Characterization of Genes Involved in the Formation of Aroma Volatiles in 'Charentais' Melon Fruit

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Abstract

Volatiles esters impart distinct characteristics to the fruit quality. 'Charentais' cantaloupe melon (*Cucumis melo* 'cantalupensis') is characterized by abundant sweetness and aromatic flavour. Plant alcohol acyl transferase (*AAT*) genes have been identified and shown to be involved in aromas production. Recently, two cDNAs (*Cm-AAT$_1$* and *Cm-AAT$_2$*) putatively involved in the formation of aroma volatile esters have been isolated from melon fruit. *Cm-AAT$_1$* protein exhibit alcohol acyl transferase activity while no such activity could be detected for *Cm-AAT$_2$*. Two new cDNAs (*Cm-AAT$_3$* and *Cm-AAT$_4$*) have been isolated from melon fruit that showed 69% and 36% similarity, respectively, with *Cm-AAT$_1$*. The percentage similarity over the whole amino acid sequence between them is 34%. *Cm-AAT$_3$* and *Cm-AAT$_4$* show the highest similarity to the tobacco *Nt-HSR201* protein and a rose alcohol acyltransferase *Rh-AAT$_1$*, respectively. All *Cm-AATs* genes, share three conserved regions common to the BAHD acyltransferase gene superfamily. Heterologous expression in yeast revealed that some of the encoded proteins have a wide range of specificity while others are specific to a narrow range of substrates.

INTRODUCTION

Aroma production is one of the most important parameters that determines the quality of fruit. In 'Charentais' cantaloupe melon (*Cucumis melo* 'cantalupensis Naud'), aroma volatiles comprise a complex mixture of volatiles compounds among which esters are quantitatively the most important and are key contributors to the unique aroma of ripe melon fruit (Bauchot et al., 1998). The esterification step is catalysed by alcohol acyltransferase enzymes (AAT) (El Yahyaoui et al., 2002).

Two genes (*Cm-AAT$_1$* and *Cm-AAT$_2$*) have previously been isolated from 'Charentais' melon fruit that share strong sequence homology but only one of them, *Cm-AAT$_1$*, has been involved in the synthesis of aroma volatile esters (El Yahyaoui et al., 2002). Two more genes have been isolated putatively involved in ester formation in melon (*Cm-AAT$_3$* and *Cm-AAT$_4$*). The present study consists in the molecular analysis of the *Cm-AAT$_{1-4}$* cDNAs and in the biochemical characterisation of the encoded proteins after expression in yeast.

MATERIALS AND METHODS

Plant Material

'Charentais' cantaloupe melon plants (*Cucumis melo* 'cantalupensis') were grown in a greenhouse under hydroponic conditions. Young leaves were collected for genomic DNA extraction in order to make a DNA library for the isolation of *Cm-AAT* genes. Fruits at climacteric stage, i.e. 30 d post-anthesis were also collected in order to extract total RNA to prepare a cDNA library to check if *Cm-AAT* genes isolated from the genomic library are transcriptionally actives.

Isolation of *Cm-AAT* cDNAs

The coding sequences of *Cm-AAT$_3$* and *Cm-AAT$_4$* have been isolated and

completed from the genomic DNA library using the Universal Genome Walker™ kit (Clontech), following the manufacturer's protocol with modifications (Siebert et al., 1995). Transcriptional expression of the genes was checked by PCR on the cDNA.

Sequence Alignment and Phylogenetic Analysis

Alignments of the predicted protein sequences were performed using CLUSTALX (Thompson et al., 1997; 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. A neighbour-joining tree is shown, with bootstrap confidence values (1000 replications) shown for neighbour-joining algorithms. The tree was visualised with the TREEVIEW program (Page, 1996).

Cloning of *Cm-AAT$_3$* and *Cm-AAT$_4$* in Yeast

Both *Cm-AAT$_3$* and *Cm-AAT$_4$* cDNAs were cloned in a pYES2.1 TOPO expression vector and transferred into the *Saccharomyces cerevisiae* INVSC1 cell line. Strains harbouring the correct constructions were grown (until OD_{600} of ~ 4.0) in selective medium (SC-U) with 2% galactose as inducer according to Invitrogen recommendations.

