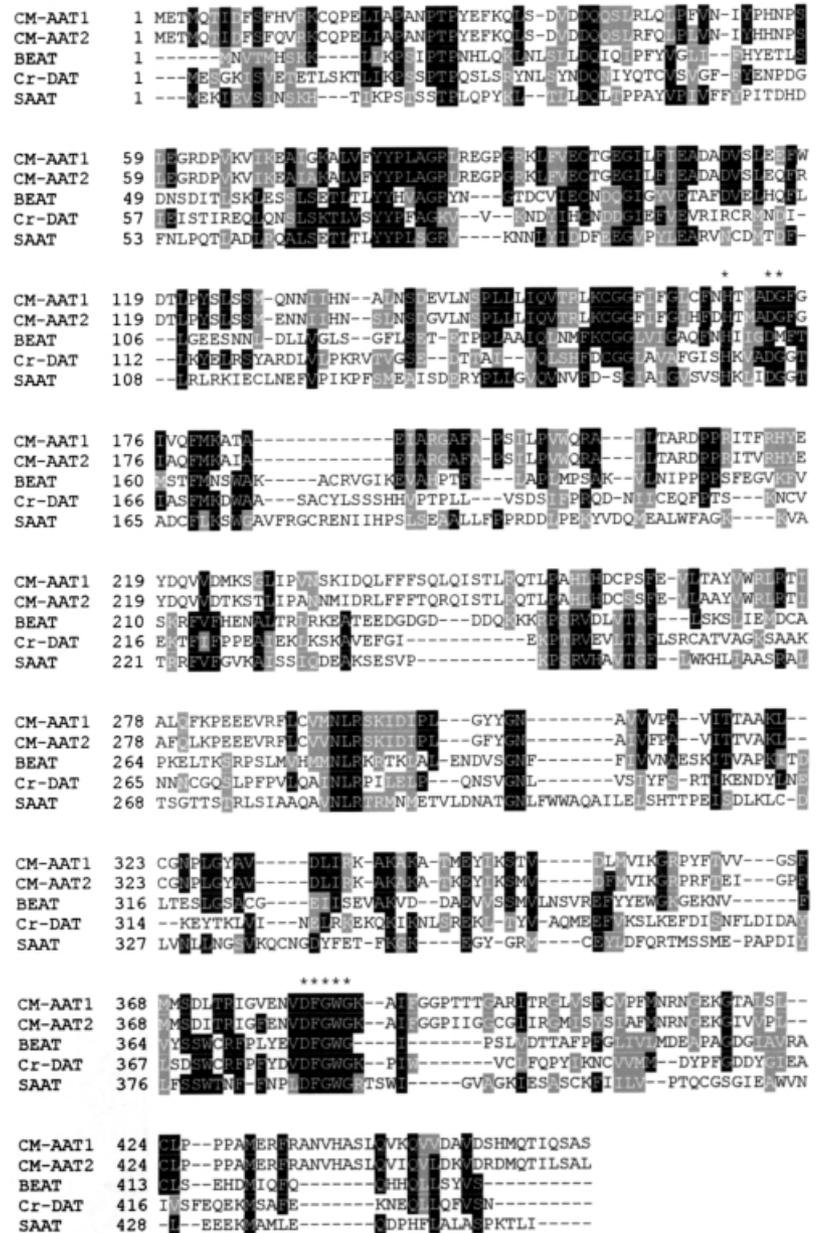


Fig. 1. Multiple alignment of melon CM-AAT1 (accession number, CAA94432) and CM-AAT2 (accession number AF468022) protein sequences with characterized *O*-acyl-transferases encoded by the BEAT gene of *Clarkia breweri* (accession number, AAC18062), strawberry SAAT (accession number, AAF04784), and the Cr-DAT gene of *Catharanthus roseus* (accession number, AAC99311). Sequences were aligned with PIMA1.4 (<http://dot.imgen.bcm.tmc.edu:9331/multialign/multialign.html>) and BOXSHADE3.21 programs (http://www.ch.embnet.org/software/BOX_form.html). Black and grey boxes contain residues that are identical and similar, respectively. Asterisks indicate the positions of the conserved regions of plant acyl-transferases considered as playing a role in activity.

However, in the BEAT sequence, which encodes an enzyme involved in scent production, glycine was substituted by methionine in the conserved triad H-x-x-DG. In yeast AATs [15], only the HxxxDG sequence element has been conserved suggesting that this element is involved in acyl-transfer from the acyl-CoA to alcohol.

