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Aroma Development during Ripening of *Fragaria chiloensis* Fruit and Participation of an Alcohol Acyltransferase (FcAAT1) Gene

Miriam González, Carlos Gaete-Eastman, Mónika Valdenegro, Carlos R. Figueroa, Lida Fuentes, Raúl Herrera, and María Alejandra Moya-León*

Laboratorio de Fisiología Vegetal y Genética Molecular, Instituto de Biología Vegetal y Biotecnología, Universidad de Talca, Casilla 747, Talca, Chile

Fragaria chiloensis is characterized for having great aroma and flavor properties. Using headspace-SPME different volatile compounds were identified and quantified during development and ripening of the fruit. The headspace was dominated by esters, butyl acetate, ethyl acetate, ethyl butanoate and ethyl hexanoate being the most abundant in fully ripe fruit. As esters are important for aroma and synthesized through alcohol acyltransferases (AAT), a full-length cDNA (*FcAAT1*) was isolated from *F. chiloensis* fruit which displayed the three motifs characteristic of most AATs. As the production of esters increased during ripening, a clear increment in *FcAAT1* transcripts was observed in fruit tissue. A good correlation was found between AAT activity and the total content of esters, especially with acetates and hexanoates. Aroma-related esters displayed during ripening the same production profile as AAT activity. Therefore it can be suggested that the *FcAAT1* gene may have a significant role in ester production of *F. chiloensis* fruit.

KEYWORDS: Alcohol acyltransferase; aroma; esters; *Fragaria chiloensis*; fruit ripening; Chilean strawberry

INTRODUCTION

Fragaria chiloensis L. (Duch.), the native Chilean strawberry, is considered to have potential to become a new exotic fruit species that could add to the diversity of berry fruits that Chile can export. Although populations of *F. chiloensis* have been used since 1000 years ago by the indigenous "Mapuches" in the central-south part of Chile (*1*), with the introduction of modern commercial cultivars of strawberry (*Fragaria* × *ananassa* Duch.) the improvement of *F. chiloensis* was not continued. Nowadays, *F.* × *ananassa* is the strawberry species grown worldwide, developed after the fortuitous cross of two wild octoploid species, *Fragaria virginiana* Duch. and *F. chiloensis* (*1*). *F. chiloensis*, the mother of the commercial strawberry, is characterized for having great aroma and flavor properties, a remarkable pest and disease resistance spectrum, resistance to salinity and drought and low nutrient needs, along with other characteristics that make it an attractive species for plant research (*2*). Indeed, research on this species has been focused on its development as a new exotic berry fruit, as well as for the genetic improvement of *F.* × *ananassa* (*2*).

Among the many quality parameters defining the desirability of fruit and vegetables, there are important nonvisual characteristics which influence the final acceptance by consumers, such as texture, nutritional value, flavor and aroma (*3*). In strawberry, appearance (color, size, form, absence of damage), firmness, flavor (solid soluble, titratable acidity, aromatic compounds) and nutritional value (vitamins and antioxidant content) define fruit quality (*4*). Aroma is an important trait to consider in a strawberry breeding program if the goal is to improve fruit quality. Fruit aroma is determined by a large number of volatile compounds, and their production is dependent on many factors, such as cultivar, maturity, and storage conditions (*5*). The molecular mechanism by which fruit flavor and aroma compounds are gained and lost during evolution and domestication are largely unknown.

Commercial strawberries produce numerous volatile compounds including esters, aldehydes, ketones, alcohols, terpenes, furanones, and sulfur compounds (*6*, *7*). Esters are quantitatively and qualitatively the most abundant class of compounds, comprising from 25% to 90% of the total volatiles in fresh ripe fruit (*8*). Esters provide the fruity and floral notes of fresh ripe fruit while the sweet fragrance is determined by furanones (*9*−*11*). Among the hundreds of volatile compounds produced by a strawberry fruit, only a small portion contribute to its aroma and flavor (*5*). Compounds like ethyl butanoate, Furaneol and ethyl hexanoate contribute importantly to the aroma of *F.* × *ananassa* fruit, in addition to methyl butanoate, linalool, 2-heptanone and 2-methyl butanoate (*12*). To date, there is scarce information regarding the aroma compounds produced by *F. chiloensis* fruit (*13*).

Volatile content increases rapidly as commercial strawberry fruit ripens (*8*, *14*, *15*). There are differences in the production of

*Corresponding author. Tel: + 56 71 200280. Fax: + 56 71 200276. E-mail: alemoya@utalca.cl.

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volatiles among strawberry cultivars, both quantitative and qualitative; although the chemical composition of volatiles is dominated by methyl and ethyl esters, the abundance of each ester varied within cultivars (*5*). On the other hand, diverse aroma patterns are observed between cultivated and wild type species, the wild species being those with the highest concentration of volatiles and better aroma properties, therefore the flavor quality also differs significantly between them (*16*). Reports indicate that the aroma profile of *F. chiloensis* fruit differs from that of *F.* × *ananassa* (*13*).

Esters are generated by esterification reactions between alcohols and acyl-CoAs, derived from both fatty acid and amino acid metabolism, in a reaction catalyzed by the enzyme alcohol acyltransferase (AAT; EC 2.3.1.84) (*17*, *18*). AATs from strawberry fruit have been characterized at the biochemical and molecular level, and differences in substrate specificity between different genotypes have been demonstrated (*19*−*22*). There is an increase in AAT activity along the ripening of strawberry fruit (*20*). Cultivars with high AAT activity resulted in higher ester production and subsequently in fruits with enhanced aroma (*20*). In general terms, breeding programs have historically focused on yield, fruit size, shape, shelf life characteristics and disease resistance, having unintended negative consequences on strawberry flavor and aroma (*6*). Nevertheless, postharvest characteristics, like flavor and aroma, are nowadays taken into consideration in breeding programs; however, as flavor is a complex and multigenic trait, it provides a unique challenge to breeders.

In this work, we report the profile of volatile compounds of *F. chiloensis* fruit and their evolution during fruit development and ripening. Emphasis was placed on ester biosynthesis, considering that these are key aroma compounds. An AAT gene was isolated (*FcAAT1*) and characterized; its expression was analyzed and discussed in relation to the production of esters by the fruit. As far as we know this is the first report that establishes a relationship between ester formation and AAT gene expression during ripening of the Chilean strawberry fruit.

MATERIALS AND METHODS

Plant Material. Chilean strawberries (*F. chiloensis* spp. *chiloensis*) were cultivated in a commercial field located at Contulmo, Biobío Region, Chile (latitude 38° 04′ 8.6″ S; longitude 73° 14.2′ 96″ W). The plants correspond to the Contulmo accession of *F. chiloensis* and represented macro propagated material done by farmers. *F. chiloensis* fruit from different plants were harvested on the 10th of January 2008 and transported immediately to the University of Talca. Uniform fruit and without defects was classified into four ripening stages according to Figueroa et al. (*23*): C1, small fruit with green receptacle and green achenes; C2, large fruit with green receptacle and red achenes; C3, turning stage, white receptacle and red achenes; and C4, ripe fruit with pink receptacle and red achenes. Three replicates for each ripening stage were employed on quality assessments. Other plant tissues were collected from the same plants (runners, leaves, flowers and stems), which were frozen under liquid nitrogen and stored at −80 °C until use.

