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RESEARCH PAPER

The fruit ripening-related gene *FaAAT2* encodes an acyl transferase involved in strawberry aroma biogenesis

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Abstract

Short-chain esters contribute to the blend of volatiles that define the strawberry aroma. The last step in their biosynthesis involves an alcohol acyltransferase that catalyses the esterification of an acyl moiety of acyl-CoA with an alcohol. This study identified a novel strawberry alcohol acyltransferase gene (*FaAAT2*) whose expression pattern during fruit receptacle growth and ripening is in accordance with the production of esters throughout strawberry fruit ripening. The full-length *FaAAT2* cDNA was cloned and expressed in *Escherichia coli* and its activity was analysed with acyl-CoA and alcohol substrates. The semi-purified FaAAT2 enzyme had activity with C1–C8 straight-chain alcohols and aromatic alcohols in the presence of acetyl-CoA. Cinnamyl alcohol was the most efficient acyl acceptor. When *FaAAT2* expression was transiently downregulated in the fruit receptacle by agroinfiltration, the volatile ester production was significantly reduced in strawberry fruit. The results suggest that *FaAAT2* plays a significant role in the production of esters that contribute to the final strawberry fruit flavour.

Key words: Alcohol acyltransferase, BADH, fruit aroma, strawberry, volatile compounds.

Introduction

Volatile esters play several roles in higher plants. They make flowers more attractive to pollinators and dispersing animals, act as protectants against pathogens by inducing several important plant defence pathways, and contribute to the aroma of ripe fruit (D'Auria *et al.*, 2007). These compounds are produced by all soft fruit species during ripening and play an important role determining the final sensory quality of fruit (Manríquez *et al.*, 2006). In fruits like apple (*Malus domestica*), pear (*Pyrus communis*), and banana (*Musa sapientum*), esters are the main components of their characteristic aroma, while in strawberry they only contribute to the blend of volatiles that define the aroma (Beekwilder *et al.*, 2004). The strawberry aroma is determined by more than 300 compounds consisting of alcohols, aldehydes, esters, sulphur compounds, and furanone derivatives (Zabetakis and Holden, 1997), but only about 20 of these compounds actually contribute to its aroma and flavour (Forney *et al.*, 2000). Some authors consider methyl butanoate, ethyl butanoate, methyl hexanoate, hexyl acetate, and ethyl hexanoate in addition to 2,5-dimethyl-4-hydroxy-3(2H)-furanone (HDMF, furaneol) and its methyl ether as the most important constituents of the characteristic strawberry aroma (Larsen *et al.*, 1992; Jetti *et al.*, 2007). Analysis of volatiles from fruit of different *Fragaria* varieties has identified some common esters (ethyl acetate, methyl butanoate, 2-methylbutyl acetate, octyl acetate, octyl butanoate, hexyl acetate, ethyl heptanoate, 2-hexenyl butanoate, benzyl acetate, and hexyl 2-methyl butanoate) in both *Fragaria chiloensis* fruits and several *Fragaria* × *ananassa* cultivars (Drawert *et al.*, 1973; Hirvi and Honkanen, 1982). However, some esters found in *F. chiloensis* (hexyl propanoate, ethyl 4-decenoate,

2-phenylethyl propanoate, and ethyl 2,4-decadienoate) have not been described previously in *F.* × *ananassa* (Pyysalo *et al.*, 1979; Zabetakis *et al.*, 1997; Azodanlou *et al.*, 2003; Berna *et al.*, 2007; González *et al.*, 2009).

The last step in the biosynthesis of volatile esters is catalysed by alcohol acyltransferase (AAT), a key enzyme in aroma biochemistry (Fellman *et al.*, 2000). This enzyme catalyses the esterification of an acyl moiety from acyl-CoA onto an alcohol (Yamashita *et al.*, 1977; Aharoni *et al.*, 2000; Olías *et al.*, 2002; Beekwilder *et al.*, 2004). The formation of a broad range of esters in the different types of fruit results from the combination of different alcohols and acyl-CoAs (Schwab, 2003). The volatile compounds produced by a fruit are determined by substrate availability and not only by AAT specificity (Tressl and Drawert, 1973; Knee and Hatfield, 1981; De Pooter *et al.*, 1983; Jayanty *et al.*, 2002; Schwab, 2003), although this enzyme participates in the rate-limiting step in ester biosynthesis (Defilippi *et al.*, 2005).

Due to their role in ester biosynthesis, several AAT enzymes and their corresponding genes have been studied in some fruit species with high commercial interest such as banana (Beekwilder *et al.*, 2004), apple (Souleyre *et al.*, 2005; Li *et al.*, 2006), melon (*Cucumis melo*) (Yahyaoui *et al.*, 2002; El-Sharkawy *et al.*, 2005), apricot (*Prunus armeniaca* L.) (González-Agüero *et al.*, 2009), grape (*Vitis vinifera* L.) (Wang and De Luca, 2005; Kalua and Boss, 2009), papaya (*Vasconcellea pubescens*) (Balbontín *et al.*, 2010), peach (*Prunus persica*) (Zhang *et al.*, 2010), and strawberry (Pérez *et al.*, 1993; Aharoni *et al.*, 2000; Olías *et al.*, 2002; González *et al.*, 2009). The expression of *AAT* always increased in these fruits throughout ripening and after harvest correlating with the total content of esters, thus suggesting that this gene family could be responsible for the production of important esters related to ripe fruit aroma (Aharoni *et al.*, 2000; Yahyaoui *et al.*, 2002; González *et al.*, 2009). Moreover, alcohol levels in peach fruit decreased during post-harvest ripening, apparently by their use as substrates for the formation of different esters (Zhang *et al.*, 2010).