AAT Activity Assay with Recombinant Proteins

The pellet from 25 mL of induced culture from centrifugation at 1800g for 10 min was resuspended in buffer A (Tris/HCl 50 mM pH=7.5, DTT 1 mM) and mechanically ground in liquid nitrogen for 2 min and stored at -80ºC until needed (El Yahyaoui et al., 2002). Then, the powder was thawed, vortexed for 1 min and centrifuged at 13000 rpm for 15 min at 4ºC. Total proteins were quantified according to Bradford (1976). AAT activity was measured according to Harada et al. (1985) with modifications. The protein extract (50 µL) was adjusted to 1 mL final volume with buffer A and complemented with 2 mM alcohol and 250 µM acyl-CoA. The mixture was incubated at 30ºC for 20 min and the reaction stopped by addition of 0.1 g citric acid and 0.37 g KCl. After 10 min agitation in a sealed 10 mL tube, the SPME fibre was introduced and held in the tube for 10 min. The esters absorbed on the fibre were then analysed by GC-FID (Hewlett-Packard gas chromatograph, model 5890) at a constant temperature of 25ºC using 5 $\mu L \cdot L^{-1}$ methylbenzoate as internal standard. The column consisted in a HP-Innowax cross-linked polyethylene glycol column (30 m x 0.25 mm x 0.25 µm). Injector and detector temperatures were 250ºC. The oven temperature was programmed from 40ºC (1 min) to 60ºC (1 min) at a rate of $2ºC \cdot min^{-1}$ and finally to 190ºC (5 min) at a rate of $10ºC \cdot min^{-1}$. Nitrogen was used as a carrier gas at 100 kPa. Compounds were identified by comparison of retention times with authentic standards and quantification was achieved by constructing a response curve for each ester. When pure authentic product was not available, quantification was based on the enhancement of the peak between 20 and 40 min of enzymatic reaction and on the response curves of esters of the same family (El Yahyaoui et al., 2002).

RESULTS AND DISCUSSION

Sequence Analysis

Cm-AAT$_3$ and *Cm-AAT$_4$* encode proteins of 459 and 479 amino acids with a theoretical molecular weight of 51.1 and 55.0 kDa and a pI of 7.64 and 7.62, respectively. Nevertheless both of them belong to the BAHD superfamily, since they display the highly conserved motifs HXXXD located in the centre of the amino acid sequence, and DFGWG placed near the C- terminal, both present in the enzymes of the O-acyltransferases family (Fig. 1). A third motif somewhat less conserved, LXXyypXaGr, is present near the N terminus (Aharoni et al., 2000; St-Pierre and De Luca, 2000). Yeast AATs contain the first conserved motif only, suggesting that this element is involved in the catalytic activity of the protein, i.e in the acyl-transfer from the acyl-CoA to alcohol (Yoshimoto et al.,

1999).

The relationships between the predicted amino acid sequences, as indicated by percentage similarity over the whole sequence, are presented in Table 1. *Cm-AAT$_3$* has strong similarity with *Cm-AAT$_1$* and *Cm-AAT$_2$* (69% and 71%, respectively) previously isolated in cantaloupe 'Charentais' melon (El Yahyaoui et al., 2002). Contrary, *Cm-AAT$_4$* displays only 36% and 34% amino acid similarity with *Cm-AAT$_1$* and *Cm-AAT$_2$*, respectively. The amino acid sequence similarity between *Cm-AAT$_3$* and *Cm-AAT$_4$* is rather low (34%) as shown in Table 1.

The comparison of the predicted amino acid sequences of *Cm-AAT$_3$* and *Cm-AAT$_4$* with known BAHD *AAT* genes from other sources (Table 1) indicates that *Cm-AAT$_3$* is most similar to a tobacco protein, *Nt-HSR201*, involved in the hypersensitivity response (84% similarity; Czernic et al., 1996), and to a lesser extent to the benzoyl-coenzyme A: benzyl alcohol benzoyl transferases *BEBT* from *Clarkia breweri* (78% similarity; D'Auria et al., 2002). *Cm-AAT$_4$* showed a rather low degree of homology with other *AAT* genes from the BAHD family. The closest AAT protein is an acyltransferases isolated from *Rosa hybrida Rh-AAT$_1$* (45% similarity; Shalit et al., 2003) and a strawberry *SAAT* (43% similarity; Aharoni et al., 2000).