Sampling and Analysis of Volatile Compounds by Headspace-SPME. Fruit from each ripening stage was analyzed for weight, length and diameter. Three replicates of approximately 30 g each (4 intact fruits for C1 stage; 3 for stage C2; and 2 for C3 and C4 stages) were employed for volatile analysis. Each replicate was introduced into a tightly closed container (400 mL) fitted with a rubber septum and incubated at 20 °C for 2 h prior to SPME (solid phase microextraction) sampling. An SPME portable field sampler of 65 μm film thickness of polydimethylsiloxane/divinylbenzene (PDMS/DVB) on a Stableflex fiber (Supelco, USA) was inserted into the chamber through the septum, and exposed to the headspace for 30 min. Volatile compounds adsorbed by partition on the SPME fiber were thermally desorbed in the injector port of the GC at 220 °C (split ratio 6.4). A Perkin-Elmer Clarus 500 gas chromatograph equipped with a SPB-5 capillary column (Supelco, 30 m × 0.25 mm i.d., 0.25 μm film thickness) and a flame ionization detector (FID) was used (*24*). Injector and detector temperatures were 200 and 250 °C, respectively. The oven temperature was held at 50 °C for 2 min, and then raised to 220 at 10 °C min^{-1} and held for 2 min, using helium as a carrier gas (50.3 cm s^{-1}). Quantification of compounds was based on GC-FID peak integration data and by constructing calibration curves with standards under our experimental conditions, from which response factors were obtained. Known amounts of 1,2-dichlorobenzene (0.5 μL) were added to the chambers as internal standard to check the adsorption capacity of each fiber, and results were expressed as pg g FW^{-1}.

GC−MS analyses were performed on a Perkin-Elmer Autosystem XL/Turbo Mass 4.1 gas chromatograph, fitted with a MDN-5 column (Supelco, 30 m × 0.25 mm i.d., 0.25 μm film thickness) (*24*). Temperature programming was the same as for GC-FID chromatogram; injector temperature was 200 °C; transfer line, 300 °C; and helium as carrier gas (40.2 cm s^{-1}). Mass spectra were obtained by electron ionization at 70 eV with a spectral range of 40−250 m/z. Compound identification was carried out by matching their mass spectra with those of reference compounds stored under the NIST mass spectra library, and whenever it was possible MS identifications were confirmed by comparing their retention times (t_R) with authentic reference compounds obtained from Sigma, Aldrich or Fluka. A linear retention index (LRI) was calculated for each compound using the retention times of a homologous series of C9−C22 *n*-alkanes analyzed under the same conditions.

Fruit Quality Assessment. Firmness was measured using the Firm Tech II (BioWorks Inc., USA) provided with a flat tip of 2 cm. Two measurements on each equatorial side were performed on each fruit. The mean of each replicate (18 fruits per stage) was recorded and expressed as newtons (N) ± standard error (SE).

After firmness measurement the peduncle and calyx of each fruit were removed, and the fruit was cut into pieces, frozen under liquid nitrogen and stored at −80 °C until use. Two grams of fruit tissue from each replicate was homogenized in water in a Waring blender and adjusted to 25 mL final volume. The mixture was filtrated through miracloth, and the juice was analyzed for soluble solids concentration (SSC), pH, and titratable acidity (TA). SSC was determined at 20 °C using a hand-held temperature compensated refractometer (Atago Co., Tokyo, Japan), and expressed as g g^{-1} of fresh weight. The pH of the juice was recorded with a pH meter (Pasco Scientific, PS-2117). TA was determined by titration of an aliquot of 5 mL of strawberry juice with 20 mM NaOH until reaching pH 8.2 and expressed as mequiv of citric acid g FW^{-1}.

Assay of AAT Activity. Frozen fruit 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 0.1 M Tris-HCl buffer (pH 8) containing 1 M KCl, 0.1% (v/v) Triton X-100. The mixture was stirred for 20 min at 4 °C, filtrated through miracloth and centrifuged (11000*g* for 20 min). The supernatant was desalted through a Sephadex G-25 gel filtration column (PD-10 Pharmacia) in the presence of buffer 50 mM Tris-HCl (pH 7.5) containing 10% (v/v) glycerol and 0.5 mM DTT.

AAT activity was quantified by its ability to convert butanol and acetyl-CoA into butyl acetate (*25*). The reaction was performed in 500 μL total volume in the presence of 10 mM butanol, 490 μM acetyl-CoA, 50 mM Tris-HCl (pH 7.5) buffer containing 10% (v/v) glycerol. The reaction was initiated by the addition of 300 μL of protein extract and the mixture incubated at 30 °C for 2 h. 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 1,2-dichlorobenzene as internal standard (10 μL of a 1/5000 dilution in pentane). 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 butyl acetate were done by GC-FID as described before. A calibration curve with butyl acetate was prepared. AAT enzyme activity was expressed as nmol of butyl acetate produced per h and per μg of protein. Protein content was determined (*26*) using BSA as standard. Determinations were performed in triplicate.

Isolation of AAT Gene from *F. chiloensis*. Total RNA (1 μg) was extracted from a ripe fruit according to Chang et al. (*27*), then treated with DNase I amplification grade (Invitrogen), and cDNA synthesized using the BD SMART PCR (Clontech) kit according to the manufacturer's instructions. Primers for the isolation of alcohol acyl transferase cDNA sequence from *F. chiloensis* were designed using the *F.* × *ananassa* AAT sequence (GenBank accession number AF193789). Primer sequences were AATF = 5′-AAGTCCTGGGGTGCTGTTT-3′ and AAT R = 5′ CCCTCTTTACCCTTGAAAGTCTC-3′. The PCR product was cloned onto pSCA using the StrataClone PCR Cloning kit according to the manufacturer's instructions (Stratagene, La Jolla, CA) and sequenced at Macrogen Inc. (Seoul, Korea). In order to complete the gene sequence, specific internal primers were designed for 5′ and 3′RACE-PCR based on the sequence obtained (AAT-RACE-5′ = 5′-AACGCTCATCAGA-TATTGCTTCCA-3′ and AAT-RACE-3′ = 5′-GGATGGGGGAGGA-CATCATGGATT-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 as described above.

The nucleotide and deduced amino acid sequences were analyzed using Vector NTI Advance v10 software (Invitrogen, 2007). The similarity search and signal peptide predictions were 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, respectively (*28*). The multialignment of amino acid sequences was performed using BioEdit Sequence Alignment Editor v7.0 software (*29*). The phylogenetic tree was built using MEGA software (version 4; http://www.megasoftware.net) (*30*) using the neighbor-joining method and Bootstrap analysis (1000 replicates).

