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ARTICLE

VpAAT1, a Gene Encoding an Alcohol Acyltransferase, Is Involved in Ester Biosynthesis during Ripening of Mountain Papaya Fruit

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Mountain papaya (*Vasconcellea pubescens*) is a climacteric fruit that develops a strong and characteristic aroma during ripening. Esters are the main volatile compounds produced by the fruit, and most of them are dependent on ethylene. As esters are synthesized through alcohol acyltransferases (AAT), a full-length cDNA (*VpAAT1*) was isolated that displayed the characteristic motifs of most plant acyltransferases. The full-length cDNA sequence was cloned and expressed in yeasts, obtaining a functional enzyme with high AAT activity toward the formation of benzyl acetate. The transcript accumulation pattern provided by qPCR analysis showed that the *VpAAT1* gene is expressed exclusively in fruit tissues and that a high level of transcripts is accumulated during ripening. The increase in *VpAAT1* transcripts in fruit is coincident with the increase in AAT activity; transcript accumulation is induced by ethylene, and it is avoided by 1-methylcyclopropene (1-MCP) treatment. The data indicate that *VpAAT1* is involved in aroma formation and that ethylene plays a major role in regulating its expression.

KEYWORDS: Alcohol acyltransferase; aroma; ester production; fruit ripening; 1-methylcyclopropene; *Vasconcellea pubescens*

INTRODUCTION

Mountain or highland papaya (*Vasconcellea pubescens*) is a diploid ($2n = 18$) dicotyledoneous species. It is native to the Andean regions of South America and belongs to the Caricaceae family, which includes the *Carica* and *Vasconcellea* genera (*1*, *2*). The fruit is climacteric and exhibits a characteristic rise in ethylene production during ripening, accompanied by softening, changes in color, and development of a strong and characteristic aroma (*3*−*5*). The main volatile compounds produced by the fruit are esters and alcohols (*4*). Most of the esters found in mountain papaya fruit are potent odor compounds, and the dynamic of their production during ripening has been previously reported (*4*). In addition, the treatment of mountain papaya fruit with 1-methylcyclopropene (1-MCP), a strong inhibitor of ethylene action (*6*), has been shown to inhibit ethylene production and the production of aroma volatile compounds in mountain papaya fruit, particularly esters (*4*). Thus, most esters identified in mountain papaya displayed a clear modulation by ethylene.

Esters are produced from alcohols and acyl-CoAs through the action of alcohol acyltransferases (AAT) (*7*). Plants contain large families of AATs, with about 61 putative members in *Arabidopsis* (*8*). AAT activity is responsible for the production of esters, and it has been measured in two main plant tissues: flowers and fruits (*9*). During the past few years, several AAT genes have been isolated and characterized from several fruit species, such as strawberry (*9*, *10*), melon (*11*, *12*), apple (*13*, *14*), banana (*7*), grape (*15*), and apricot (*16*). According to their sequences, acyltransferases dependent on coenzyme A (CoA) have been included within a wide and divergent family of proteins, the BAHD superfamily (*17*). Among them the HXXXD motif, located in the middle of the protein sequence, is highly conserved in superior plants and yeasts. The substitution of the histidine residue from this motif causes the loss of protein function (*18*), which suggests that it could be involved in the transfer of the acyl group from acyl-CoA toward an alcohol. Another highly conserved motif in higher plants in this superfamily is the DFGWG sequence, located near the carboxylic end of the protein, which seems to be involved in the maintenance of the structural integrity of the enzyme (*19*).

An important question in the field has been the identification of enzymes that are critical for production of the distinctive blend of esters characteristic of each fruit species (*13*). The available information indicates that substrate specificity of AAT enzymes seems to be wide but with differential preferences toward acyl-CoAs and alcohols. This could indicate that the particular aroma

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of each fruit or flower depends on the interaction of different types of AATs, each one with preferential, although nonexclusive, substrates. In addition, AAT sequences provide valuable information about enzyme properties, although in some cases it has been difficult to predict substrate selectivity, and minor differences in the amino acid sequence could alter the ability of a certain enzyme to produce some esters (*7*, *11*, *12*). On the other hand, substrate availability for AAT enzymes is also another aspect to consider.

In the present paper, the isolation and characterization of an AAT gene for the first time from the Caricaceae family was performed. In addition, it was demonstrated that the encoding protein is functional and active toward several substrates, and the role of ethylene on the expression of the gene was investigated.

MATERIALS AND METHODS

Plant Material. Mountain papaya (*V. pubescens*) fruit and other vegetative tissues were collected in February 2005 from orchards located at Lipimavida (34° 51′ S; 72° 08′ W; 20 m asl), on the coast of Curicó, Chile. Young expanding fruit was picked and staged as small green fruit (SGF), medium green fruit (MGF), or large green fruit (LGF), according to the guidelines of Gaete-Eastman et al. (*5*). Ripening fruit was harvested after the first signs of chlorophyll breakdown, sorted for size and then randomly divided into three lots: one lot was left intact (control fruit); the second lot was treated with 1-MCP; and the last one was treated with ethylene (Ethrel), both on the same day of harvest (day 0). After each treatment, the fruit was allowed to ripen at 20 °C in separate rooms.

1-MCP and Ethylene Treatments. Fruit was placed inside an airtight chamber of 0.28 m^3 and treated with 0.3 μL L^{-1} 1-MCP for 16 h at 20 °C. One hundred and fifty milligrams of EthylBloc (0.14% 1-MCP) was dissolved in 20 mL of 0.9% (w/v) sodium hydroxide to allow the release of 1-MCP gas. The chamber was immediately closed after EthylBloc application, and it was opened only after 16 h to remove the 1-MCP-treated fruit, which was subsequently left at 20 °C. For ethylene treatment, the fruit was dipped during 5 min in 25 L of an aqueous solution of 2 g L^{-1} Ethrel 48 SL (Bayer CropScience S.A.; ethephon, 2-chloroethyl phosphonic acid), allowed to dry at room temperature, and then left at 20 °C in separate rooms.

Sampling of fruit from each treatment (ethylene, 1-MCP, and nontreated) was performed every 2 days (starting on day 1) until completing 13 days at 20 °C. Three replicates of four fruits were randomly selected and assessed for volatiles and ethylene (*4*). In addition, on each sampling date, pulp tissue from each fruit was immediately frozen in liquid nitrogen and stored at −80 °C until required.

Ethylene Production Rate. The procedure employed was described previously (*4*). Briefly, the fruit was introduced into airtight respiration chambers (6 L), and after 3 h of incubation at 20 °C, 1 mL gas samples were withdrawn from the headspace and quantified for ethylene in a Perkin-Elmer (Clarus 500) gas chromatograph. Three independent ethylene samples were taken per chamber, and results were expressed as means ± standard error (SE) (μL kg^{1-} h^{-1}).