The AAT proteins share several common motifs with the BAHD superfamily of acyltransferases (D'Auria, 2006). Among them, the HXXXD motif located in the middle of the protein sequence is highly conserved in higher plants and yeasts and has been suggested to be involved in the catalytic mechanism (D'Auria, 2006). In fact, the replacement of the histidine residue in this motif causes the loss of protein function (Bayer *et al.*, 2004). Another highly conserved motif in AAT proteins is the DFGWG sequence. This motif is located near the carboxylic end of the protein and, apparently, has a structural function keeping the conformational integrity of the enzyme structure (El-Sharkawy *et al.*, 2005; D'Auria, 2006).

Several *AAT* genes have been isolated from *F.* × *ananassa* cv. Elsanta (*SAAT*) (Aharoni *et al.*, 2000), wild strawberry *F. vesca* (*VAAT*) (Beekwilder *et al.*, 2004), and *F. chiloensis* (*FcAAT1*) (González *et al.*, 2009). In all cases, these genes reached their maximum transcript levels in red-ripened fruits, suggesting that their corresponding enzymes are involved in the biosynthesis of volatile esters in the strawberry fruit receptacle. The SAAT enzyme prefers medium-chain aliphatic alcohols in combination with different acyl-CoAs as substrates. In contrast, with the tertiary monoterpenol linalool as substrate, activity was insignificant. Although the phylogenetic analysis indicated that VAAT and SAAT are closely related, their enzymic activities were quite different. In this way, VAAT is more active towards short-chain alcohol substrates, which are not preferred by SAAT (Beekwilder *et al.*, 2004). These results correlate with the substrate preference observed for different AAT enzymes isolated from both wild and cultivated varieties (Olías *et al.*, 2002).

Furthermore, it has been shown that AAT proteins from different sources, such as banana and melon (BanAAT and CmAAT1, respectively), have a similar preference towards alcohols and similar activity profiles (Beekwilder *et al.*, 2004; El-Sharkawy *et al.*, 2005). This enzymic behaviour has been ascribed to a special form of convergent evolution, in which new enzymes with the same function appeared in different plants from a shared pool of related enzymes with similar functions while related members (as VAAT and SAAT) show important differences in their specificity (Pichersky and Gang, 2000).

This study reports the identification and characterization of a new strawberry ripening-related *AAT* gene, *FaAAT2*. The semi-purified recombinant enzyme has been obtained and its enzymic affinity for different alcohols and acyl-CoA substrates studied. This study also provides evidence for the possible role of FaAAT2 in the biosynthesis of esters that contribute to the strawberry fruit flavour through the transitory silencing of *FaAAT2* expression in the strawberry fruit receptacle.

Materials and methods

Plant material

Strawberry plants (*F.* × *ananassa* Duch. cv. Camarosa, an octoploid cultivar; *F. vesca*; *F. chiloensis*; and *F. virginiana*) were grown under field conditions in Huelva (southwest Spain). *F.* × *ananassa* fruits were harvested at different developmental stages: small-sized green fruits (G1, 2–3 g), middle-sized green fruits (G2, 3–5 g), full-sized green fruits (G3, 4–7 g), white fruits (W, 5–8 g), full-ripe red fruits (R, 6–10 g), overripe fruits (OR, 6–10 g), and senescent fruits (SN, 6–10 g). Vegetative tissues, such as stolons, roots, crowns, and expanding leaves, were also harvested. All tissue and fruits samples were immediately frozen in liquid nitrogen and stored at –80 °C. Strawberry plants (*F.* × *ananassa* Duch. cv. Elsanta) used for agroinfiltration were grown in a greenhouse in Freising (Germany). This cultivar was selected for the transitory silencing because the agroinfiltration method renders better and with more repetitive results than in other *F.* × *ananassa* cultivars tested, such as Camarosa, Chandler, and Candonga. Besides, the Elsanta fruits better survive to the agroinfiltration technique than the fruits of other varieties.

Auxin treatment

Achenes of two sets of 50 middle-sized green fruits (G2) each, still attached to the plant, were carefully removed using the tip of

a scalpel blade. One set of deachened G2 fruits were treated with the synthetic auxin 1-naphthalenacetic acid (NAA) in lanolin paste (1 ml) with 1 mM NAA in 1% (w/v) dimethyl sulphoxide. The other set of G2 deachened fruits (control group) were treated with the same paste, but without NAA. Both treatments were applied over the whole fruit surface. All fruits were harvested 5 days after treatment, immediately frozen in liquid nitrogen, and stored at –80 °C. During the course of the assays, the fruits reached the G3–W developmental stage.

RNA isolation

Total RNA was isolated from independent pools of strawberry fruits at different growth and ripening stages and from vegetative tissues according to Asif *et al.* (2000). Achenes were always removed from fruit samples and only receptacle RNA was extracted and purified. The total RNA obtained was treated with DNase I (RNase free) (Invitrogen) to remove genomic DNA contamination and purified by using the RNeasy Mini kit (Qiagen). RNA samples were considered DNA-free when no amplicons corresponding to the analysed genes were observed using RNA as template in a standard PCR.