The phylogenetic tree of the acyl-transferase family (Fig. 2) show three groups of protein sequences. The first group comprises the two yeast AATs, ATF1 and ATF2. The second is composed of three proteins characterized as *O*-transferases, *Catharanthus roseus* Cr-deacetylindoline acetyl-transferase (DAT), strawberry AAT (SAAT) and *Clarkia* BEAT. The melon AATs are included in a third group and are closely related to the tobacco hypersensitivity-related (hsr)201 protein and the NHCBT of *Arabidopsis*, characterized as an *N*-transferase. CM-AAT1 and CM-AAT2 are therefore related to a wide family of

multifunctional plant acyl-transferases that participate in the biosynthesis of esters [9,16], and defence compounds [17,18]. This acyl-transferase gene family is very large. In *Arabidopsis* for instance, it is composed of 90 members that underwent divergent evolution [19]. The function of only a very few of them has been identified so far. It is notable that the melon and strawberry AATs are located in two separate groups (Fig. 2).

CM-AAT1 and CM-AAT2 gene expression

RT-PCR studies indicated that both genes are specifically expressed in fruit. Vegetative tissues such as leaves, stems and roots exhibited no expression even when treated with ethylene (not shown). This is in agreement with the previous data [4,10] on the *Mel2* gene (corresponding to *CM-AAT1*). The expression of the strawberry *SAAT* was also fruit

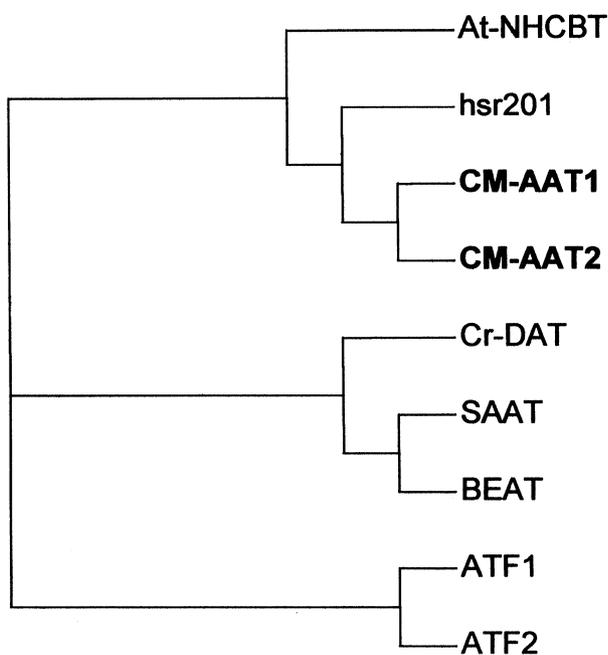


Fig. 2. Dendrogram of full-length deduced amino acid sequences of *CM-AAT1* and *CM-AAT2* and homologues, including: *Arabidopsis* anthranilate NHCBT, *Nicotiana tabacum* hsr201, *Saccharomyces* alcohol acetyl-transferases (ATF1 and ATF2), *Catharanthus roseus* Cr-DAT, *Clarkia* BEAT, and *Strawberry* SAAT. The accession numbers are as in Fig. 1 plus, ATF1 (6324953), ATF2 (7493829), NHCBT (CAB62598) and hsr201 (CAA64636). The dendrogram was created by using CLUSTAL-X alignment [36] and TREEVIEW32 (<http://taxonomy.zoology.gla.ac.uk/rod/treeview/treeview.html>).

specific [9]. Other *O*-acetyl-transferases also show organ-specific expression in leaves [12] and flowers [20]. As observed by Aggelis *et al.* [4,10], *CM-AAT1* showed increased mRNA expression in WT fruit between 32 and 41 days after pollination (DAP) and then declined (Fig. 3A). Treating fruit with the ethylene antagonist 1-MCP 3 days before harvest at 37 DAP resulted in a