Isolation of *VpAAT1* Gene from *V. pubescens*. To isolate the AAT gene, total RNA (1 μg) was extracted from 6 g of pulp tissue of a ripe mountain papaya fruit (day 5 of storage) using an adapted version of the CTAB method (*20*). Then the RNA was treated with DNase I amplification grade (Invitrogen) and cDNA synthesized using a BD SMART PCR (Clontech) kit according to the manufacturer's instructions. Degenerate primers (VpAAT1 F = 5′-TACTAYCCHYTHKCYGGAAG-3′ and VpAAT1deg R = 5′-GBCYKYCCCCATCCAAA-3′) were designed to match AATs conserved regions. PCR runs were performed, and PCR product was cloned onto pCR 2.1 TOPO (Invitrogen) and manually sequenced through ALFexpress II (Amersham Biosciences) using a thermosequenase dye terminator kit (Amersham Biosciences). To complete the gene sequence, specific internal primers were designed for 5′ and 3′ RACE-PCR on the basis of the isolated sequence (VpAAT1RACE F = 5′-TCCATTGGCCGGACGTCTCAGGG-3; VpAAT1RACE R = 5′-ATGGCAGCCATCT CACTGGGACC-3′). RACE-PCR runs were performed using the BD SMART RACE cDNA amplification kit (Clontech), according to the manufacturer's instructions. The PCR products amplified were cloned and sequenced by Macrogen Inc. (Seoul, Korea). The full-length cDNA sequence was amplified by using primers specially designed to match the initial and final transcription zones of the gene (Vp-AAT1 F = 5′-ATGGCAGAGAAAGCTAGTTC-3′; Vp-AAT1 R = 5′-AATGGCGGACACAATAAAGA-3′) and then sequenced.

The nucleotide and deduced amino acid sequences were analyzed using Vector NTI Advance 9.0 software (Invitrogen, 2003). The similarity analysis was performed using the local alignment tool (BLAST, National Center for Biotechnology Information, Bethesda, MD) and the web-based tool Wolf PSORT World Wide Web Prediction Server (*21*). The multiple alignment of amino acid sequences was performed using the software BioEdit Sequence Alignment Editor v7.0 (*22*). The phylogenetic tree was built using MEGA software (version 4; http://www.megasoftware.net) (*23*) using the maximum parsimony method and Bootstrap analysis (1000 replicates).

DNA Gel-Blot Analysis. Mountain papaya genomic DNA was extracted from a pool of young leaves (2 g) as described by Murray and Thompson (*24*). Probe for DNA gel-blot analysis of *VpAAT1* was designed from the 3′-end portion of the ORF sequence. The 108 bp probe (from position 1308 to 1415 in *VpAAT1* sequence) was prepared through PCR reaction with the primers QAAT1-F (5′-AGGAGAGAAAGG-GATCGTAG-3′) and QAAT1-R (5′-TCAGCGGAAACAAGTAG-TTG-3′), and radiolabeled using [α-^{32}P]dCTP (Easytides, NEN Life Sciences Products). For Southern blots, 20 μg of genomic DNA was digested with *Bam*HI, *Hin*dIII, *Eco*RV, and *Eco*RI, fractioned on a 0.7% agarose gel, and transferred to Hybond-N+ membranes (Amersham Biosciences) using 20× SSC as blotting buffer. Membranes were prehybridized at 42 °C for 4 h in a solution containing 50% deionized formamide, 1% (w/v) SDS, 5× SSCE, 5× Denhart's solution, and 100 μg mL^{-1} denatured salmon sperm DNA. The hybridization step was carried out overnight at 42 °C with denatured ^{32}P-labeled probe with gentle agitation. Washings were performed with 2× SSC containing 0.1% (w/v) SDS for 15 min at 42 °C, followed by three washes with 1× SSC plus 0.1% (w/v) SDS during 15 min at 50 °C. The blots were exposed, and autoradiograms were scanned in a densitometer (FLA-5100 Imaging System, Fujifilm, Japan).

Real-Time (qPCR) Expression Analysis. Total RNA was extracted from 6 g of mountain papaya fruit pulp (control, 1-MCP and ethylene-treated papaya fruits) after 1, 3, 5, 7, 9, and 13 days at 20 °C using an adapted version of the CTAB method (*20*). In addition, total RNA was also extracted from other vegetative tissues: flower, leaf, root, and stem. Total RNA was treated with DNase I amplification grade (Invitrogen) and cleaned using the RNeasy Plant Mini Kit (Qiagen, Germany). First-strand cDNA synthesis was performed using an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA) following the manufacturer's instructions. Three biological replicates for each sampling date were employed. Specific primers for a divergent 3′-end region of *VpAAT1* and the α-elongation factor 1 (*VpEF1*-α; as internal control) genes were designed using Vector NTI v9, with high stringency to avoid amplification of nonspecific PCR products. Primers were tested by RT-PCR and the amplification products sequenced to ensure their specificity. Primer pair sequences were QAAT1-F and QAAT1-R (described above) and EF1-F (5′-TCAATGAACCCAAGAGGCCATCC-3′) and EF1-R (5′-CACG-TCCCAC TGGAACAGTTCCA-3′).

The amplicon sizes were 108 bp for the AAT gene and 96 bp for *VpEF1*-α. The amplification reactions were performed using Brilliant SYBR Green QPCR Master Mix (Stratagene) according to the manufacturer's instructions in a DNA engine Opticon 2 Real-Time PCR System (MJ Research, Watertown, MA). PCR conditions were as follows: 94 °C for 10 min; 40 cycles of 94 °C for 15 s, 60 °C for 15 s, and 72 °C for 20 s; melting curve from 58 to 95 °C at 0.5 °C increments. A dilution series was built to estimate the amplification efficiency using a cDNA mix as template prepared from control fruit samples (1−13 days of storage). Each reaction was performed in triplicate, and a negative water control was included in each run. Fluorescence was measured at the end of each annealing step. The amplification efficiency was estimated through a melting curve, and amplification products were visualized on agarose gels (1.5% w/v). The relative expression levels were first normalized against the *VpEF1*-α gene and using nontreated fruit samples from day 1 as calibrator, with a nominal value of 1. The method described by Pfaffl (*25*) was used to make all calculations.

Expression of Recombinant VpAAT1. The full-length sequence of VpAAT1 was initially cloned into *Escherichia coli* TOP 10 One Shot (Invitrogen) to select colonies with the right orientation. The insert was then cloned in the pYES2.1 TOPO-TA cloning vector following the instructions provided by the manufacturer (Invitrogen). The construct was used to transform the *Saccharomyces cerevisiae* cell line INVSc1. Transformed yeasts were grown at 30 °C in selective medium (SC-U) with 2% galactose as inducer of the recombinant protein expression, with constant stirring and bubbling of sterile air until the OD_{600} of the culture reached <1 U.