Cloning and sequence analysis of full-length cDNA of FaAAT2

The full-length cDNA of *FaAAT2* (JN089766) was isolated from total RNA of strawberry red fruit by rapid amplification of cDNA ends (RACE) using a Marathon cDNA amplification kit (BD Biosciences), according to the manufacturer's instructions (www.clontech.com). The primers FaAAT2-forward (5′-TGTG GAGGTGAGAGGACGACCCC-3′) and FaAAT2-reverse (5′-TGGCAAGCATACTGGCACCAAGATTTC-3′) were used for 3′ and 5′ RACE amplifications, respectively. All PCR fragments obtained were separated by gel electrophoresis, purified using the QIAquick Gel Extraction kit (Qiagen), and cloned into pGEM-T Easy vector (Promega) for sequencing. The full-length cDNA fragment was amplified by using primers specially designed to match the initial and final transcription zones of the gene (FaAAT2-FL-forward: 5′-TGTCCCATTCATCATGTCTTACA AGAA-3′; FaAAT2-FL-reverse: 5′-TGCCATCTAAATAGCC-TCCAAAAGAAC-3′) and then sequenced.

The nucleotide and deduced amino acid sequences were analysed using the Lasergene software package (DNASTAR, USA). Similarity analysis was performed using the alignment tool (BLAST, National Center for Biotechnology Information, Bethesda, MD, http://www.ncbi.nlm.nlm.gov/BLAST).

Expression of recombinant FaAAT2 protein in Escherichia coli

The vector pGEX-4T-1 (Amersham) was used for the expression of recombinant FaAAT2 protein in *E. coli* cells (Blb21 Gold DE3 strain, Stratagene). The empty pGEX-4T-1 vector served as control for the experiments. The FaAAT2 coding region was amplified using the primer FaAAT-prot-forward (5′-CGGAATT CTCTTACAAGAACAATC-3′) and FaAAT-prot-reverse (5′-CTACTAACCAGAGCTCACTGAGCTCAAAG-3′). These oligonucleotides introduced an *Eco*RI and a *Xho*I restriction site at the 5′ and 3′ ends of the *FaAAT2* PCR fragment, respectively, which were complementary to the restriction sites in the vector. The forward primer eliminated the native methionine ATG codon of *FaAAT2* to obtain a frame fusion protein at the N-terminus. This peptide included an ATG translation initiation codon for glutathione S-transferase (GST). The expression construct (pGEX-4T-1-*FaAAT2*) was verified by restriction enzyme analysis and DNA sequencing before transformation into *E. coli* Blb21 cells.

For recombinant FaAAT2 protein expression, 300 µl of an overnight liquid culture of transformed *E. coli* cells were grown in fresh Luria-Bertani medium (300 ml) supplied with ampicillin (50 µl/ml) at 37 °C until $OD_{600} = 0.6$. Then, the culture was equilibrated to 16 °C and induced with 1 mM isopropyl-β-D-thiogalactoside. After 12 hours of growth at 16 °C, cells were harvested by centrifugation and the pellet was frozen for 15 min at –80 °C. After this time, the cells were resuspended in 8 ml of 1 × ice-cold wash buffer (4.3 mM $Na_2HPO_4.7H_2O$, 1.47 mM KH_2PO_4, 0.137 M NaCl, 2.7 mM KCl, pH 7.3) and sonicated on ice at least three times for 30 s with 10% energy using a sonicator (Sonopuls GM 2017, Bandelin). The sonicated cells were centrifuged at 4 °C and 20 000 *g* for 20–30 min. The soluble protein fraction (supernatant) was incubated with GST-sepharose (Novogen) for at least 30 min at 4 °C with continuous agitation and then centrifuged at 4 °C and 800 *g* for 3 min. The protein attached to the sepharose was washed three times with 8 ml of 1 × ice-cold wash buffer at 4 °C and 800 *g* for 5 min. Finally, the sepharose-bound protein was released by incubating for 5 min in 200 µl of 1 × elution buffer (50 mM TRIS-HCl, 1 mM glutathione, pH 8) at room temperature and centrifuged again at 800 *g* for 5 min. The eluate was quantified by Bradford's method (Bradford, 1976) and analysed by SDS-PAGE (12% Progel TRIS-Glycine, 1.0 mm, Anamed).

Enzymic characterization of the recombinant FaAAT2 protein

The activity of recombinant semi-purified FaAAT2 protein was quantified by its ability to convert different alcohols and acyl-CoA substrates into the corresponding esters. Several *E. coli* cultures expressing pGEX-4T-1-*FaAAT2* and pGEX-4T-1 empty vector (control) were independently used for biotransformation assays. 20 ml aliquots from both induced *E. coli* cultures were assayed with different alcohols (10 µM each) in plugged glass flasks. Straight-chain alcohols (methanol, ethanol, propanol, 2-propanol, butanol, 2-butanol, pentanol, hexanol, heptanol, hex-2(E)-enol, hex-3(Z)-enol, octanol, oct-1-en-3-ol, nonanol, and decanol), branched-chain alcohols (3-methylbutanol), terpene alcohols (geraniol, linalool, farnesol, nerol, and nerolidol), aromatic alcohols (benzyl alcohol, 2-phenylethanol, eugenol, and cinnamyl alcohol), and furfuryl alcohol (all from Sigma-Aldrich) were included as potential substrates. The activity assay was performed at 16 °C under agitation (120 rpm) for 18 h. Each culture included nonane (1 mM) as internal standard. Esters were extracted with hexane and analysed by gas chromatography-mass spectrometry (GC-MS).