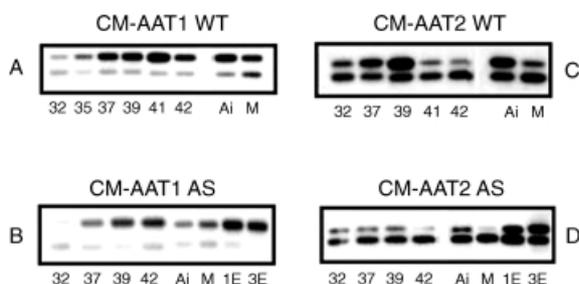


Fig. 3. Expression pattern of *CM-AAT1* (A, B) and *CM-AAT2* (C, D) genes during fruit development and ripening between 32 and 42 days of wild type (WT) and antisense ACC oxidase (AS) melons. Some of the fruit were treated on the vine with 50 $\mu\text{L}\cdot\text{L}^{-1}$ ethylene for 24 h (line 1E) or 72 h (line 3E) or with 1 $\mu\text{L}\cdot\text{L}^{-1}$ 1-MCP (line M) or with air (line Ai) for 72 h before harvest at 37 DAP. The upper and lower bands correspond to the *CM-AAT1* or *CM-AAT2* genes and actin, respectively.

substantial reduction of transcript level. The pattern of *CM-AAT2* mRNA expression was similar to that of *CM-AAT1* except that expression peaked at 39 DAP instead of 41 DAP. Shalit *et al.* [21] have also demonstrated an increase of AAT activity during ripening of an aromatic variety of melon. In AS fruit where ethylene production had been strongly reduced, expression was either severely (*CM-AAT2*) or weakly (*CM-AAT1*) inhibited (Figs 3B and D). In agreement with the present data, a survey of genes differentially expressed in AS and WT melons showed that a cDNA called *RM5* and corresponding to *CM-AAT1* showed ethylene-dependent expression [22]. Treatment of AS fruit with 1-MCP gave no additional inhibition for *CM-AAT1* while it completely suppressed *CM-AAT2* expression (Fig. 3D), indicating that ethylene alone could account for the regulation of *CM-AAT2*, while other developmental factors are involved in addition to ethylene in the regulation of *CM-AAT1*. Application of the 1-MCP to WT fruits strongly inhibited the expression of both genes (Figs 3A and C). Treating AS fruit with ethylene resulted in a strong stimulation of expression of both genes after 1 or 3 days of treatment. Ethylene may also be involved in the expression of the *hsr201* gene of tobacco, a member of the same family whose expression is stimulated during infection by pathogens [17]. No information exists on the role of ethylene on the expression of the strawberry *SAAT* [9]. However due to the nonclimacteric character of strawberry ripening, it may be speculated that ethylene is probably not involved in its expression.

Search for alcohol acyl-transferase activity of *CM-AAT1* and *CM-AAT2* recombinant proteins

None of the recombinant proteins produced in *Escherichia coli* exhibited AAT activity under various conditions of protein concentration, incubation time, or protein extraction method (sonication, mechanical grinding, lysozyme lysis). In addition, no activity was found towards the formation of benzoyl anthranilate although both sequences showed homology to anthranilate benzoyl-transferase genes [23,24]. The production of recombinant proteins was then attempted in yeast. In that case, the *CM-AAT1*-transformed yeast in culture evolved, in the absence of any exogenous precursor, a strong aroma of banana, but not the control cells transformed with the vector only. No such smell was encountered in *CM-AAT2*-transformed yeast and the GC pattern of the culture medium was identical to control cells even after addition of a variety of alcohols. Analysis of the culture medium of *CM-AAT1*-transformed cells revealed a high production of isoamyl acetate, responsible for the strong banana aroma (280-fold higher than control), phenyl-2-ethyl acetate (300-fold higher than control) and other minor unidentified volatiles (Fig. 4). The synthesis of these two esters is achieved through the acetylation of endogenous isoamyl alcohol and phenyl-2-ethanol and is an indicator of the expression of an AAT activity. In addition, in agreement with the synthesis of esters, a lower level of isoamyl alcohol was found in the medium of *CM-AAT1*-transformed cells as compared with the medium of control cells. Feeding *CM-AAT1*-transformed yeast with benzyl alcohol produce high amounts of benzyl acetate (14-fold higher than control) (Fig. 4D,E). All of these observations support the conclusion that the *CM-AAT1* recombinant