Purification of Recombinant VpAAT1. The purification of the recombinant protein was carried out according to the method of El-Sharkawy et al. (*11*) with some modifications. Six flasks containing 100 mL of yeast culture induced with galactose (OD_{600} of 0.8) were centrifuged (1800*g* for 5 min at room temperature), and the cells collected from each flask were resuspended in 2 mL of 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 10 min and stored at −80 °C until needed. To extract AAT enzyme, the powder was thawed and centrifuged at 10000*g* for 20 min at 4 °C. The crude extract obtained was purified through a BD Talon Metal Affinity column (BD Biosciences), an affinity column designed to purify polyhistidine-tagged proteins, according to the manufacturer's protocol. The recombinant protein was eluted with buffer A containing 150 mM imidazole. Proteins were quantified according to the Bradford method (*26*) and visualized through 10% SDS-PAGE gels.

Assay of AAT Activity. AAT activity of recombinant VpAAT1 protein was quantified by its ability to convert alcohols and acyl-CoAs into the corresponding ester (*10*, *27*). AAT activity was assayed in 500 μL of total volume in the presence of 2 mM alcohol−250 μM acyl-CoA in 50 mM Tris-HCl (pH 7.5) buffer containing 10% (v/v) glycerol and 1 mM dithiothreitol (DTT) (*12*). A set of three acyl-CoAs (acetyl-, butanoyl-, and hexanoyl-CoA) and nine different alcohols were assayed: ethanol, methanol, butanol, hexanol, octanol, benzyl alcohol, geranyl alcohol, 1-phenylethanol, and cinnamyl alcohol. Each reaction assay contains the mixture of one alcohol and one acyl-CoA per time. The reaction was initiated by the addition of 200 μL of purified protein (10−15 μg), and the mixture was incubated at 30 °C for 2.5 h in sealed Eppendorf tubes. The reaction was stopped by the addition of 50 mg of citric acid and 185 mg of KCl, and after mixing during several minutes, the supernatant was transferred to a glass vial, which was sealed after the addition of 0.5 μL of 1,2-dichlorobenzene as internal control. The solution was stirred during 15 min at room temperature; meanwhile, the volatiles produced during the enzymatic reaction were released into the headspace and adsorbed onto an SPME fiber (PDMS/DVB). The separation and quantification of each ester was done by a gas chromatograph fitted with a flame ionization detector (GC-FID) (Perkin-Elmer, Clarus 500) (*4*). The separation and quantification of each ester was done by a GC-FID following the procedure described by Balbontín et al. (*4*). A calibration curve was prepared for each ester. AAT enzyme activity was expressed as picokatals (pmol s^{-1}) per milligram of protein. Protein content was determined using BSA as standard (*26*). Determinations were performed in triplicate and expressed as mean ± SE.

AAT activity was also assayed in mountain papaya pulp samples obtained during ripening at 20 °C. Frozen pulp tissue (10 g) was homogenized in a mortar with the help of liquid nitrogen in the presence of 0.2 g of PVPP and 20 mL of buffer 100 mM Tris-HCl (pH 8)−1 M KCl, 0.1% (v/v) Triton X-100, containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and 2 μM leupeptin as protease inhibitors. The mixture was stirred for 20 min at 4 °C, filtered through Miracloth, and centrifuged (10000*g* for 20 min). The supernatant was desalted through a Sephadex G-25 gel filtration column (PD-10 Pharmacia) in the presence of 50 mM Tris-HCl (pH 7.5) buffer containing 10% (v/v) glycerol and 0.5 mM DTT. AAT activity was quantified by its ability to convert acetyl-CoA and hexanol into hexyl acetate (*10*, *27*). The reaction was performed as described above in the presence of 10 mM hexanol−490 μM acetyl-CoA−50 mM Tris-HCl (pH 7.5) buffer, 10% (v/v) glycerol, and 0.5 mM DTT. The reaction was initiated by the addition of 300 μL of protein extract and the mixture incubated at 30 °C for 2 h. A calibration curve was prepared with hexyl acetate.

Statistical Analysis. The experiment was conducted using a complete random design with three replicates. Statistical analyses were performed using the SPSS v. 14 package. Analysis of variance was performed, and significant differences were determined at $P \leq 0.05$ (LSD test).

RESULTS

***VpAAT1* Shares High Similarity with AATs from the BAHD Family.** With the aim to isolate ripening-related AAT genes from mountain papaya fruit, degenerate primers were designed on the basis of two conserved regions in the BAHD superfamily gene sequences: the motif YYPLAGRL, located close to the N-terminal, and the DFGWGR motif, at the C-terminal end (*9*). By PCR a fragment of 1200 bp was amplified with high homology to other AAT sequences, which was used as a template to design internal primers for 5′ and 3′ RACE-PCR runs. Two fragments of 1300 and 800 bp were obtained using these primers. A composite cDNA sequence of 1622 bp called *VpAAT1* (GeneBank accession no. FJ548611) was generated from all fragments.

Analysis of the *VpAAT1* sequence revealed an ORF of 1383 bp and a deduced amino acid sequence of 463 amino acids with a molecular weight of 51.4 kDa (**Figure 1A**). The sequence also contains 60 and 173 bp of 5′- and 3′-UTR, respectively. *VpAAT1* shares the characteristic motifs found in other plant acyltransferases, including the active site motif HXXXDG (amino acids 166−171). In *VpAAT1* the His and Asp residues are conserved; however, the Gly is replaced by Ala. Some AATs also have Ala in this position, but it is not known if this change could affect the activity or substrate preferences (*13*). There is also another highly conserved motif located toward the carboxylic end formed by five amino acids, DFGWG (amino acids 381−385). *VpAAT1* also exhibits a third less conserved motif, LXXyyplaGR (amino acids 75−84), located near the amino-terminal end, which is common among AATs involved in the synthesis of volatile compounds in fruits and flowers. Finally, it is important to highlight the presence of Thr in position 266, which has been shown to be essential for enzyme activity (*12*).

Sequence Comparison and Phylogenic Analysis. The VpAAT1 deduced amino acid sequence was aligned and compared with 15 plant acyltransferases (**Figure 1A**). The highest similarity was found between VpAAT1 and CmAAT3 isolated from *Cucumis melo* (*12*) and BEBT (benzoyl coenzymeA: benzyl alcohol benzoyl transferase) isolated from *Clarkia breweri* (*28*), with 67 and 61% identity at the amino acid level, respectively. A phylogenic tree was built from the previous multiple alignment, and the grouping pattern provides three main subgroups (**Figure 1B**). VpAAT1 was clustered into subgroup III with AATs related to the synthesis of esters in melon and *Clarkia* (CmAAT3 and CbBEBT). The same cluster also incorporates AATs isolated from apple (MpAAT1), pear (PcAAT1), banana (MsAAT1) (*7*), and melon (CmAAT1 and CmAAT2) (*11*). Subgroup II comprises AATs isolated from rose (*29*) and different strawberry species, such as *Fragaria* × *ananassa* (*9*), *Fragaria vesca* (*7*), and *Fragaria chiloensis* (*10*); finally, subgroup I was formed only by *Cm*-AAT4 from melon (*12*).