Genomic DNA Isolation and DNA Gel-Blot Analysis. Chilean strawberry genomic DNA was extracted from a pool of young leaves (2 g) as described by Murray and Thompson (*31*). For DNA gel blots, 20 μg aliquots of genomic DNA from Chilean strawberry were digested with the restriction enzymes *Bam*HI, *Hin*dIII, *Eco*RV and *Eco*RI, fractioned on a 0.7% agarose gel, and transferred to Hybond-N^+ membranes (Amersham Biosciences, U.K.) using 20× SSC as blotting buffer. Membranes were prehybridized at 42 °C for 4 h in a solution containing 50% deionized formamide, 1% 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 in SSC, containing 0.1% (w/v) SDS: 2× SSC for 15 min at 42 °C and three times for 15 min at 50 °C with 1× SSC. The blots were exposed, and autoradiograms were scanned in a densitometer (FLA-5100 Imaging System, Fujifilm, Japan).

Probe for DNA gel-blot analysis of *FcAAT1* was designed from a 148 bp region of the gene (nt 1287−1434), consistent in a 3′ end portion of 101 bp plus a 3′-UTR sequence of 47 bp. The probe was prepared through PCR reactions with the specific primers qAAT-F/qAAT-R (described in qPCR section) and radiolabeled using [α-^{32}P]dCTP (Easytides, NEN Life Sciences Products, USA).

Expression Analysis by Real Time PCR (qPCR). Total RNA was extracted from 8 g of *F. chiloensis* fruit bulk prepared for each developmental stage and other vegetative tissues. It was treated with DNase I amplification grade (Invitrogen), and cleaned using an RNeasy Plant Mini Kit (Qiagen). 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 fruit stage were used. Specific primers for the UTR-3′ region of *FcAAT1* and the glyceraldehyde 3-phosphate dehydrogenase (Fc*GAPDH1*; as internal control) genes were designed using Vector NTI v10, with high stringency to avoid amplification of unspecific PCR products and secondary structure. Primers were tested by RT-PCR and the amplification products were sequenced in order to check product identity. Primer pair sequences were as follows: qAAT-F (5′- CTGGAATT-GAAGCGTGGGTGAATC-3′), qAAT-R (5′-AAATCGAAACATTG-CACGAGCCAC 3′), GAPDH1-F (5′-TCCATCACTGCCACCCA-GAAGACTG-3′) and GAPDH1-R (5′- AGCAGGCAGAACCTTTCC-GACAG-3′).

The amplicon sizes were 149 bp for the *FcAAT1* gene and 96 bp for *FcGAPDH1*. The amplification reactions were performed using Brilliant SYBR Green QPCR Master Mix (Stratagene, La Jolla, CA) 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

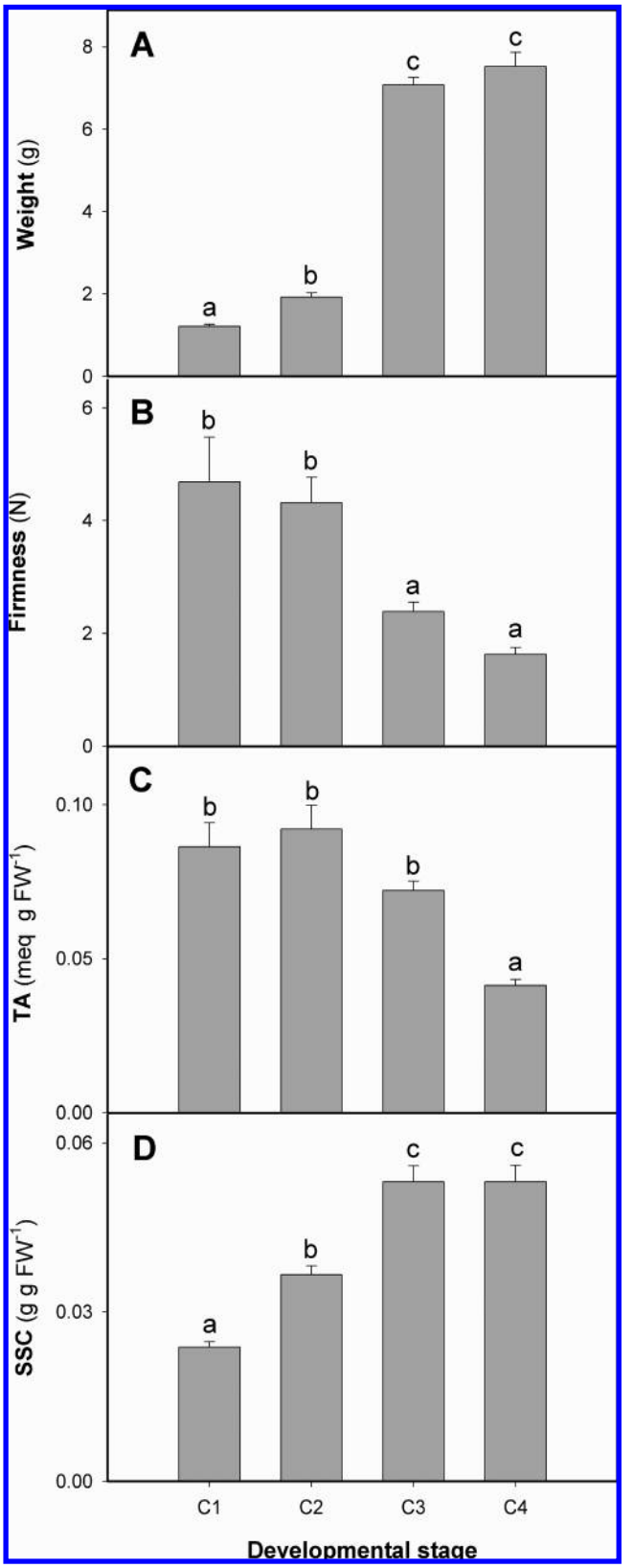

Figure 1. Changes in weight (**A**), firmness (**B**), total titratable acidity (TA) (**C**) and soluble solid content (SSC) (**D**) during the development of *F. chiloensis* fruit. Data corresponded to the mean ± SE. Different letters indicate differences between stages (P = 0.05).