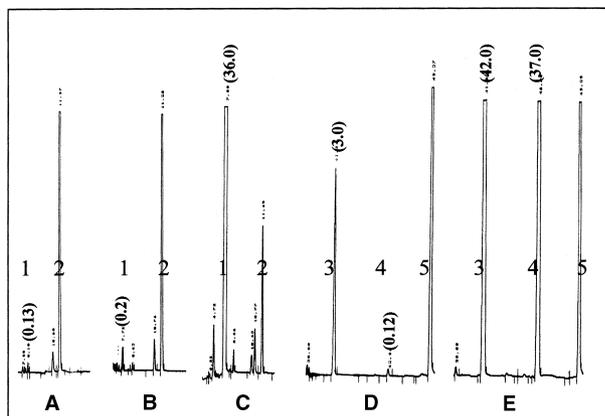


Fig. 4. Volatile compounds extracted from the culture medium of control yeasts transformed with the vector only (A and D), with the vector harbouring the *CM-AAT1* gene (C and E) and the *CM-AAT2* gene (B). The medium was either not complemented with any precursors (A, B, and C) or complemented with 50 $\mu\text{L}\cdot\text{L}^{-1}$ benzoyl alcohol (D and E). Ten mL of each spent medium was extracted with 500 μL pentane, and 1 μL was injected into a GC. 1, Isoamyl acetate; 2, isoamyl alcohol; 3, benzyl acetate; 4, phenyl-2-ethyl acetate; 5, benzyl alcohol. Values within parentheses correspond to the concentration of esters in the culture medium ($\text{mg}\cdot\text{L}^{-1}$).

protein has AAT activity. This was confirmed by measuring *in vitro* activity. However, no activity of the recombinant CM-AAT2 protein could be found using a number of substrates including: ethanol, butanol, isoamyl alcohol, 2-methylbutanol, *cis*-2-hexenol and benzyl alcohol (in the presence of acetyl-CoA); ethanol, isoamyl alcohol and benzyl alcohol (in the presence of propionyl-CoA, isovaleryl-CoA, *n*-butyryl-CoA, isobutyryl-CoA, hexanoyl-CoA, and benzoyl-CoA). This is the first gene of this type functionally identified in climacteric fruit. The only other AAT gene so far identified was in the strawberry [9]. CM-AAT2, which has strong homology to CM-AAT1, exhibited no acyl-transferase activity while the *SAAT* gene of strawberry which is by far more divergent showed such activity. This could be explained by an evolutionary process whereby the two genes evolved towards two different pathways [19]. In these conditions, the absence of correlation between sequence homologies and substrate specificity would not be surprising. The absence of activity found upon expression in *E. coli* may be due to a requirement for specific post-translational modification of the protein although several acyl-transferases had been successfully expressed in *E. coli* [9,24,25]. A potential glycosylation site (NHTM amino acids 167–170) that may be crucial for activity has been identified in the protein sequence.

Effect of pH and various effectors on recombinant CM-AAT1

CM-AAT1 protein was active over the pH range 6–8 consistent with the previous studies on banana [26], strawberry [27], melon [8] and yeast [28]. Na^+ and Mg^{2+} stimulated AAT activity by 100% and 150%, respectively. K^+ had the same effect as Na^+ (data not shown). The

optimum concentration was half for MgCl_2 (50 mM) as compared to NaCl (100 mM). At MgCl_2 concentrations > 50 mM, activity decreased sharply, whereas at NaCl concentrations of 100–500 mM activity was almost stable. These data are different from those obtained with banana AAT [26] where 10^{-3} to 10^{-1} M NaCl and MgCl_2 had an inhibitory effect. Akita *et al.* [28] reported a slight effect of Mg^{2+} (10% increase) in sake yeast AAT activity. However, excess Mg^{2+} , but not excess Na^+ , caused a decrease in AAT activity. A partial inhibition by Mg^{2+} was reported in AAT of *Neurospora* sp. [29] and brewer's yeast [30]. This could cause acetyl-CoA precipitation, thereby reducing the availability of the substrate [31]. Up to 5 mM, dithiothreitol had no obvious effect but > 5 mM was inhibitory, reaching 60% inhibition at 50 mM dithiothreitol, indicating an important role for the disulfide bonds in activity. Harada *et al.* [26] observed similar inhibition of banana AAT activity with reducing agents. Dimethylpolycarbonate, an inhibitor of histidine-based enzymes [32] was very slightly stimulating up to 10 mM but became strongly inhibitory above this concentration.