AAT Activity of *Cm-AAT$_3$* and *Cm-AAT$_4$* Recombinant Proteins In Vivo

In vivo bioconversion of alcohols and organic acids into volatiles esters in the culture medium of transformed yeast was performed by addition of precursors (alcohol at 2 mM and organic acids at 1 mM final concentration) when the yeast culture reached an OD_{600} of ~ 4.0. Yeast harbouring the pYES 2.1 TOPO/*Cm-AAT$_3$* or *Cm-AAT$_4$* constructs exhibited activity whereas yeast transformed with the pYES 2.1 TOPO vector without insert did not show any AAT activity. For example, the chromatographic profiles of in vivo bioconversion of benzyl alcohol and acetic acid (added as sodium salt) presented in Fig. 2 clearly showed the formation of benzyl acetate in the *Cm-AAT$_3$* transformed yeast, while no such formation could be observed in the yeast with auto-ligated vector.

AAT Activity of *Cm-AAT$_3$* and *Cm-AAT$_4$* Recombinant Proteins In Vitro

Substrate specificity of all four recombinant *Cm-AATs* was assayed in vitro using protein extracts. Table 2 showed that *Cm-AAT$_1$* is capable of catalysing the formation of esters from a wide range of alcohols and acyl-CoAs, with a maximum activity for acetyl-CoA: (*E*)-hex-2-enol acetyltransferases. *Cm-AAT$_2$* has never shown any AAT activity with the whole set of substrates. *Cm-AAT$_3$* was capable of catalysing the formation of esters from a wide range of alcohols and acyl-CoAs, but its maximum activity is by far of the benzyl alcohol acetyl transferases type. This activity was higher than the activity of hexanol acetyl transferases (150-fold) and ethanol benzoyl transferases (4-fold).

Cm-AAT$_4$ activity is highly selective with only three types of alcohol-acyl transferases activities: hexanol acetyl transferases, (*E*)-hex-2-enol acetyl transferases, and benzyl alcohol acetyl transferases. The (*E*)-hex-2-enol acetyl transferases activity is by far the most important with around 40-fold higher activity than the other two (Table 2).

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Tables

Table 1. Percentage of amino acid sequence similarity between the predicted full length AAT genes $CmAAT_1$, $Cm\text{-}AAT_2$, $Cm\text{-}AAT_3$, $Cm\text{-}AAT_4$, Nt-HSR201, BEBT, SAAT, BEAT, $Rh\text{-}AAT_1$ and Cr-DAT.

	Cm-AAT$_1$	*Cm-AAT$_2$*	*Cm-AAT$_3$*	*Cm-AAT$_4$*
$Cm\text{-}AAT_1$				
$Cm\text{-}AAT_2$	89			
$Cm\text{-}AAT_3$	69	71		
$Cm\text{-}AAT_4$	36	34	34	
Nt-HSR201	70	73	84	33
BEBT	64	66	78	33
SAAT	34	34	34	43
BEAT	32	31	31	35
$Rh\text{-}AAT_1$	35	34	34	45
Cr-DAT	32	33	32	39

Table 2. Substrate specificity of the recombinant proteins $Cm\text{-}AAT_1$, $Cm\text{-}AAT_2$, $Cm\text{-}AAT_3$ and $Cm\text{-}AAT_4$ towards different types of alcohols and acyl-CoAs. Activity was measured in yeast protein extracts. Activity is expressed as $nkat \cdot g^{-1}$ protein as the mean ± SE of three replicates. ND, non detectable.