Table 1. Abundance of Main Volatile Compounds (pg g^{-1}) Produced by *Fragaria chiloensis* Fruit at Different Ripening Stages

					ripening stage			
compound[a]		t_R[b]	LRI[c]	MW	C1	C2	C3	C4
esters								
ethyl acetate [d]	ea	1.60		88.1	4.13 ± 2.11	21.68 ± 7.95	41.09 ± 4.09	52.26 ± 0.01
ethyl butanoate [d]	eb	3.33		116.1	0.00 ± 0.00	2.11 ± 0.66	1.78 ± 0.07	2.45 ± 0.00
butyl acetate [d,e]	ba	3.54		116.0	4.47 ± 0.35	38.64 ± 10.07	130.98 ± 11.25	165.65 ± 11.92
ethyl 2-methyl butanoate [d]	e2mb	4.34		130.0	0.90 ± 0.06	1.53 ± 0.06	0.42 ± 0.05	0.44 ± 0.00
methyl hexanoate [d,e]	mhx	5.43	913	130.9	0.57 ± 0.15	1.13 ± 0.28	0.83 ± 0.24	0.95 ± 0.25
ethyl hexanoate [d,e]	ehx	6.50	976	144.0	0.40 ± 0.11	0.57 ± 0.45	0.64 ± 0.05	1.60 ± 0.44
hexenyl acetate [d,e]	hxna	6.80	993	142.1	0.85 ± 0.31	1.64 ± 0.62	0.05 ± 0.01	0.06 ± 0.04
hexyl acetate [d,e]	hxa	7.07	1008	144.0	3.24 ± 0.30	2.64 ± 0.69	0.55 ± 0.04	0.46 ± 0.13
ethyl heptanoate [d]	ehp	8.60	1092	158.2	0.10 ± 0.03	0.17 ± 0.08	0.09 ± 0.01	0.06 ± 0.04
hexyl propanoate [f]	hxpr	8.64	1094	158.0	0.45 ± 0.16	0.43 ± 0.27	0.07 ± 0.01	0.02 ± 0.01
hexyl butanoate [f]	hxb	9.54	1148	172.1	0.02 ± 0.01	0.04 ± 0.02	0.01 ± 0.00	0.03 ± 0.01
2-hexenyl butanoate [f]	2hxnb	9.92	1171	170.0	0.02 ± 0.01	0.03 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
benzyl acetate [d]	bna	10.34	1196	150.2	0.07 ± 0.02	0.05 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
octyl acetate [d,e]	oa	10.45	1203	172.0	0.07 ± 0.02	0.10 ± 0.02	0.11 ± 0.01	0.10 ± 0.02
hexyl 2-methyl butanoate [f]	hx2mb	10.71	1221	186.3	0.01 ± 0.00	0.03 ± 0.01	0.03 ± 0.00	0.03 ± 0.01
phenyl ethyl acetate [d]	pea	11.62	1282	164.0	0.04 ± 0.01	0.06 ± 0.01	0.05 ± 0.01	0.05 ± 0.01
octyl butanoate [f]	ob	12.24	1325	200.0	0.00 ± 0.00	0.03 ± 0.01	0.01 ± 0.00	0.02 ± 0.00
ethyl 4-decenoate [f]	e4dec	13.00	1376	200.0	0.02 ± 0.00	0.04 ± 0.02	0.03 ± 0.01	0.03 ± 0.01
2-phenylethyl propanoate [f]	2-pepr	13.28	1395	178.2	0.00 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
ethyl 2,4-decadienoate [f]	e2.4dec	14.26	1466	196.0	0.01 ± 0.00	0.01 ± 0.01	0.01 ± 0.00	0.01 ± 0.00
total acetates					12.87	64.81	172.85	221.96
total propanoates					0.45	0.44	0.08	0.03
total butanoates					0.95	3.77	2.25	2.96
total hexanoates					0.98	1.71	1.48	2.55
total heptanoates					0.10	0.17	0.10	0.10
total others					0.02	0.04	0.04	0.04
total esters					15.34	70.94	176.80	227.64
alcohols								
1-butanol [d]	bol	2.00		74.0	11.51 ± 3.16	7.74 ± 2.00	3.04 ± 0.30	1.20 ± 0.15
1-hexanol [d,e]	hxol	4.40		102.0	6.03 ±	3.01 ±	2.64 ±	2.56 ±
1-heptanol [d]	pol	6.38	967	116.2	28.12 ± 7.44	52.83 ± 10.80	68.54 ± 7.37	72.54 ± 10.24
1-octanol [d,e]	ocol	8.18	1069	130.2	0.11 ± 0.05	0.20 ± 0.10	0.05 ± 0.02	0.00 ± 0.00
total alcohols					45.36	63.78	74.27	76.30
ketones								
2-heptanone [f]	2hpt	5.08		114.0	5.06 ± 0.02	5.04 ± 0.02	5.02 ± 0.02	5.01 ± 0.00
total general					65.76	139.76	256.09	308.95

[a] Response factors (× 10^3): ea, 2236.38; eb, ba, 91.97; e2mb, 65.64; mhx, 24.35; ehx, hxa, 7.02; hxna, 8.46; ehp, 2.27; hxpr, 2.31; hxb, 0.81; 2hxnb, 0.93; bna, oa, 0.79; hx2mb, 0.30; pea, 1.46; ob, 2-pepr; 0.12; e4dec, 0.14; e2.4dec, 0.16; 2hpt, 112.350; bol, 708.77; hxol, 410.89; pol, 10.00; ocol, 23.00. [b] t_R values correspond to GC-FID chromatographic separation. [c] LRI: linear retention index. [d] Compound identified by GC−MS and confirmed by using commercial standards. [e] Compound described previously in *F. chiloensis* (*13*). [f] Compound tentatively identified by GC−MS.

for 20 s; and a melting curve from 58 to 95 °C at 0.5 °C increments. A dilution series was built to estimate the amplification efficiency using cDNA mix from fruit samples as template. Each reaction was performed in triplicate, and a negative water control was included in each run. Fluorescence was measured at the end of each extension 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 *FcGAPDH1* gene and using fruit sample from stage C1 as calibrator, with a nominal value of 1. The method described by Pfaffl (*32*) was used to make all calculations.

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 for quality assessment; Tukey test for volatile measurement and AAT activity; Scheirer−Ray−Hare test, an extension of the Kruskal−Wallis test, for the expression analysis of FcAAT1) (*33*). To provide a global overview of volatile compounds, principal component analyses (PCA) were performed.

RESULTS

Characterization of Fruit Stages. Four different developmental stages were defined in *F. chiloensis* fruit following the earlier classification made by Figueroa et al. (*23*). While stages C1 and C2 correspond to developing fruit, stages C3 and C4 correspond to turning fruit and fully ripe fruit, respectively. As development takes place, the size of the fruit constantly increases, however after the C3 stage the growing of the fruit ceases (**Figure 1A**). As ripening progresses, firmness and titratable acidity reduction were observed (**Figure 1B** and **1C**), while total soluble solids increased constantly from the C1 to the C3 stage (**Figure 1D**).