Activity of CM-AAT1 protein towards various substrates *in vitro*

The substrate specificity of CM-AAT1 was assessed *in vitro* by incubating yeast protein extracts in the presence of different alcohols and acyl-CoAs (Table 1). Protein extracts of yeast transformed with the vector only produced very low amounts of esters (hexyl acetate 90 $\text{pmol}\cdot\text{h}^{-1}\cdot\mu\text{g}^{-1}$ protein) as compared to CM-AAT1-transformed yeast (1400 $\text{pmol}\cdot\text{h}^{-1}\cdot\mu\text{g}^{-1}$ protein). The same trend was observed with other substrates (data not shown). Table 1 shows that the recombinant protein was capable of producing esters from a wide range of combinations of alcohols and acyl-CoAs with the exception of ethanol, nonanol, and linalol from acetyl-CoA, and ethanol, *cis/trans*-3-hexenol, heptanol and nonanol when tested with propionyl-CoA. The highest activity found for CM-AAT1 (1400 $\text{pmol}\cdot\text{h}^{-1}\cdot\mu\text{g}^{-1}$ total proteins for acetylation of hexanol) was very similar to the highest activity of the purified recombinant AAT of strawberry (≈ 1600 $\text{pmol}\cdot\text{h}^{-1}\cdot\mu\text{g}^{-1}$ enzyme for acetylation of octanol).

In respect of aliphatic ester production, it was found that the longer the carbon chain of the alcohol, the higher the AAT activity. The activity of ester formation was in increasing order: hexyl acetate > butyl acetate; hexyl propionate > butyl propionate; and hexyl hexanoate > butyl hexanoate. The results are in agreement with those obtained with the strawberry [9] and yeast AATs [33]. CM-AAT1 was capable of accepting branched alcohols such as 2- and 3-methylbutyl alcohol (also named amyl and isoamyl alcohols). The position effect of the methyl group on activity was weak for acetyl-CoA and propionyl-CoA-derived esters with only $\approx 15\%$ higher activity with 2-methyl than 3-methyl compounds. It was more pronounced for hexanoyl-CoA-derived esters, with 43% higher activity with 2-methyl compounds.

In the case of aromatic alcohols, 2-phenylethanol was a better substrate than 1-phenyl-1-ethanol. The production of the corresponding esters, 2-phenylethyl acetate, 2-phenylethyl propionate and 2-phenylethyl hexanoate were three-, two- and fivefold higher, respectively, than esters derived from 1-phenylethanol in the same order.

Table 1. Substrate specificity of the recombinant CM-AAT1 enzyme towards different types of alcohols and acyl-CoAs. Activity was measured in yeast protein extracts. Activity is expressed in $\text{pmol}\cdot\text{h}^{-1}\cdot\mu\text{g}^{-1}$ protein as the mean \pm SD of three replicates. TR, present at trace amounts; ND, non detectable; NT, not tested; +, reported in the literature; NR, not reported in the literature [5,6,35].

Alcohols	Acyl-CoA	Esters reported in melon	Propionyl- CoA	Esters reported in melon	Hexanoyl- CoA	Esters reported in melon
Ethanol	TR	+	ND	+	TR	+
Butanol	383 \pm 12	+	535 \pm 6	+	500 \pm 19	+
Hexanol	1263 \pm 35	+	1386 \pm 21	NR	1883 \pm 270	NR
Heptanol	1310 \pm 135	+	ND	NR	NT	NR
Nonanol	ND	+	ND	NR	NT	NR
2-Methylbutanol	916 \pm 35	+	1015 \pm 17	NR	1434 \pm 21	+
3-Methylbutanol	796 \pm 4	+	875 \pm 69	NR	1000 \pm 36	NR
3-Met-2-buten-1-ol	610 \pm 70	+	NT	NR	NT	NR
Linalol	ND	NR	NT	NR	NT	NR
<i>cis</i> -2-Hexenol	1000 \pm 3	NR	670 \pm 10	NR	814 \pm 32	NR
<i>trans</i> -2-Hexenol	1400 \pm 15	NR	1285 \pm 73	NR	2050 \pm 75	NR
<i>cis</i> -3-Hexenol	1270 \pm 33	+	ND	NR	1960 \pm 33	NR
<i>trans</i> -3-Hexenol	850 \pm 19	+	ND	NR	1393 \pm 123	NR
Benzyl alcohol	555 \pm 48	+	1032 \pm 47	+	935 \pm 85	NR
1-Phenyl ethanol	322 \pm 8	+	865 \pm 23	NR	390 \pm 49	NR
2-Phenyl ethanol	1323 \pm 160	+	1760 \pm 100	+	1915 \pm 42	NR

Table 2. Kinetic properties of recombinant CM-AAT1 protein. The reaction conditions were as described in Material and methods.