AAT activity was also tested with semi-purified recombinant FaAAT2 protein (2 µg) in 500 µl of total volume in the presence of 20 mM alcohol and 0.1 mM acyl-CoA in 50 mM TRIS-HCl buffer (pH 7.5) containing 10% (v/v) glycerol and 1 mM dithiothreitol (DTT). These reactions were performed in triplicate. A set of different acyl-CoAs (acetyl-, propionyl-, butyryl-, and hexanoyl-CoA) and alcohol substrates (methanol, ethanol, 1-propanol, butanol, pentanol, hexanol, heptanol, geraniol, benzyl alcohol, 2-phenylethanol, eugenol, and cinnamyl alcohol) were tested each reaction including one alcohol and one acyl-CoA. Reactions were always initiated by adding semi-purified recombinant FaAAT2 protein and then incubated with continuous agitation at 30 °C for 20 min. After incubation, 1 mM nonane was added as internal standard and the resulting esters extracted with hexane and analysed by GC-MS. The GC-MS method is very sensitive and flexible and has been optimized in the study laboratory for the quantification of fruit volatiles. Nonane was added at the end of the incubation period to avoid errors caused by loss of the standard. The enzymic activity was expressed as nmol substrate turned over s^{-1} (mg protein)$^{-1}$. K_{cat} values were determined by dividing V_{max} values by the protein concentration of the semi-purified enzyme in the reaction mixture.

GC-MS analysis was performed using a high polarity capillary column (ZB-WAX column, internal diameter 60 m × 0.32 mm, 0.25 µm, J & W Scientific, Folsom, CA, USA) and a low polarity capillary column (HP-5MS column, internal diameter 30 m × 0.25 mm, 0.5 µm, J & W Scientific). Chromatography conditions were as follows: injector, 250 °C; initial oven temperature, 40 °C held for 2 min, increased by 2 °C min^{-1} to 60 °C, then

increased by 5 °C min^{-1} to 220 °C, and held for 2 min; carrier gas, nitrogen at a flow rate of 1 ml min^{-1}. MS parameters were as follows: ion source, 230 °C; electron energy, 70 eV; multiplier voltage, 1247 V; GC-MS interface zone, 280 °C; scan range of 30–250 mass units. Volatiles were identified by comparison of their retention times and mass spectra with those of authentic standards from Sigma-Aldrich and published data. Concentrations of esters were calculated according to the internal standard method with the peak area of the internal standard, the peak area of the ester, and the standard curves of the authentic ester. A mass spectral survey was performed using the NIST Mass Spectral Search program (version 2.0, 2002).

Analysis of volatile compounds from strawberry fruits

Frozen deachened tissue (1 g) was ground in liquid nitrogen and transferred as aqueous solution to a vial containing 1 ml of a 35% (w/v) sodium chloride solution. As internal standard, 50 µl of 1,2-dimethoxyethane (0.965 nmol l^{-1}) was added to the sample. For manual solid-phase microextraction analyses, samples were equilibrated at 30 °C for 30 min and then exposed to a fibre coated with 85 µm of polydimethylsiloxane and carboxen (57295-U, Supelco, Dreieich, Germany). The fibre was exposed for 30 min to the headspace above the solution. Vials were closed with polytetrafluoroethylene/butyl rubber septa caps. The volatiles were subsequently desorbed for 10 min at 220 °C, separated by GC-MS, and identified as described above.

The levels of volatile alcohols and esters of both *F.* × *ananassa* cv. Camarosa and *F. vesca* varieties were analysed in different fruit developmental stages: green (G), white (W), and red (R).

Expression analysis by quantitative real-time PCR

Expression analysis of *FaAAT2* (JN089766) was performed by qRT-PCR using the iCycler system. First-strand cDNA was obtained using 2 µg total RNA and the iScript kit (BioRad) according to the manufacturer's instructions. The PCR reaction mix consisted of 25 µl of a mixture containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM each sequence-specific primer, 3 µl SYBR Green I (1:15000 diluted), 3 µl transcribed cDNA, and 0.5 U *Taq* polymerase (Biotools). The following PCR program was run: initial denaturation step at 94 °C for 2 min; 40 cycles of denaturation at 94 °C for 15 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; final extension step at 72 °C for 5 min. These conditions generated specific PCR products of the desired length (Supplementary Table S1, available at *JXB* online). No primer dimers were present. Investigated transcripts showed optimal PCR efficiencies, in the range of 0.25–2 µg of total RNA input with high linearity. The primers for quantitative amplification for *FaAAT2* were FaAAT2-forward and FaAAT2-reverse and for *SAAT* were 5′-GGAGGACATCATGGATTGGAGTTGC-3′ and 5′-GGGGATCTTGTTCTAGCATAGCC-3′. Each reaction was performed at least in triplicate and the corresponding C_t values were normalized using the C_t value corresponding to an interspacer 26S–18S strawberry RNA sequence (as a housekeeping gene). All these values were then used to determine the relative increase or decrease of *FaAAT2* expression in the samples with respect to the control according to Pedersen (2001). Preliminary transcriptome analyses by microarrays of green strawberry fruits versus red fruits, vegetative tissues, and deachened fruits allowed the identification of a group of housekeeping genes whose expression was constant in all these situations (unpublished data). The stable expression of these genes was afterwards corroborated by qRT-PCR (Supplementary Table S2). When the expression of these genes was analysed by qRT-PCR and normalized between them, it was found that the interspacer 26S–18S was the sequence with the least variability (data not shown). Thus, the interspacer 26S–18S region (primers 5′-ACCGTTGATTCGCACAATTGGTCATCG-3′ and 5′-TACTGCGGGTCGGCAATCGGACG-3′), used widely as the normalizing sequence (Griesser *et al.*, 2008; Encinas-Villarejo *et al.*, 2009; Muñoz *et al.*, 2010; Quesada *et al.*, 2009; Cruz-Rus *et al.*, 2011), was selected as control for its stable expression throughout the different experimental conditions tested. The efficiency of each particular qRT-PCR and the melting curves of the products were also analysed to ensure the existence of a single amplification peak corresponding to a unique molecular species (*FaAAT2* T_m = 87 °C, *SAAT* T_m = 83.5 °C, and the *interspacer 26S–18S* sequence T_m = 91.5 °C).