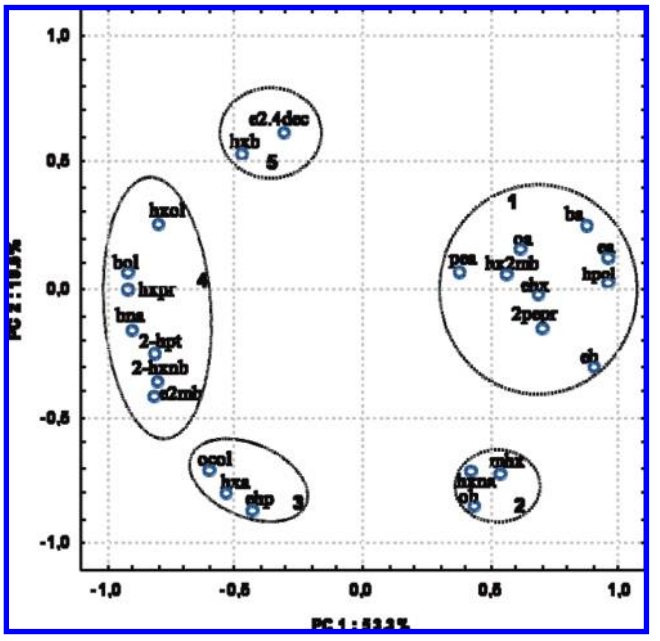

Figure 2. Loading plot of the first two principal components of PCA for volatiles produced during development of the Chilean strawberry fruit. Labels of volatile compounds as indicated in **Table 1**. PC1 and PC2 accounted for 72.8% of the variance.

Fruit firmness was markedly reduced between the C2 and C3 stages as previously described (*23*).

Production of Volatiles and AAT Activity during *F. chiloensis* Development. Many different volatile compounds were identified in the headspace of intact Chilean strawberry fruit, and 25 of them were quantified. The main compounds found were esters, although some alcohols and ketones were also identified (**Table 1**). Total esters comprised 23.3%, 50.8%, 69.0% and 73.4% of total volatiles in C1, C2, C3 and C4 stages, respectively. The most abundant esters present in fully ripe fruit comprised 98% of total esters, and they have aroma properties with fruity notes: butyl acetate, ethyl acetate, ethyl butanoate and ethyl hexanoate. Among esters, acetates were the most abundant followed by butanoates and hexanoates. Acetates increased their concentration during ripening of the fruit, with maximum production rates at the C4 stage, while butanoates and hexanoates increased between the C1 and C2 stages, remaining high in the following ripening stages.

Total alcohols are abundant in *F. chiloensis* fruit, comprising 69.0%, 45.6%, 29.0% and 25.0% of total volatiles in each developmental stage, respectively. Heptanol is the major alcohol found, followed by butanol and hexanol. Heptanol increased its concentration between stages C1 and C2, and remained high and almost constant during fruit ripening (Table**1**). The other alcohols (butanol, hexanol and octanol) displayed a reduction in their content during fruit development and ripening, simultaneously with the increment of acetate esters, butanoates and hexanoates.

To provide a general overview of the volatiles, a principal component analysis (PCA) was performed (**Figure 2**). The volatiles produced by the Chilean strawberry fruit were classified into five groups, and the first two components were able to account for 72.8% of the variance. The production profiles for selected volatiles belonging to different groups are shown in **Figure 3**.

Group 1 of compounds comprises volatiles that increased their concentration during development of *F. chiloensis* fruit, with maximum levels in fully ripened fruit, and includes compounds such as ethyl acetate, ethyl butanoate, butyl acetate, ethyl

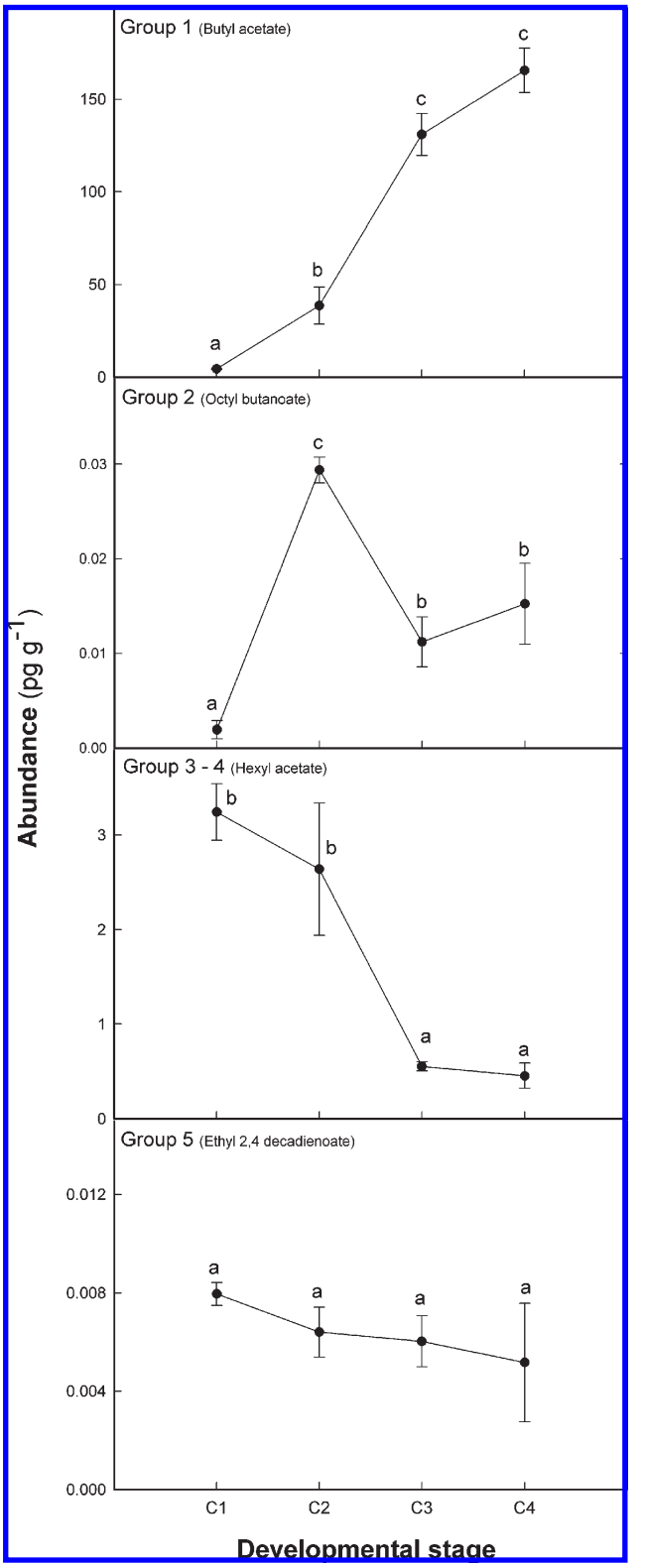

Figure 3. Production profiles of selected esters during development of *F. chiloensis* fruit. Volatiles belong to different groups according to PCA. The production of volatile compounds was assayed in three replicates per fruit stage, and data corresponds to the mean ± SE. Different letters indicate differences between stages ($P = 0.05$).

hexanoate, octyl acetate, phenyl ethyl acetate, ethyl 4-decenoate, 1-heptanol, hexyl 2-methyl butanoate and 2-phenylethyl propanoate. Group 2 involves compounds that increased their concentration between C1 and C2 stages and decreased after