DNA Gel-Blot Analysis. To analyze the complexity of mountain papaya's AAT family, genomic analysis was performed using DNA gel blots. By using a probe that matches the 3′ end of the coding sequence of the *VpAAT1* gene (**Figure 1A**), a divergent region of AAT single-hybridization fragments were detected in DNA digested with *Hin*dIII, *Eco*RV, and *Eco*RI enzymes, all of them ranging between 2.3 and 9.4 kb. Only *Eco*RI has a restriction site at nucleotide 403 of the *VpAAT1* sequence. Analysis of *Bam*HI-digested DNA revealed two hybridizing bands around 9.4 kb and over 6.6 kb (**Figure 2**). Considering that no restriction sites for *Bam*HI are included in the coding sequence, its restriction pattern could suggest two possibilities according to the location

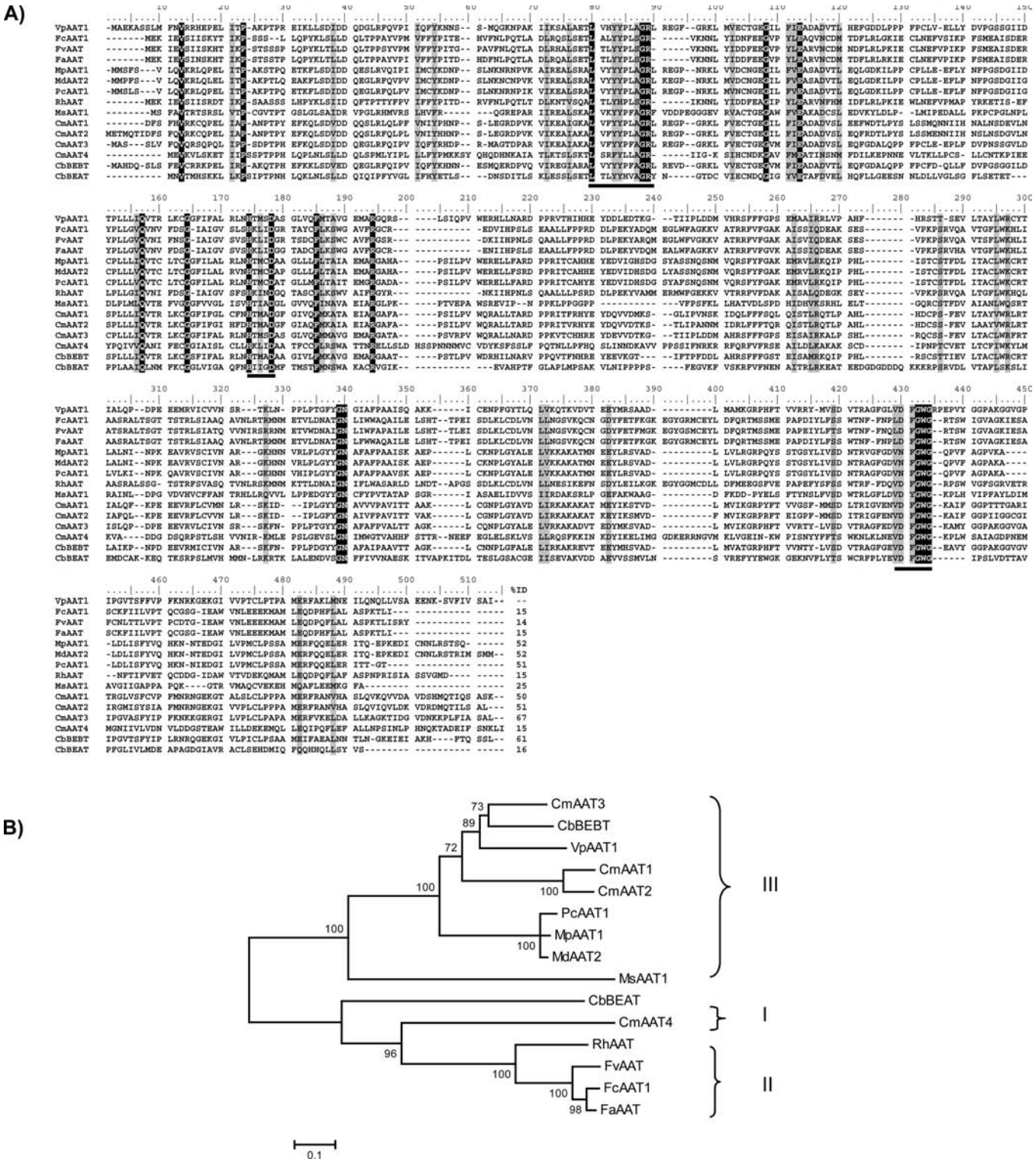

Figure 1. (**A**) Alignment of the deduced VpAAT1 sequence with other acyltransferases of known function from different floral and fruit species. Gaps are indicated by dashes, letters with black background are identical amino acids, and letters with gray background are similar amino acids. The three motifs that are characteristic of most AATs are underlined: LVHYYPLAGR, HTMSD (related to the catalytic activity and conserved within the BAHD acyltransferase family), and DFGWG (highly conserved within the BAHD protein family and apparently required for conformation integrity of the protein structure). Sequences correspond to GenBank data library accession numbers: *Cm* (*Cucumis melo*) AAT1 (CAA94432), AAT2 (AAL77060), AAT3 (AAW51125), AAT4 (AAW51126); Cb (*Clarkia breweri*) BEBT (AAN09796), BEAT (AAF04787); Fa (*Fragaria* × *ananassa*), AAT (AAG13130); Fc (*Fragaria chiloensis*) AAT1 (FJ548610); Fv (*Fragaria vesca*) AAT (AAN07090); Md (*Malus domestica*) AAT2 (AAS79797); Mp (*Malus pumila*) AAT1 (AAU14879); Ms (*Musa sapientum*) AAT1 (CAC09063); *Pc* (*Pyrus comunis*) AAT1 (AAS48090); Rh (*Rosa hybrida*) AAT (AAW31948); Vp (*Vasconcellea pubescens*) AAT1 (FJ548611). Sequences were aligned using Bioedit Sequence Alignment Editor v 7.0. At the end of alignment, the percentage of identity with *VpAAT1* is shown. (**B**) Phylogenic analysis of VpAAT1. The phylogenic tree was built using MEGA software (version 4). Numbers on branches indicate bootstrap values (as a percentage). Sequences are the same used in **A** (see above).