Generation of a FaAAT2 *RNA interference construct and transfection of strawberry fruit by agroinfiltration*

FaAAT2 was cloned into pFRN binary vector (courtesy of Dr. Marten Denekamp from Department of Molecular Cell Biology, University of Utrecht, The Netherlands) using the Gateway technology (Invitrogen). A 517-bp region from *FaAAT2* cDNA (cv. Camarosa), which is non-conserved between this and *SAAT* (Supplementary Fig. S1), was PCR amplified for use as an RNA interference (RNAi) fragment in the silencing construct, using the primers 5′-GTAACAGTCCGCTGGGATATGCAG-3′ (forward) and 5′-GTGAAGAAGTAAAACGTGATTGCCATCTAAA-3′ (reverse). The resulting fragment was cloned into pCR8/GW/TOPO (Invitrogen) and subsequently transferred to the Gateway pFRN vector by specific recombination between both plasmids using LR clonase (Invitrogen). The generated RNAi construct (pFRN-*FaAAT2*) was tested by sequencing and restriction analyses prior to transformation of strawberry plants. The pFRN-*FaAAT2* construct generates RNAi directed against the endogenous *FaAAT2*.

Agrobacterium tumefaciens strain AGL0 (Lazo *et al.*, 1991) containing the pFRN-*FaAAT2* or empty pFRN was grown at 28 °C in fresh Luria-Bertani medium supplemented with appropriate antibiotics. When the culture reached an OD_{600} of about 0.8, *Agrobacterium* cells were harvested and resuspended in modified Murashige and Skoog salts (10 mM morpholine ethanesulphonic acid pH 5.6, 20 g sucrose l^{-1}, and 200 µM acetosyringone) according to Spolaore *et al.* (2001). Both *Agrobacterium* suspensions were evenly and independently injected with a sterile 1 ml hypodermic syringe into the base of the entire fruits once (*F.* × *ananassa* cv. Elsanta) while they were still attached to the plant 14 days after pollination (Hoffmann *et al*, 2006, G3 stage), the time point at which *FaAAT2* expression starts. About 10–14 days after treatment, the fruits infiltrated with the silencing or the control constructs were harvested and analysed by qRT-PCR and GC-MS. A total of 15–25 strawberry plants and 30–40 agroinjected fruits were inoculated and analysed, respectively. The transitory silencing technique was used as an alternative approach to the stable and permanent transformation (transgenic plants). It has been widely demonstrated that this technique mimics the permanent silencing and gives very clear, repetitive, and positive results (Hoffmann *et al.*, 2006, 2011; Muñoz *et al.*, 2010; Schwab *et al.*, 2011). This system works very similarly to the virus-induced gene silencing technology recently reported and successfully used for functional genomics studies in strawberry fruits (Chai *et al.*, 2011; Jia *et al.*, 2011). Both approaches are also very fast, open, new, and robust possibilities for functional genomics studies in the strawberry fruit ripening process, whereas stable transformation takes 2 years time from the beginning of the process until the plants render fruits.

The percentage of silencing was determined by comparing the amount of *FaAAT2* transcripts in pFRN-*FaAAT2* agroinjected fruits with those observed in pFRN vector agroinfiltrated fruits.

Results

Isolation and sequence analysis of FaAAT2

The complete DNA sequence of the putative full-length *FaAAT2* cDNA (JN089766) was amplified by 3′- and