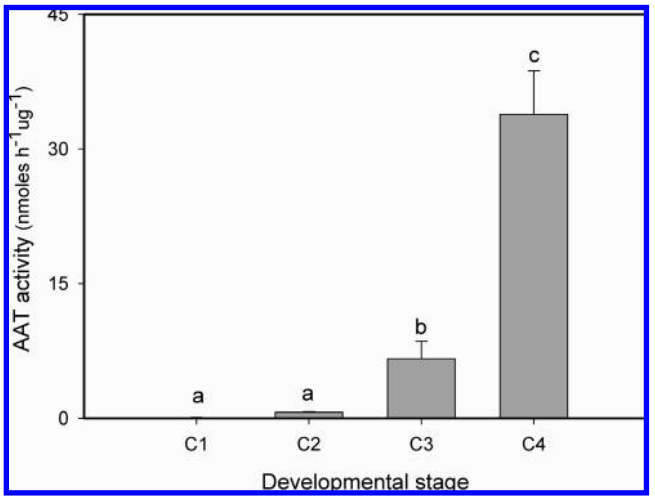

Figure 4. Changes in AAT activity during development of *F. chiloensis* fruit. Activity data corresponded to the mean ± SE of three replicates. Different letters indicate differences between stages ($P = 0.05$).

that: methyl hexanoate, octyl butanoate and hexenyl acetate. Group 3 comprises compounds such as 1-octanol, hexyl acetate and ethyl heptanoate, while group 4 includes compounds like 1-butanol, benzyl acetate, hexyl propanoate, 2-heptanone, 2-hexenyl butanoate, ethyl 2-methyl butanoate and 1-hexanol. Compounds from groups 3 and 4 showed a constant reduction between stages C1 and C3, remaining almost constant after that or displaying a small increase at stage C4. The final group (group 5) comprises volatiles that almost do not change their concentration during development such as ethyl 2,4-decadienoate and hexyl butanoate.

Since esters are important for aroma and a significant increase in total ester production was observed during development of the Chilean strawberry fruit, we focused our analysis on ester biosynthesis by the AAT enzyme. *In vitro* AAT activity, assayed by the capacity to produced butyl acetate, showed an important increment during *F. chiloensis* development, with a high level of activity at C3 and C4 stages (**Figure 4**). A good correlation was found between AAT activity and the total content of esters ($r = 0.81$); especially a high correlation was found between AAT activity and acetates ($r = 0.81$) and hexanoates ($r = 0.93$).

Isolation of *FcAAT1* and Phylogenetic Analysis. An AAT gene was isolated from a ripe *F. chiloensis* fruit using specific primers matching an AAT gene from *F.* × *ananassa*. A fragment of 545 bp with high homology to other AAT genes from the *Fragaria* genus was obtained, and used as template to design internal primers for 5′ and 3′-RACE-PCR. A 793 bp 5′ RACE fragment (with 291 bp overlap with the original fragment) and a 781 bp 3′-RACE fragment (with 215 bp overlap) were isolated using these primers. Therefore, a composite cDNA sequence of 1,620 bp called *FcAAT1* (GenBank accession number FJ548611) was generated after assembling of all fragments. Analysis of the *FcAAT1* sequence revealed an ORF of 1,384 bp, and a deduced polypeptide sequence of 450 amino acids with a molecular weight of 50.4 kDa. The sequence also contained 35 bp and 234 bp of 5′ and 3′-UTR, respectively. The coding region displayed the three motifs which are characteristic of most AAT previously described: LALYYPLSGR, HKLID and DFGWG (**Figure 5A**). A multiple alignment of *FcAAT1* with other fruit-specific AAT sequences showed a low level of amino acid conservation, restrained to the functionally and structurally important motifs.

A phylogenetic tree was built from the multiple alignment of deduced amino acid sequences of fourteen AATs (**Figure 5B**). The clustering pattern visualized in the phylogenetic tree reproduces three subgroups. Interestingly, the grouping pattern incorporates *FcAAT1* into subgroup II, next to other AATs from the *Fragaria* genus, and in a divergent position with respect to some ripening-related AATs from other fruit species (subgroup III) such as melon, pear and apples.

DNA Gel-Blot Analysis. In order to analyze the complexity of Chilean strawberry's alcohol acyltransferase gene family, DNA gel-blot of genomic DNA digested with four different restriction enzymes was hybridized with a specific probe obtained from the divergent region of *FcAAT1* (**Figure 6**). DNA gel-blot analysis revealed at least two hybridizing bands with all the restriction enzymes tested, all of them ranging between 2.0 kb and 23.1 kb approximately. The digestion product of *Bam*HI showed two hybridizing bands over 9.4 kb while the other three enzymes showed smaller bands. *Hin*dIII restriction pattern exhibited three major hybridizing bands and one weaker around 2.4 kb, while *Eco*RI digestion showed one major (around 5.0 kb) and four weaker bands. An *in silico* analysis of the cDNA showed no restriction sites for *Bam*HI, *Eco*RI and *Eco*RV, although two restriction sites were found for *Hin*dIII.

***FcAAT1* Expression Analysis during Fruit Development.** The expression of *FcAAT1* was analyzed by qPCR in fruit samples at different developmental stages, as well as in different vegetative tissues (**Figure 7**). Almost no transcript accumulation was observed in fruit at the first stage of development; however, the expression level increased concomitantly with the ripening progress, showing a high level of *FcAAT1* transcripts at C3 and C4 stages (**Figure 7A**). In the other vegetative tissues analyzed, flowers, runners, stem and leaves, a very low transcript accumulation was observed compared to ripe *F. chiloensis* fruit (**Figure 7B**).

DISCUSSION

Chilean strawberry is a new and commercially promising fruit crop in Chile, but unfortunately little information exists concerning its ripening physiology. Our research group has initiated a study on aspects which are relevant for fruit quality, such as softening (*23*,*34*) and aroma formation. Among the volatiles found in intact *F. chiloensis* fruit, esters are important due to their abundance and aroma properties. Therefore, we decided to analyze the production profile of esters during development of the fruit.

Using headspace-SPME we were able to identify more than 40 different volatile compounds in intact *F. chiloensis* fruit, 25 of them were quantified. Volatile components of the Chilean strawberry fruit were dominated by esters, mainly by butyl acetate and ethyl acetate, which increased during ripening. Among the volatiles quantified, only six esters and two alcohols were reported previously in *F. chiloensis* fruit (*13*). Early studies performed in fruit extracts reported differences in the aroma patterns between different strawberry cultivars (*35*), and also differences between *F.* × *ananassa* and wild species such as *F. chiloensis* (*13*). Our results with intact fruit confirm that issue. Some esters found in *F. chiloensis* fruit like ethyl acetate, methyl butanoate, 2-methyl acetate, octyl acetate, octyl butanoate, hexyl acetate, ethyl heptanoate, 2-hexenyl butanoate, benzyl acetate and hexyl 2-methyl butanoate were also described in several *F.* × *ananassa* cultivars (*7*, *9*, *36*, *37*). However, we found in *F. chiloensis* some esters which have not been described previously in *F.* × *ananassa*, such as hexyl propanoate, ethyl 4-decenoate, 2-phenylethyl propanoate and ethyl 2,4 decadienoate; nevertheless they have been described before in apple fruit and strawberry wine (*38*, *39*) On the other hand, the same type of alcohols was found in both species, *F.* × *ananassa* and *F. chiloensis*.