Alcohol	*Acyl-CoA*	*Ester*	*CmAAT$_1$*	*CmAAT$_2$*	*CmAAT$_3$*	*CmAAT$_4$*
Ethanol	Acetyl-CoA	Ethyl acetate	N.D.	N.D.	N.D.	N.D.
Butanol	Acetyl-CoA	Butyl acetate	1.4±0.2	N.D.	2.1±0.2	N.D.
Isoamyl alcohol	Acetyl-CoA	Isoamyl acetate	4.6±0.5	N.D.	7.9±0.8	N.D.
Hexanol	Acetyl-CoA	Hexyl acetate	6.8±0.5	N.D.	0.9±0.05	2.3±0.4
(*E*)-hex-2-enol	Acetyl-CoA	(*E*)-hex-2-enyl acetate	20.7±2.5	N.D.	2.9±0.3	43.3±4.7
Benzyl alcohol	Acetyl-CoA	Benzyl acetate	9.0±0.6	N.D.	124.5±1.4	3.3±0.4
Ethanol	Hexanoyl-CoA	Ethyl hexanoate	N.D.	N.D.	0.5±0.06	N.D.
Butanol	Hexanoyl-CoA	Butyl hexanoate	N.D.	N.D.	7.5±1.3	N.D.
Isoamyl alcohol	Hexanoyl-CoA	Isoamyl hexanoate	0.5±0.06	N.D.	1.3±0.3	N.D.
Hexanol	Hexanoyl-CoA	Hexyl hexanoate	0.2±0.02	N.D.	N.D.	N.D.
Isoamyl alcohol	Isovaleryl-CoA	Isoamyl isovalerate	N.D.	N.D.	14.8±1.4	N.D.
Ethanol	Benzoyl-CoA	Ethyl benzoate	N.D.	N.D.	26.5±1.1	N.D.
Ethanol	Propionyl-CoA	Ethyl propionate	N.D.	N.D.	N.D.	N.D.
Butanol	Propionyl-CoA	Butyl propionate	3.1±0.1	N.D.	7.4±0.4	N.D.
Isoamyl alcohol	Propionyl-CoA	Isoamyl propionate	3.4±0.4	N.D.	14.0±1.5	N.D.
Hexanol	Propionyl-CoA	Hexyl propionate	10.8±0.6	N.D.	2.1±0.1	N.D.