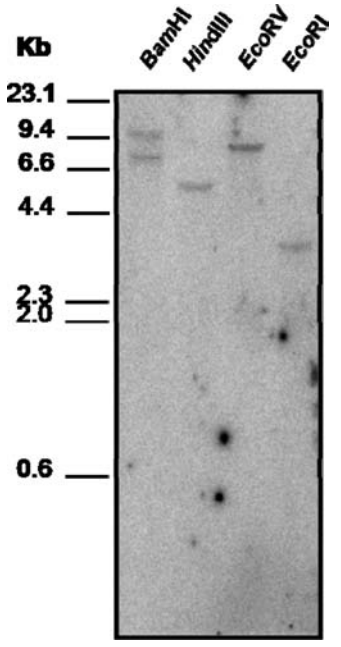

Figure 2. DNA gel-blot analysis of genomic DNA from mountain papaya. Genomic DNA (20 μg per lane) was digested with the indicated restriction enzyme and hybridized with the corresponding ^{32}P-labeled probe for *VpAAT1*. Hybridization and stringency conditions were as described under Materials and Methods.

of *Bam*HI polymorphism. The *Bam*HI restriction site could be located either in an intron of the *VpAAT1* gene or outside the gene. In both cases, this result demonstrates the heterogozity of the plant species and the presence of two different alleles of *VpAAT1* gene in the mountain papaya genome. With the aim to localize other AAT genes in mountain papaya, further experiments should be performed with a probe containing an AAT conserved region.

Transcriptional Analysis of *VpAAT1*. Mountain papaya fruit displayed the typical climacteric rise in ethylene production during ripening at 20 °C (**Figure 3A**). Treatment of the fruit with 1-MCP inhibited the ethylene production rise by the fruit during ripening, whereas ethylene treatment produces the advancement in time of the climacteric phase development. To estimate the level of *VpAAT1* transcript accumulation during ripening of mountain papaya fruit, qPCR analyses were performed (**Figure 3B**). In control fruit a notorious increment in the level of *VpAAT1* transcripts was observed during ripening, reaching the highest level at day 5 and decreasing after that. In 1-MCP-treated fruit *VpAAT1* transcripts remained almost constant after treatment, with levels similar to that of day 1. In samples treated with ethylene a clear increment in transcript accumulation was shown during the first days of storage, reaching the maximum level at day 3 and decreasing until the end of the storage period.

Accumulation of *VpAAT1* transcripts was analyzed in several vegetative tissues and fruits at different developmental stages. *VpAAT1* transcripts were detected only in fruit tissues, not being expressed in other vegetative tissues (**Figure 3C**). A very low transcript level was observed at the large green fruit stage compared to ripe fruit.

AAT Activity during Ripening of Mountain Papaya Fruit. AAT activity showed an increment during ripening of mountain papaya fruit (**Figure 4**), reaching the maximum level after 3−5 days of storage, followed by a small decline in AAT activity during the remaining days. AAT activity was assayed by its capacity to produce hexyl acetate, one of the main esters

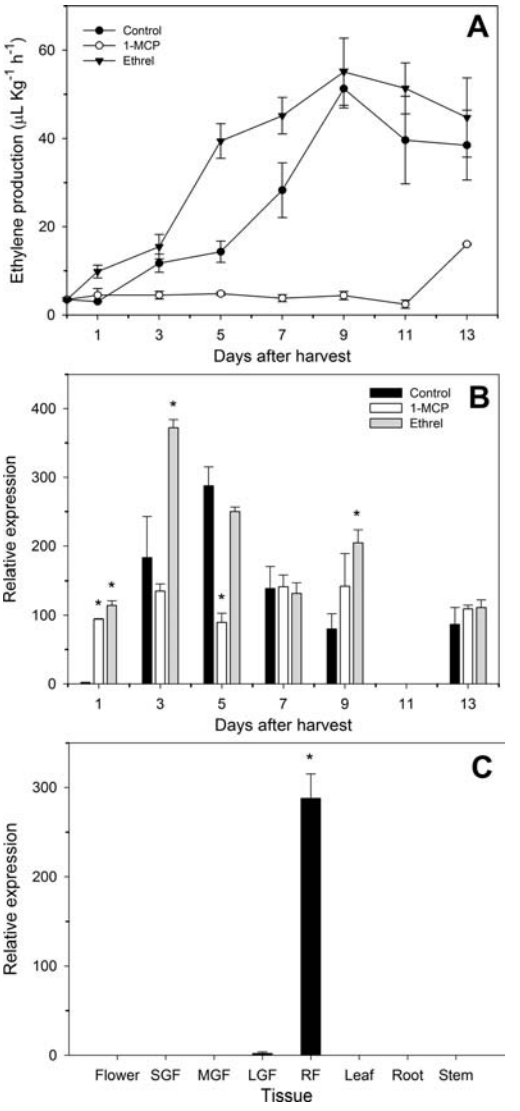

Figure 3. (**A**) Changes in ethylene production during ripening of mountain papaya fruit. Fruits harvested at the breaker stage were divided into three lots: one was kept untreated (control); another was treated with 1-MCP (0.3 μL L^{-1} for 16 h at 20 °C) on the same day of harvest; and the last one was treated with 2 g L^{-1} Ethrel. After the treatments, the fruit was allowed to ripen at 20 °C. Three replicates of four fruits chosen at random were analyzed every 2 days. The data correspond to the mean ± SE. (**B**) Changes in *VpAAT1* mRNA abundance during ripening of mountain papaya fruit measured by qPCR. Samples were collected after 1, 3, 5, 7, 9, and 13 days of storage at 20 °C. The expression data correspond to means of three replicates, normalized against *VpEF1*-α abundance, using a control sample from day 1 as calibrator, and expressed in arbitrary units ± SE. Asterisks indicate significant differences between control and treatments at the same storage day ($P \leq 0.05$). (**C**) *VpAAT1* mRNA abundance in vegetative tissues and fruit samples at different developmental stages. Expression analysis of *VpAAT1* by qPCR was performed in flower, small-size green fruit (SGF), medium-size green fruit (MGF), large-size green fruit (LGF), ripe fruit (RF, corresponding to control fruit on day 5), leaf, root, and stem. The expression data are means of three replicates, normalized to *VpEF1*-α abundance, using a control fruit sample from day 1 as calibrator, and expressed in arbitrary units ± SE. Asterisks indicate significant differences between tissues ($P \leq 0.05$).

produced by mountain papaya fruit. The same profile of AAT activity was observed when activity was assayed by its capacity to produce benzyl acetate (data not shown), an ester that is absent in

mountain papaya's aroma, although maximum activity recorded (3.2 pkat mg^{-1} of protein) was lower than for hexyl acetate.