5′-RACE and contained 1656 bp with an open reading frame (ORF) of 1176 bp encoding a polypeptide of 392 amino acid residues with a predicted molecular mass of 43.2 kDa (Supplementary Fig. S4). Although the length of *FaAAT2* cDNA was shorter than other plant AAT, it was similar to other AAT from different strawberry varieties as *F.x chiloensis* (*FcAAT1*: 1620 bp; ORF 1384; 450 amino acids, 50.4 kDa) and *F.x camarosa* cv. Elsanta (*SAAT*: 1618 bp; 452 amino acids, 50.7 kDa). The sequence also contained 62 and 417 bp of 5′- and 3′-untranscribed region, respectively. Both RACE experiment and bioinformatic analysis of *FaAAT2* cDNA were repeated several times with the same results confirming that the cDNA isolated was a full-length cDNA that contains the entire ORF corresponding to the FaAAT2 protein. The sequence alignment of the putative FaAAT2 protein and other plant acyltransferases showed that all share the characteristic HXXXDG (residues 174–179), DFGWG (residues 422–426), and LXXYYPLAGR (residues 80–89) motifs of the BAHD protein family (Supplementary Fig. S3). In addition, the corresponding *AAT2* full-length cDNA from the wild variety *F. vesca* (*FvAAT2*) was isolated in order to compare *FaAAT2* with its closest orthologue. Sequence comparison of *FaAAT2* and *FvAAT2* showed a sequence similarity of 99.6% (Supplementary Fig. S2).

The phylogenetic tree of selected members of the acyl transferase family provides four main subgroups of protein sequences (Fig. 1). The FaAAT2 protein clustered in subgroup III close to other AATs related to the synthesis of esters in melon (CmAAT1–3), papaya (VpAAT1), and *Clarkia breweri* (CbBEBT). However, the highest similarity at the amino acid level was found between FaAAT2 and MdAAT2 (*Malus domestica*), MpAAT1 (*Malus pumilla*), and PcAAT1 (*P. communis*) with 62, 61, and 62% similarity, respectively.

AAT proteins recently discovered in different strawberry species such as *F.* × *ananassa* cv. Elsanta (SAAT), *F. vesca* (VAAT), and *F. chiloensis* (FcAAT1) belong to a different subgroup than FaAAT2. The comparison of the FaAAT2 sequence with the SAAT, VAAT, and FcAAT1 sequences revealed 21, 22, and 22% similarity at the amino acid level, respectively. These results indicate that the FaAAT2 protein is different from the other AAT proteins previously described in strawberry fruit.

Gene expression studies

Transcript analyses in fruits of the cultivated *F.* × *ananassa* cv. Camarosa variety and the wild *F. vesca* variety showed that *FaAAT2* expression was quite low in the early stages of fruit development followed by a significant increase during the ripening process, which starts at the white stage of receptacle ripening (Fig. 2A and B). The maximum level of *FaAAT2* transcripts was observed in overripe fruit receptacles, with a slight decrease in expression in the senescent stage. However, the relative expression level of *FaAAT2* in fruits of *F. vesca* was much lower than in the cultivated variety. A comparison of *SAAT* and *FaAAT2* transcript levels in fruits of *F.* × *ananassa* cv. Camarosa (Fig. 2A and 2C) and *F. vesca* (Fig. 2B and D) showed that *SAAT* and *FaAAT2* have similar expression patterns but in *F. vesca* the relative expression level of *SAAT* was significantly higher than that of *FaAAT2*.

FaAAT2 and *SAAT* were weakly expressed in achenes (Fig. 3) in all fruit ripening stages. Besides, *FaAAT2* was barely expressed in vegetative tissues and roots (Fig. 4A) but the production of *FaAAT2* transcripts increased dramatically in the ripe fruit receptacle indicating that the expression of *FaAAT2* was quite specific of the fruit receptacle (Fig. 4B).

The comparative gene expression analysis in receptacle of control fruits and deachened green fruits demonstrated a clear increase of *FaAAT2* expression in deachened fruits that was reverted after the external application of auxin (NAA) on deachened fruits (Fig. 5). This result showed that *FaAAT2* expression is negatively regulated by auxins

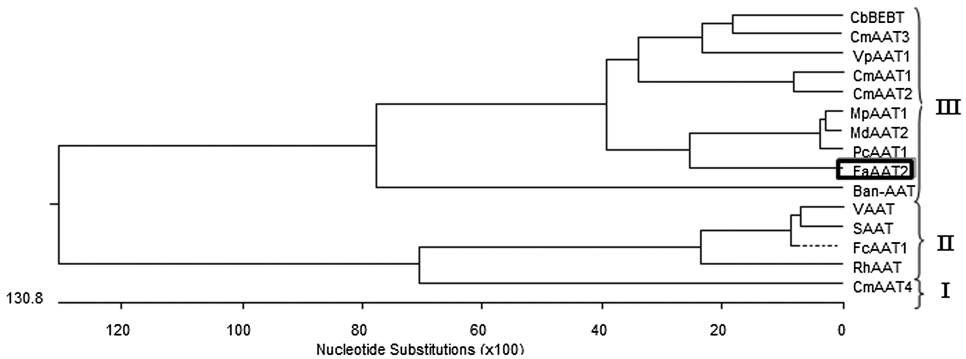

Fig. 1. Phylogenetic analysis of the FaAAT2 protein. Full-length protein sequences of 15 alcohol acyl transferases were aligned using MegAlign for Windows 32 version 5.00 (DNASTAR). The length of each pair of branches represents the distance between sequence pairs and the scale below indicates the number of nucleotide substitutions for both DNA and protein sequences. GenBank accession numbers: CAC09063 (Ban-AAT1), AAN09796 (CbBEBT), CAA94432 (CmAAT1), AAL77060 (CmAAT2), AAW51125 (CmAAT3), AAW51126 (CmAAT4), JN089766 (FaAAT2), FJ548610 (FcAAT1), AAN07090 (FvAAT), AAS79797 (MdAAT2), AAU14879 (MpAAT1), AAS48090 (PcAAT1), AAW31948 (RhAAT), AAG13130 (SAAT), FJ548610 (VpAAT1). Sequences are the same as those used in Supplementary Fig. S3.