Figures

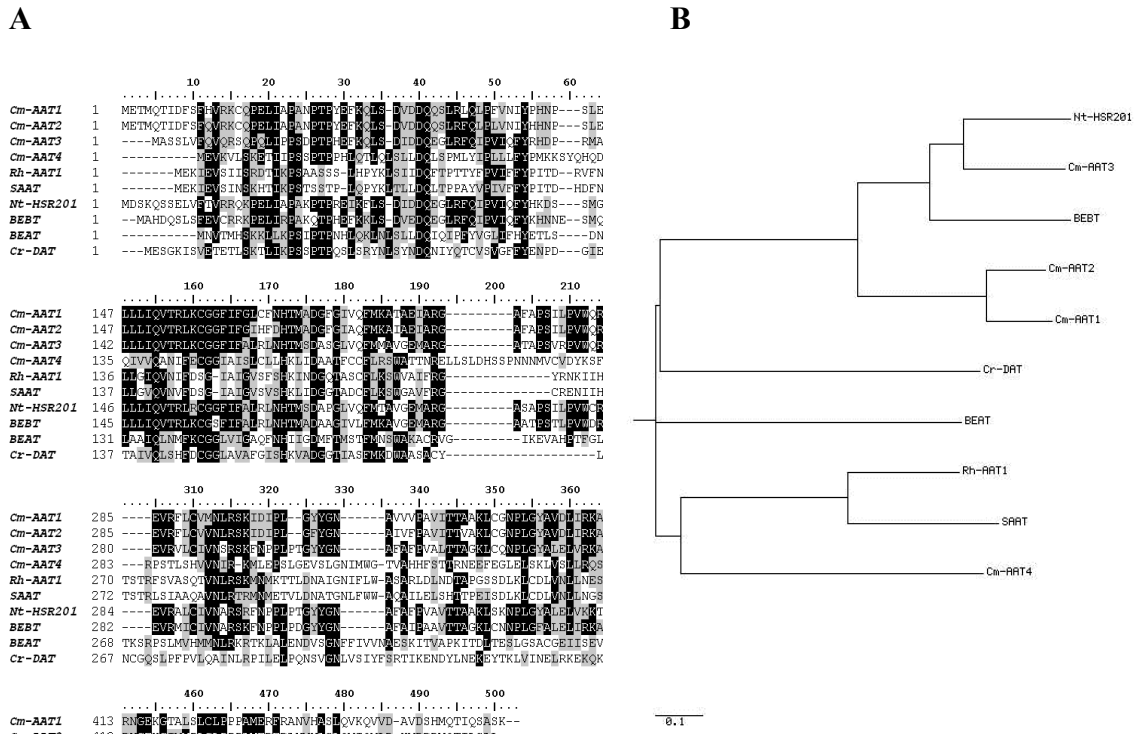

Fig. 1. (A) Amino acid sequence alignment of melon *Cm-AAT$_1$* (Z70521), *Cm-AAT$_2$* (AF468022), *Cm-AAT$_3$*, *Cm-AAT$_4$*, *Rosa hybrida Rh-AAT$_1$* (BQ106456), strawberry alcohol acyltransferase *SAAT* (AF193789), tobacco *Nt-HSR201* (AF500202), *Clarkia breweri BEBT* (AF500200), *BEAT* (AAC18062), and *Catharantus roseus* deacetylvindoline 4-O-acetyltransferase from *Cr-DAT* (AF053307) using the ClustalX program. Conserved residues are shaded in black. Amino acids shaded in black represent identical matches; grey shaded boxes represent conservative changes. Asterisk indicate the three conserved motifs mentioned in the text. (B) Phylogenetic relationships between the different AATs genes based on predicted amino acid sequence using TreeView program.

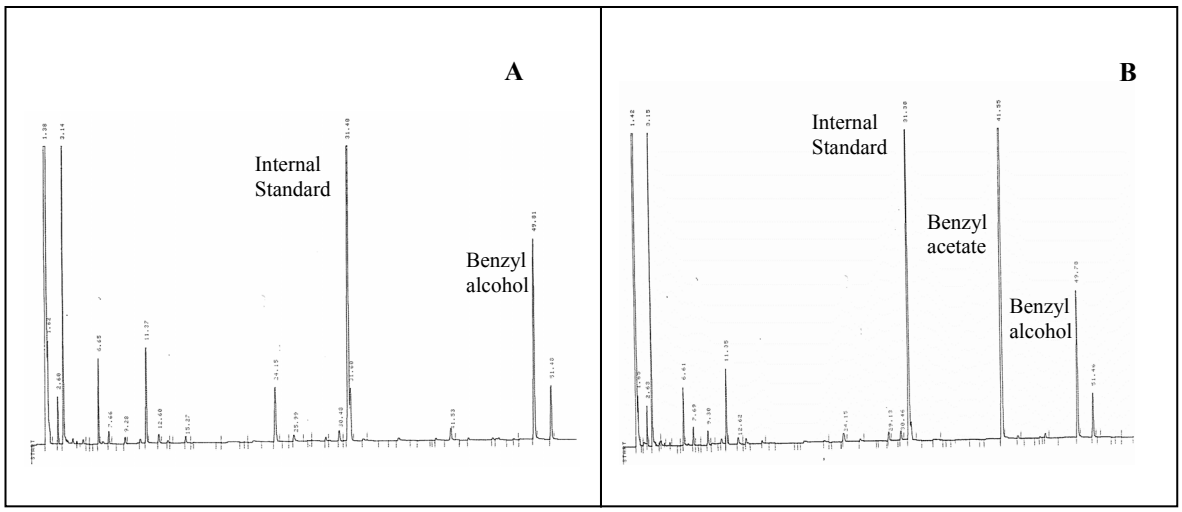

Fig. 2. Volatile compounds analysed by SPME-GC-FID from 10 mL culture medium of control yeasts transformed with the vector only (A) and with the vector harbouring the Cm-AAT_3 gene (B). The medium was complemented with benzyl alcohol 2 mM and acetate 1 mM, and 5 $\mu L \cdot L^{-1}$ internal standard was added for the GC-FID measurements.