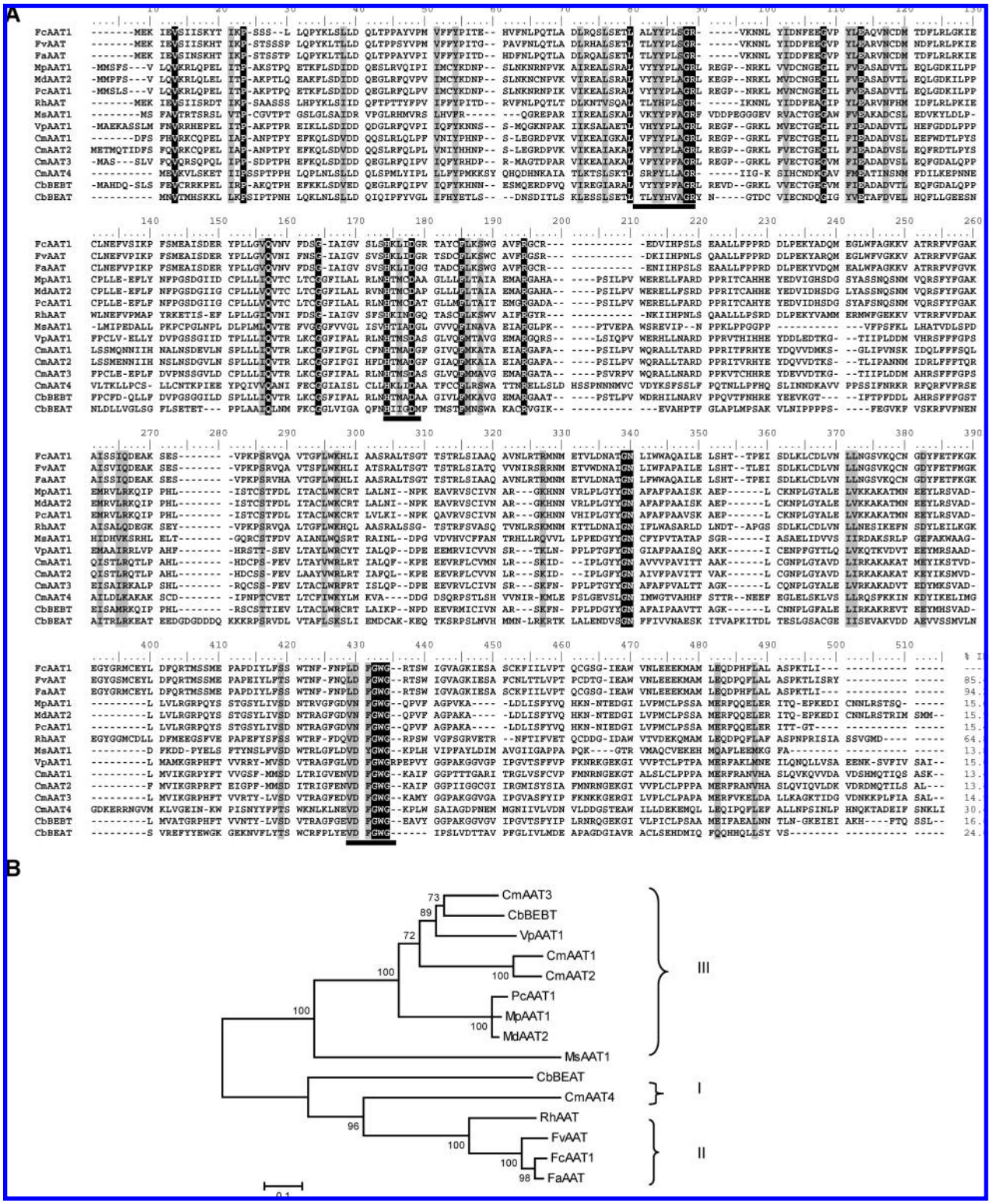

Figure 5. (**A**) Alignment of the deduced *FcAAT1* full length amino acid sequence with AATs from other sources. Gaps are indicated by dashes, letters with black background are identical amino acids, and letters with gray background are similar amino acids. The three motifs which are characteristic of most AATs are indicated: LALYYPLSGR, HKLID (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. (**B**) Phylogenetic analysis of *FchAAT1*. The phylogenetic tree built using MEGA software (version 3.1; http://www.megasoftware.net). Numbers on branches indicate bootstrap values (as a percentage). Sequences are the same used in panel **A** (see above).

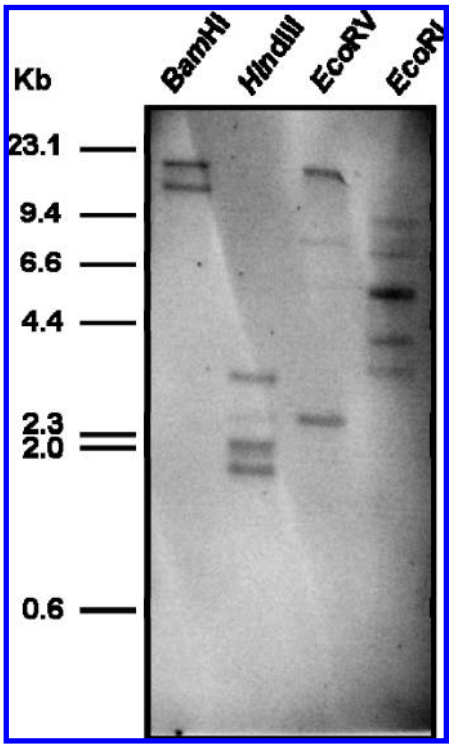

Figure 6. DNA gel-blot analysis of genomic DNA from *F. chiloensis*. Genomic DNA (20 μg per lane) was digested with the indicated restriction enzymes and hybridized with the corresponding ^{32}P-labeled specific probe for 3′ UTR *FcAAT1*. Hybridization and stringency conditions as described in Materials and Methods.