Co-substrate S1 (variable concentration)	Co-substrate S2 (saturating concentration)	Apparent K_m (S1)	V_{max} ($\text{pmol}\cdot\text{h}^{-1}\cdot\mu\text{g}^{-1}$ protein)
1-Butanol	Acetyl-CoA	8.0 mM	400
1-Hexanol	Acetyl-CoA	1.4 mM	1200
Acetyl-CoA	1-Butanol	100 μM	350
Acetyl-CoA	1-Hexanol	85 μM	1100
Hexanoyl-CoA	1-Butanol	90 μM	350

The position effect of the branched or aromatic residue was amplified in the presence of hexanoyl-CoA as a cosubstrate as compared with acetyl-CoA. Also, the size of the aromatic residue seems to be important with an acetylation of 2-phenylethanol being higher than that of benzyl alcohol. In contrast, Dudareva *et al.* [16] reported that 2-phenylethanol was 10 times less acetylated than benzyl alcohol by the *Clarkia* BEAT enzyme.

The acetylation of hexanol and *cis*-3-hexenol were similar for CM-AAT1 while hexanol was a better substrate than *cis*-3-hexenol in the case of SAAT. Acetylation of *trans*-3-hexenol and hexanoylation of *cis*-2-hexenol was the lowest among hexenol isomers for CM-AAT1. Among the isomers of hexenol, *trans*-2-hexenol was a better substrate than *cis*-2-hexenol whatever the acyl-CoA was, but *trans*-3-hexenol was less efficient than *cis*-3-hexenol. Intriguingly, no activity was detected with *cis/trans*-3-hexenol and with propionyl-CoA. The acetylation of hexanol and *cis*-3-hexenol was similar and acetylation of *trans*-3-hexenol was the lowest among these isomers, although hexanoylation of *cis*-2-hexenol was the lowest.

It is important to emphasize that the recombinant CM-AAT1 was unable to acylate ethanol, while some esters of ethanol are abundant in the volatiles evolved by fruit [5,34], suggesting the presence of other AATs in the melon.

Conversely, CM-AAT1 was capable of producing a large number of esters, mainly from propionyl- and hexanoyl-CoA, that have not been detected in fruit (Table 1), indicating that the availability of some acyl donors is a limiting factor *in vivo*.

Table 2 shows some kinetic properties of the recombinant CM-AAT1 protein for some of the substrates. Under fixed concentrations of butanol and hexanol (40 mM) the apparent K_m for acetyl-CoA were similar (100 μM and 85 μM , respectively) and in the same order as those reported for the recombinant strawberry SAAT [9], partially purified AAT of strawberry [27] and banana [26]. The yeast AAT exhibits higher affinity towards acetyl-CoA with an apparent K_m of 25 μM [35]. Kinetic studies using a fixed concentration of acetyl-CoA (250 μM) indicated that the apparent K_m for butanol was much higher (8 mM) than for hexanol (1.4 mM). The K_m for octanol, hexanol and butanol of strawberry SAAT were 5.7, 8.9 and 46 mM, respectively. The K_m values towards acetyl- and hexanoyl-CoA were similar (between 85 and 100 μM). These data show that K_m values towards alcohols were much more variable than towards acetyl-CoA and therefore that the affinity for alcohols rather than for the acyl residues was crucial in the level of activity. In addition, values for V_{max} of CM-AAT1 were more strongly affected by the nature of the alcohol

than of the acyl moiety. A competing reaction between butanol and hexanol in the presence of acetyl-CoA was made by supplying both alcohols at 20 mM to the same reaction tube. This resulted in 10-fold higher production of hexyl acetate than butyl acetate (data not shown) indicating that hexanol is a much better substrate than butanol. Such a ratio was not observed when the two alcohols were incubated separately.

CONCLUSIONS

CM-AAT1 and *CM-AAT2* are fruit specific and ethylene-regulated genes that belong to a large acyl-transferase multifunctional gene family. Despite their strong sequence homology, they do not share the same activity. *CM-AAT1* is capable of transferring acyl residues into a variety of alcohols and *CM-AAT2* is inactive towards the same substrates. *CM-AAT1* has the same enzyme activity as a strawberry SAAT characterized by Aharoni *et al.* [9] although they share only 21% sequence identity. *CM-AAT1* probably plays a major role in generating a wide range of esters derived from aliphatic, branched and aromatic alcohols that are produced in large quantities by Charentais melon fruit during ripening. However, *CM-AAT1* was also capable of producing *in vitro* a large number of esters that have not been reported in melon fruit, mainly propanoate and hexanoate esters, indicating that the corresponding acyl donors could limit the production of some esters *in vivo*. Conversely, the failure of *CM-AAT1* to acylate ethanol, while ethyl esters are produced by melon fruit, suggests the involvement of other AAT(s) in these reactions.

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