Functionality of the *VpAAT1* Gene. With the aim to evaluate if VpAAT1 protein is functional, the full-length cDNA sequence was cloned and expressed in yeasts. Several transformants were analyzed, and the correct orientation of the insert was checked by means of PCR. The recombinant protein expressed in yeast cells (61.2 pkat mg^{-1} of protein) was partially purified (450 times) through an affinity column (BD Talon), obtaining an enzyme with high specific AAT activity (27548.1 pkat mg^{-1}) toward the formation of benzyl acetate. It is important to note that the ability to synthesize esters was notably diminished in cultures with $OD_{600} > 1$. The activity of the purified enzyme was also assayed in the presence of different combinations of acyl-CoAs and alcohols as substrates (**Table 1**). The results obtained indicate that the recombinant protein is able to synthesize a wide range of esters when the substrates are provided, including some characteristic aroma compounds from mountain papaya fruit. A marked preference was observed toward the formation of particular compounds: VpAAT1 displayed a strong activity toward the formation of benzyl acetate, followed by the ability to synthesize methyl hexanoate, geranyl acetate, and cinnamyl acetate. VpAAT1 showed a moderate activity toward the synthesis of phenylethyl acetate, ethyl hexanoate, and butyl butanoate, and a small activity toward the synthesis of ethyl butanoate, octyl acetate, butyl hexanoate, and hexyl acetate. No activity was registered for the formation of hexyl hexanoate (**Table 1**).

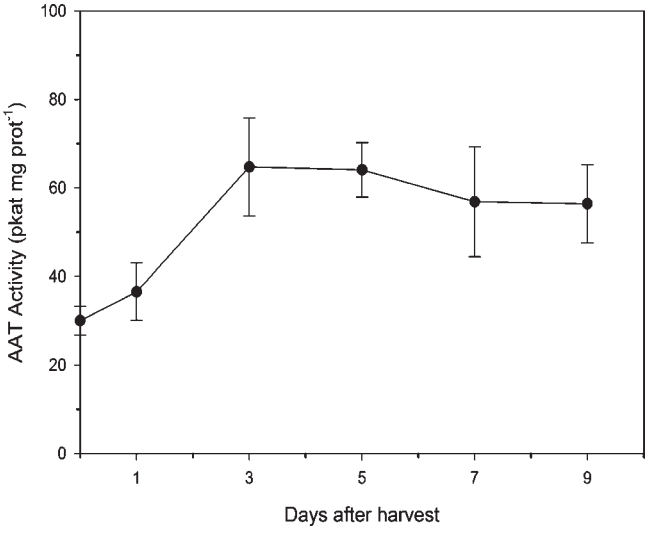

Figure 4. Change in AAT activity during ripening of mountain papaya fruit. Papaya fruit harvested at the breaker stage was allowed to ripen at 20 °C. Fruit extracts were prepared, and AAT activity was quantified by its ability to convert acetyl-CoA and hexanol into hexyl acetate. Data correspond to the mean ± SE of three replicates.

DISCUSSION

Mountain papaya is a climacteric fruit that develops an interesting and characteristic aroma during ripening, which is mainly due to esters (*4*). Most of the esters identified in mountain papaya fruit are modulated by ethylene, showing an increase in production during ripening and in response to ethylene treatment, and a strong reduction in response to 1-MCP treatment. Because esters are important for aroma, and a significant increase in ester production was observed during ripening of papaya fruit, our study was focused on ester biosynthesis by the AAT enzyme. AAT activity assayed by its capacity to produce hexyl acetate showed an important increment during ripening of mountain papaya fruit, with the maximum level of activity after 5 days of storage at 20 °C.

In addition, an AAT gene sequence was isolated from mountain papaya fruit (*VpAAT1*) that displays all of the conserved regions observed in other acyltransferases belonging to the BAHD superfamily. A large number of acyltransferases have been described in plants, with 61 and 91 putative members in *Arabidopsis* and *Populus*, respectively. In some fruit species, such as melon and apple, a small multigene family has been described (*12*−*14*). To determine the complexity of AAT genes present in mountain papaya's genome, DNA gel-blot analysis was performed. Our results suggest the existence of two different alleles of the *VpAAT1* gene in mountain papaya. Phylogenic analysis indicated that *VpAAT1* is close to other AAT genes belonging to subfamily III that participate in the synthesis of volatile compounds during fruit ripening, such as *CmAAT1*, *CmAAT3*, *PcAAT1*, *MpAAT1*, and *MdAAT2* (*12*). In addition, members of subfamily III have been described for their capacity to produce benzyl acetate (*28*). Interestingly, benzyl acetate has not been described in mountain papaya fruit aroma (*4*, *30*, *31*). According to our phylogenic analysis, the most similar sequences to *VpAAT1* are *CmAAT3* and *CbBEBT*, and all of them displayed the highest activity toward the synthesis of benzyl acetate (**Table 2**). CbBEBT and VpAAT1 displayed similar activity toward the synthesis of cinnamyl acetate (8.4 and 7.6%,

Table 1. Activity of VpAAT1 Recombinant Protein toward Different Acyl-CoAs and Alcohols as Substrates[a]

acyl-CoA	alcohol	ester produced	produced in papaya fruit?	AAT activity (pkat mg^{-1})
acetyl-CoA	benzyl alcohol	benzyl acetate	no	29275 ± 2593
	geranyl alcohol	geranyl acetate	no	3451 ± 1179
	cinnamyl alcohol	cinnamyl acetate	no	2219 ± 4.9
	phenylethanol	phenethyl acetate	no	805 ± 157
	octanol	octyl acetate	yes	60 ± 15.1
	hexanol	hexyl acetate	yes	20 ± 6.1
	butanol	butyl acetate	yes	1 ± 0.2
	ethanol	ethyl acetate	yes	tr
butanoyl-CoA	butanol	butyl butanoate	yes	526 ± 9.8
	ethanol	ethyl butanoate	yes	83 ± 14.7
hexanoyl-CoA	hexanol	hexyl hexanoate	no	nd
	butanol	butyl hexanoate	yes	50 ± 3.3
	ethanol	ethyl hexanoate	yes	790 ± 147
	methanol	methy hexanoate	yes	4668 ± 1737

[a] Calibration curves were prepared for each ester; R^2 values ranged between 1 and 0.93. Data correspond to mean ± SE. tr, traces; nd, nondetectable.