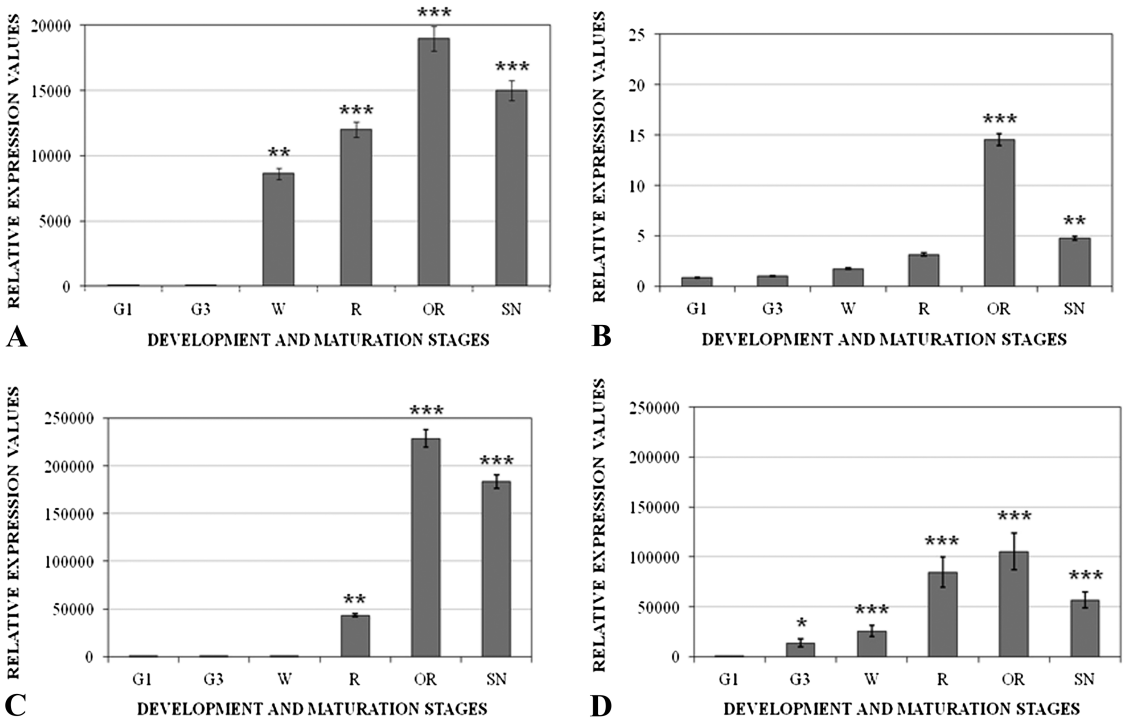

Fig. 2. Developmental expression of strawberry *FaAAT2* (A and B) and *SAAT* (C and D) in fruit receptacles of *Fragaria* × *ananassa* cv. Camarosa (A and C) and *Fragaria vesca* (B and D), respectively. Results were obtained by quantitative real-time PCR using *FaAAT2* and *SAAT* specific primers. Quantification is based on C_t values as described in Materials and Methods. The increase in the mRNA value was relative to the G1 C_t value of each experiment, which was assigned an arbitrary value equal to unity. Values are mean ± SD of five independent experiments. G1, small-sized green fruit; G3, full-sized green fruit (both stages of development); OR, overripe stage; R, red stage; SN, senescent stage; W, white stage. Statistical significance with respect to the reference sample (G1 fruits) was determined by the Student's *t*-test: ** $P < 0.01$, *** $P < 0.001$.

released from the achenes into the fruit receptacle. *FaAAT2* and *SAAT* expression was also analysed in ripe fruits of different strawberry cultivars. *FaAAT2* was highly expressed in fruits of the cultivated variety *F.* × *ananassa* cv. Camarosa, showed intermediate expression levels in the cultivars *F. virginiana* and *F. chiloensis*, and showed the lowest level of expression in the wild variety *F. vesca* (Fig. 6A and B). *SAAT* transcript levels were higher in fruits of *F. virginiana* and *F. vesca* than in *F.* × *ananassa* cv. Camarosa and *F. chiloensis* (Fig. 6C).

Enzymic characterization of the FaAAT2 protein expressed in E. coli

The coding region of the *FaAAT2* cDNA cloned in frame with GST-tag was used to express the recombinant FaAAT2 enzyme in *E. coli*. Protein SDS-PAGE analysis showed a band that could not be detected in the control samples and presumably corresponded to the semi-purified recombinant FaAAT2-GST protein (Supplementary Fig. S4). The molecular mass of the FaAAT2-GST protein was 70 kDa (43 kDa plus 27 kDa of the GST-tag) which is coincident with the estimated size deduced from the full-length cDNA corresponding to *FaAAT2* and, for this reason, the recombinant protein was not sequenced.