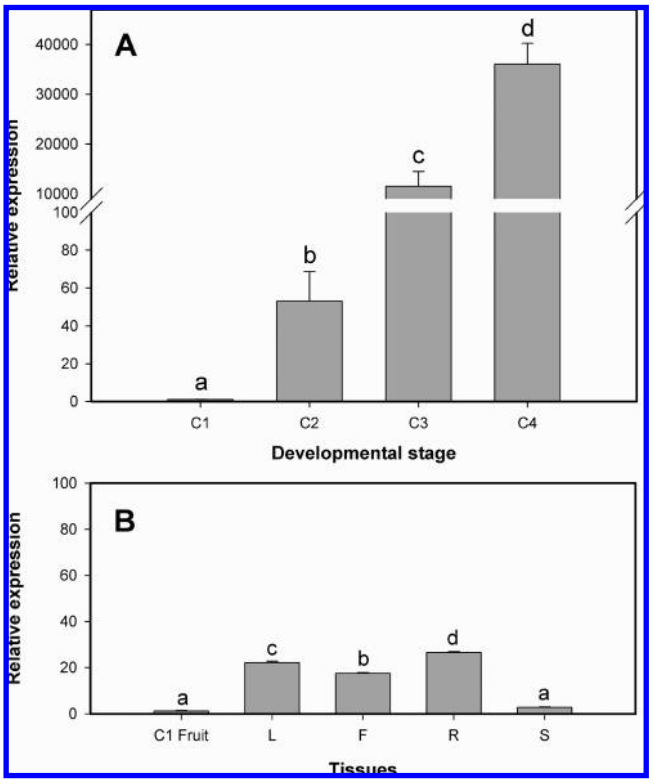

Figure 7. (**A**) Changes in *FcAAT1* mRNA abundance during development of the Chilean strawberry fruit by qPCR. The expression data corresponded to means of three replicates, normalized against *FcGAPDH1* abundance, using C1 stage fruit as calibrator, and expressed in arbitrary units ± SE. Different letters indicate differences between stages (P = 0.05). (**B**) Changes in *FcAAT1* mRNA abundance in other tissues. Expression analysis of *FcAAT1* by qPCR was performed in leaves, flowers, runners and stem. The expression data are means of three replicates, normalized to *FcGAPDH1* abundance, using C1 stage fruit as calibrator and expressed in arbitrary units ± SE. Different letters indicate differences between tissues (P = 0.05).

A prospective analysis of four wild strawberry accessions (*F. virginiana* 'W9'; *F. vesca* ssp. *vesca* 'Geising'; *F. vesca* ssp. *vesca* f. *alba*; *F. moschata* 'Cotta') in comparison to *F.* × *ananassa* cv. Elsanta, described that wild accessions had higher aroma intensities than the cultivated one (*16*). The study showed that *F. virginiana* contains 15 times higher volatiles content than *F.* × *ananassa*. The aroma profile of wild species varied qualitatively and quantitatively, however the pattern shares similarities with the aroma profile of *F. chiloensis* fruit, especially related to ester compounds. Coincidently, alcohols which are predominant in *F. chiloensis* and accumulates as fruit ripening progresses, showed the same pattern in wild strawberries (*16*).

During ripening of *F. chiloensis* fruit there was a clear increase in the total content of volatiles, both esters and alcohols. As the production of esters increases, a clear increment of both AAT activity and transcript accumulation of FcAAT1 gene was observed. A good correlation was found between AAT activity and the total content of esters, especially with acetates and hexanoates. Esters from group 1, which includes the major esters produced by *F. chiloensis*'s fruit, displayed a similar production profile during development as AAT activity, and therefore it can be suggested that AAT may have a significant role in the production of these important esters. Our findings are coincident with those reported by Perez et al. (*20*), who observed a high correlation between AAT activity and aroma profile during ripening in four cultivars of *F.* × *ananassa*. Also with Aharoni et al. (*21*) that reported that the expression of AAT increases during ripening of *F.* × *ananassa*, peaking at the full red stage with high levels of transcripts. A good correlation was observed between *FcAAT1* transcript accumulation, AAT enzyme activity and ester production by the fruit, similarly to the findings of Carbone et al. (*40*) in *F.* × *ananassa*. Although FcAAT1 gene could perfectly explained the production of esters from group 1, other AATs expressed at different development times should exist in order to explain the production of the remaining esters produced by the Chilean strawberry fruit.

Due to the high correlation between ester production and AAT activity we decided to isolate an AAT gene from *F. chiloensis* fruit (*FcAAT1*). This gene shows the nineteen amino acid residues that are absolutely conserved in the three conserved motifs which are characteristic of most AATs previously isolated from different fruit species. The first one is the less conserved motif, and it is located close to the N-terminus as previously described (*21*). The second motif, the most conserved within the BAHD acyltransferase family, is located in the middle of the sequence and it is involved in the catalytic mechanism (*41*). The last motif is also highly conserved within the BAHD protein family and is located in the C-terminus. Apparently, this last motif plays a structural function keeping the conformational integrity of the enzyme structure (*42*). Phylogenetic analysis reproduces the three subgroups previously described (*42*) and concludes that *FcAAT1* belongs to subgroup II, which comprises the sequences from *Fragaria* AAT genus and *Rosa hybrida*.

In melon fruit, AATs are encoded by a multigene family of at least four members (*42*) suggesting that AAT genes could be part of a small multigene family. DNA gel-blot analysis for AAT gene in *F. chiloensis* (**Figure 6**) confirms this hypothesis, as it indicates that at least two gene copies exists according to *Bam*HI digestion

pattern which shows two fragments over 9 kb. Moreover, the digestion pattern of *Eco*RI shows five hybridization fragments between 2.3 kb and 9.4 kb. These two enzymes do not have restriction sites on the cDNA sequence of *FcAAT1* gene. On the other hand, the 3′ end of the gene (that is recognized by the probe) is highly variable (**Figure 5A**), and therefore it is probable that these hybridization bands could represent other AAT gene family members present in the *F. chiloensis* genome. As far as we know, this is the first report that shows that the genomic organization of an AAT gene in strawberry genome is representative of a small multigene family. Other strawberry ripening-related genes, like those related to cell wall degradation such as endoglucanase, pectin methyl esterase, pectate lyase and polygalacturonase, show complex hybridization profiles indicating that they belong to large multigene families (*23*, *43*). Certainly, the octoploid condition of strawberries (*F.* × *ananassa* and *F. chiloensis*) is not directly related to the size of a determined gene family.

Transcript accumulation of *FcAAT1* increased at the same time as fruit development. *FcAAT1* is mainly expressed in fruit tissues, during the late stages of development and ripening, although there is some transcript accumulation in vegetative tissues at a similar expression level to that observed in small fruit. The strong induction of *FcAAT1* at C3 and C4 stages strongly suggests a participation in ester formation, as it has been previously described for other AATs from a wide range of fruit species. However, the study of the kinetic parameters of FcAAT1 recombinant protein will provide more information about the real role and substrate specificity of this candidate gene.

In conclusion, our study reveals an increment in AAT activity which is related to an increment in ester production during ripening of the Chilean strawberry fruit. In addition, *FcAAT1* gene is highly expressed in fruit tissue during ripening of *F. chiloensis* and it could be responsible for the production of important esters related to aroma.

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