Table 2. Comparison of AAT Activities toward Different Acyl-CoAs and Alcohol Substrates between VpAAT1 and Closely Related AATs[a]

ester produced	Vp AAT1	Cb BEBT	*Cm* AAT3	Mp AAT1	*Cm* AAT1[b]	Ban AAT
benzyl acetate	100	100	100	nr	52	2
methy hexanoate	16	nr	nr	nr	nr	nr
geranyl acetate	12	46	tr	nr	tr	88
cinnamyl acetate	8	8	tr	nr	4	100
phenethyl acetate	3	nr	nd	nr	nd	nr
ethyl hexanoate	3	nr	0	nr	1	nr
butyl butanoate	2	nr	nr	nd	nr	nr
octyl acetate	0	71	nd	nr	5	44
hexyl acetate	0	33	1	100	50	nr
butyl acetate	0	36	3	5	34	1
ethyl acetate	tr	nd	nd	nr	nd	0
ethyl butanoate	0	nr	1	nr	3	nr
hexyl hexanoate	nd	nr	1	85	90	nr
butyl hexanoate	0	nr	2	nd	4	nr

[a] The AAT activity of VpAAT1 recombinant protein was assayed by combining a set of three acyl-CoAs (acetyl-, butanoyl-, and hexanoyl-CoA) and nine different alcohols as substrates (ethanol, methanol, butanol, hexanol, octanol, benzyl alcohol, geranyl alcohol, phenylethanol, and cinnamyl alcohol). Some combinations were not quantified. Activity values for related AATs were obtained from the literature (*7*, *11*−*13*, *29*) and expressed as percentage of the maximum activity recorded for each enzyme. tr, traces; nd, nondetected; nr, not reported. [b] 100% activity of CmAAT1 is not mentioned as it corresponds to the production of *Z*-hexenyl acetate, which was not proved in VpAAT1.

respectively). On the other hand, CmAAT3 and VpAAT1 shared a low activity toward hexyl acetate formation (0.5 and 0.07%, respectively). This analysis could support the hypothesis that members from subfamily III share structural and functional characteristics.

The transcript accumulation pattern provided by qPCR analysis showed that the *VpAAT1* gene is expressed exclusively in fruit tissues and that a high level of transcripts are accumulated during ripening of mountain papaya fruit. The increase in *VpAAT1* transcripts is coincident with the increase in AAT activity during ripening of the fruit. On the other hand, transcript accumulation of *VpAAT1* is induced by ethylene, and its induction is avoided by 1-MCP treatment. The data suggest that the expression of the *VpAAT1* gene is modulated by ethylene, as has been reported for other acyltransferases involved in the synthesis of plant volatiles (*32*). Nevertheless, other regulatory mechanisms besides ethylene could not be discarded (*33*). The specific transcript accumulation of *VpAAT1* in fruit tissue has also been reported previously in other fruit species such as melon (*11*,*12*,*34*). Nevertheless, in apple the *MpAAT1* gene is expressed not only in ripening fruits but also in flowers and developing fruits (*13*). On the other hand, genes involved in aroma biosynthesis of floral species such as *C. breweri* (*28*) and *Rosa hybrida* (*29*) are expressed in their floral organs.

The functionality of VpAAT1 recombinant protein was proved, showing that it was able to use different alcohols and acyl-CoAs as substrates for the synthesis of esters. The assays performed with partially purified enzyme showed a strong activity of the enzyme toward the production of benzyl acetate, which is one of the most powerful odorant compounds used as fragrance in perfumes and foods (*35*), and it is the main ester produced by AATs belonging to subfamily III (*12*). We also found a high activity of the recombinant protein toward the formation of esters that are not normally produced by mountain papaya fruit, such as phenethyl acetate and benzyl acetate (**Table 1**). Similar findings have been reported for the formation of esters in other fruits, regarding the formation of *E*-2 hexenyl acetate, 2-ethylbutanoate, and 3-methylbutyl, geranyl, and cinnamyl acetate in melon (*12*) and geranyl, furfuryl, 2-phenylethyl, and benzyl acetate in apple (*13*). Similarly, SAAT and BanAAT1 displayed a strong preference for the synthesis of geranyl and cinnamyl acetate, respectively, which have not been described in strawberry or banana fruits (*7*). All together, this could indicate that the volatiles produced by a fruit are determined also by substrate availability and not only AAT selectivity, as has been proposed previously (*36*−*39*), albeit AAT purportedly is the rate-limiting step in ester biosynthesis (*32*).

VpAAT1 protein expressed in yeast was able to produce a wide range of esters, including those normally produced by papaya fruits and some with high aroma impact. This supports the hypothesis that VpAAT1 is involved in aroma biosynthesis in papaya fruit. However, the activity observed toward the formation of these compounds was considerably lower than that toward the synthesis of benzyl acetate, geranyl acetate, cinnamyl acetate, and methyl hexanoate. The reduced activity of VpAAT1 toward the synthesis of butyl acetate and ethyl acetate, compounds that are profusely produced in ripening papaya fruit, most probably indicates that other AAT genes, which have not been described yet, are present in the species.

In conclusion, our study strongly suggests that the *VpAAT1* gene is functional and is involved in the synthesis of esters, which are important contributors to the aroma of papaya fruit. The increase in *VpAAT1* transcripts in fruit tissue during ripening of papaya fruit is coincident with the increment in AAT activity and ester production. Finally, ethylene plays a major role modulating the expression of *VpAAT1* gene and promoting the increment in AAT activity.

LITERATURE CITED

(1) Badillo, V. M. *Carica* L. vs. Vasconcella St. Hil. (Caricaceae): con la rehabilitación de este último. *Ernstia* **2000**, *10*, 74–79.

(2) Van Droogenbroeck, B.; Breyne, P.; Goetghebeur, P.; Romeijn-Peeters, E.; Kyndt, T.; Gheysen, G. AFLP analysis of genetic relationships among papaya and its wild relatives (Caricaceae) from Ecuador. *Theor. Appl. Genet*. **2002**, *105*, 289–297.

(3) Moya-León, M. A.; Moya, M.; Herrera, R. Ripening of the Chilean papaya fruit (*Vasconcellea pubescens*) and ethylene dependence of some ripening events. *Postharvest Biol. Technol*. **2004**, *34*, 211–218.

(4) Balbontín, C.; Gaete-Eastman, C.; Vergara, M.; Herrera, R.; Moya-León, M. A. Treatment with 1-MCP and the role of ethylene in aroma development of mountain papaya fruit. *Postharvest Biol. Technol*. **2007**, *43*, 67–77.

(5) Gaete-Eastman, C.; Figueroa, C. R.; Balbontín, C.; Moya, M.; Atkinson, R. G.; Herrera, R.; Moya-León, M. A. Expression of an ethylene-related expansin gene during softening of mountain papaya fruit (*Vasconcellea pubescens*). *Postharvest Biol. Technol*. **2009**, *53*, 58–65.

(6) Blankenship, S. M.; Dole, J. M. 1-Methylcyclopropene: a review. *Postharvest Biol. Technol*. **2003**, *28*, 1–25.

(7) Beekwilder, J.; Alvarez-Huerta, M.; Neef, E.; Verstappen, F. W. A.; Bouwmeester, H. J.; Aharoni, A. Functional characterization of enzymes forming volatile esters from strawberry and banana. *Plant Physiol.* **2004**, *135*, 1865–1878.
(8) Yu, X.-H.; Gou, J.-Y.; Liu, C.-J. BAHD superfamily of acyl-CoA dependent acyltransferases in *Populus* and *Arabidopsis*: bioinformatics and gene expression. *Plant Mol Biol.* **2009**, *70*, 421–442.
(9) Aharoni, A.; Keizer, L. C. P.; Bouwmeester, H. J.; Sun, Z.; Alvarez-Huerta, M.; Verhoeven, H. A.; Blaas, 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.; O'Connell, A. P. Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. *Plant Cell* **2000**, *12*, 647–662.
(10) González, M.; Gaete-Eastman, C.; Valdenegro, M.; Figueroa, C. R.; Fuentes, L.; Herrera, R.; Moya-León, M. A. Aroma development during ripening of *F. chiloensis* fruit and participation of an alcohol acyltransferase (FcAAT1) gene. *J. Agric. Food Chem.* **2009**, *57*, 9123–9132.
(11) Yahyaoui, F. E.; Wongs-Aree, C.; Latche, A.; Hackett, R.; Grierson, D.; Pech, J. C. 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.* **2002**, *269*, 2359–2366.
(12) El-Sharkawy, I.; Manriquez, D.; Flores, F. B.; Regad, F.; Bouzayen, M.; Latche, A.; Pech, J. C. 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. *Plant Mol. Biol.* **2005**, *59*, 345–362.
(13) Souleyre, E. J.; Greenwood, D. R.; Friel, E. N.; Karunairetnam, S.; Newcomb, R. D. An alcohol acyltransferase from apple (cv. Royal Gala), MpAAT1, produces esters involved in apple fruit flavor. *FEBS J.* **2005**, *272*, 3132–3144.
(14) Li, D.; Xu, Y.; Xu, G.; Gu, L.; Li, D.; Shu, H. Molecular cloning and expression of a gene encoding alcohol acyltransferase (MdAAT2) from apple (cv. Golden Delicious). *Phytochemistry* **2006**, *67*, 658–667.
(15) Wang, J.; De Luca, V. The biosynthesis and regulation of biosynthesis of Concord grape fruit esters, including 'foxy' methyl anthranilate. *Plant J.* **2005**, *44*, 606–619.
(16) González-Aguero, M.; Troncoso, S.; Gudenschwager, O.; Campos-Vargas, R.; Moya-León, M. A.; Defilippi, B. G. Differential expression levels of aroma-related genes during ripening of apricot (*Prunus armeniaca* L.). *Plant Physiol. Biochem.* **2009**, *47*, 435–440.
(17) D'Auria, J. C. Acyltransferases in plants: a good time to be BAHD. *Curr. Opin. Plant Biol.* **2006**, *9*, 331–340.
(18) Bayer, A.; Ma, X. Y.; Stockigt, J. Acetyltransfer in natural product biosynthesis − functional cloning and molecular analysis of vinorine synthase. *Bioorg. Med. Chem.* **2004**, *12*, 2787–2795.
(19) Ma, X.; Koepke, J.; Panjikar, S.; Fritzch, G.; Stöckigt, J. Crystal structure of vinorine synthase, the first representative of the BAHD superfamily. *J. Biol. Chem.* **2005**, *280*, 13576–13583.
(20) Chang, S.; Puryear, J.; Cairney, J. A simple and efficient method for isolating RNA from pines trees. *Plant Mol. Biol. Rep.* **1993**, *11*, 113–116.
(21) Horton, P.; Park, K.-J.; Obayashi, T.; Fujita, N.; Harada, H.; Adams-Collier, C. J.; Nakai, K. WoLF PSORT: protein localization predictor. *Nucleic Acids Res.* **2007**, *35*, W585–W587.
(22) Hall, T. A. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* **1999**, *41*, 95–98.
(23) Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596–1599.
(24) Murray, M. G.; Thompson, W. F. Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res.* **1980**, *8*, 4321–4326.
(25) Pfaffl, M. W. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **2001**, *29*, e45.
(26) Bradford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein−dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.
(27) Fellman, J. K.; Mattinson, D. S.; Bostick, B. C.; Mattheis, J. P.; Patterson, M. E. Ester biosynthesis in 'Rome' apples subjected to low oxygen atmospheres. *Postharvest Biol. Technol.* **1993**, *3*, 201–214.
(28) D'Auria, J.; Chen, F.; Pichersky, E. Characterization of an acyltransferase capable of synthesizing benzylbenzoate and other volatile esters in flowers and damaged leaves of *Clarkia breweri*. *Plant Physiol.* **2002**, *130*, 466–476.
(29) Shalit, M.; Guterman, I.; Volpin, H.; Bar, E.; Tamari, T.; Menda, N.; Adam, Z.; Zamir, D.; Vainstein, A.; Weiss, D.; Pichersky, E.; Lewinsohn, E. Volatile ester formation in roses. Identification of an acetyl-coenzyme A geraniol/citronellol acetyl transferase in developing rose petals. *Plant Physiol.* **2003**, *131*, 1868–1876.
(30) Morales, A. L.; Duque, C. Aroma constituents of the fruit of the mountain papaya (*Carica pubescens*) from Colombia. *J. Agric. Food Chem.* **1987**, *35*, 538–540.
(31) Idstein, H.; Keller, T.; Schreier, P. Volatile constituents of mountain papaya (*Carica candamarcensis*, syn. *C. pubescens* Lenne et Koch) fruit. *Agric. Food Chem.* **1985**, *33*, 666–672.
(32) Defilippi, B. G.; Kader, A. A.; Dandekar, A. M. Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. *Plant Sci.* **2005**, *168*, 1199–1210.
(33) Zhu, Y.; Rudell, D. R.; Mattheis, J. P. Characterization of cultivar differences in alcohol acyltransferase and 1 aminocyclopropane-1-carboxylate synthase gene expression and volatile ester emission during apple fruit maturation and ripening. *Postharvest Biol. Technol.* **2008**, *49*, 330–339.
(34) Aggelis, A.; John, I.; Karvouny, Z.; Grierson, D. Characterization of two cDNA clones for mRNAs expressed during ripening of melon (*Cucumis melo* L.) fruits. *Plant Mol. Biol.* **1997**, *33*, 313–322.
(35) Bauer, K.; Garbe, D.; Surburg, H. *Common Fragrance and Flavor Materials*, 2nd ed.; VCH Publishers: Cambridge, U.K., 1990; pp 9, 24.
(36) Tressl, R.; Drawert, F. Biogenesis of banana volatiles. *J. Agric. Food Chem.* **1973**, *21*, 560–565.
(37) Knee, M.; Hatfield, S. G. S. The metabolism of alcohols by apple fruit tissue. *J. Sci. Food Agric.* **1981**, *32*, 593–600.
(38) De Pooter, H. L.; Montens, J. P.; Willaert, G. A.; Dirinck, P. J.; Schamp, N. M. Treatment of Golden Delicious apples with aldehydes and carboxylic acids: effect on the headspace composition. *J. Agric. Food Chem.* **1983**, *31*, 813–818.
(39) Jayanty, S.; Song, J.; Rubinstein, N. M.; Chong, A; Beaudry, R. M. Temporal relationship between ester biosynthesis and ripening events in bananas. *J. Am. Soc. Hortic. Sci.* **2002**, *127*, 998–1005.

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