Different alcohols were independently fed to *FaAAT2*-expressing *E. coli* to determine whether the recombinant FaAAT2 protein possessed enzymic activity and to clarify its substrate preference (Table 1). When alcohols such as methanol, ethanol, propanol, butanol, pentanol, hexanol, heptanol, octanol, geraniol, eugenol, benzyl alcohol, 2-phenyl ethanol, and cinnamyl alcohol were added, the formation of different types of ester could detected. Esters such as nonyl acetate, decyl acetate, farnesyl acetate, and neryl acetate were never detected. The results showed that the FaAAT2 protein can use endogenous *E. coli* acetyl-CoA when acyl-CoA substrate is not supplied exogenously. Moreover, methyl butanoate, ethyl propanoate, and hexyl hexanoate were also detected (Table 1), indicating that the FaAAT2 protein can also use longer endogenous acyl-CoA molecules as substrates. The FaAAT2 protein was able to produce esters using endogenous *E. coli* acyl-CoAs and 15 different alcohols tested in this study.

To characterize the enzymic activity of FaAAT2 recombinant protein, the FaAAT2-GST fusion protein was semi-purified from an induced crude *E. coli* extract using GST-tag resin and assessed the substrate specificity of the protein *in vitro* by supplying a range of alcohols and acyl-CoAs. The volatile esters produced were analysed by GC-MS. The kinetic properties of FaAAT2 were determined using combinations of 13 alcohols were tested at saturation concentration (20 mM) with four different acyl-CoAs as donors – acetyl-CoA, propionyl-CoA, butyryl-CoA, and hexanoyl-CoA – at

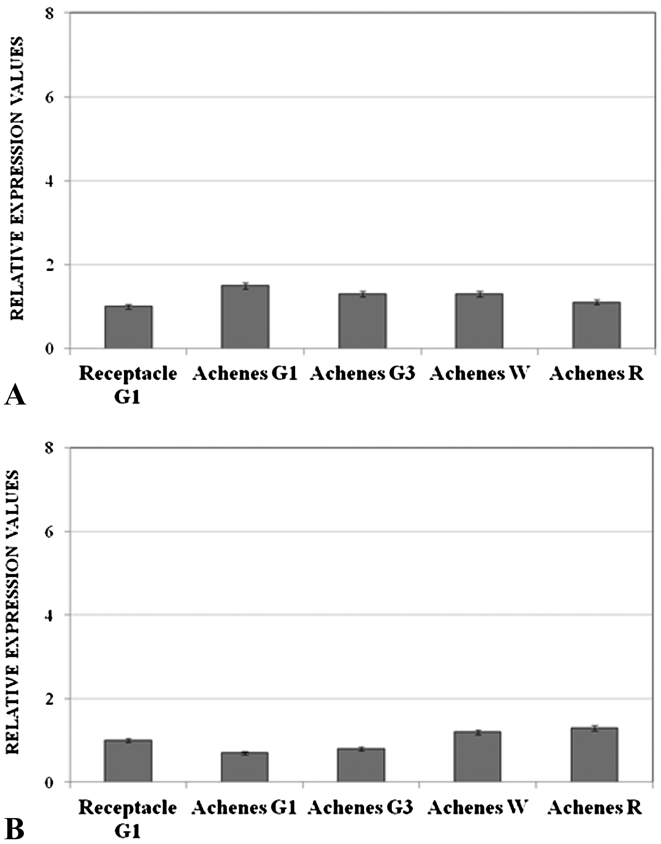

Fig. 3. Developmental expression of strawberry *FaAAT2* (A) and *SAAT* (B) in achenes of *Fragaria* × *ananassa* cv. Camarosa assessed by quantitative real-time PCR using *FaAAT2* and *SAAT* specific primers. Quantification is based on C_t values as described in Materials and Methods. The increase in the mRNA value was relative to the receptacle G1 C_t value of each experiment, which was assigned an arbitrary value equal to unity. Values are mean ± SD of five independent experiments. G1, small-sized green fruit; G3, full-sized green fruit (both stages of development); R, red stage; W, white stage.

different concentrations (Table 2). The specificity constant for acetyl-CoA was highest when cinnamyl alcohol was used as co-substrate (K_{cat}/K_m 50.53 s^{-1} μM^{-1}), whereas in the presence of hexanol and octanol K_{cat}/K_m was 42.95 s^{-1} μM^{-1} and 18.92 s^{-1} μM^{-1}, respectively. Much lower specificity constants were observed for propionyl-CoA, butyryl-CoA, and hexanoyl-CoA, independent of the alcohol used, except for eugenol. Thus, when hexanol was used as co-substrate, the specificity constant for acetyl-CoA was 42.95 s^{-1} μM^{-1}, and 5.85, 0.515, and 0.215 s^{-1} μM^{-1} for propionyl-CoA, butyryl-CoA, and hexanoyl-CoA, respectively. This suggests that acetyl-CoA is the preferred acyl donor of FaAAT2 *in vivo* (Table 3).

To study the specificity of FaAAT2 for the alcohol substrates, different concentrations of alcohols were used in combination with acetyl-CoA at saturated concentration (0.1 mM) (Table 4). The specificity constant was highest for cinnamyl alcohol (727 s^{-1} mM^{-1}) followed by hexanol (314 s^{-1} mM^{-1}), and octanol (208 s^{-1} mM^{-1}).

Determination of alcohols and volatile esters from strawberry fruit

The analysis of esters emitted during strawberry fruit ripening showed that the highest concentration of esters was always detected in the red fruit stage in *F.* × *ananassa* cv. Camarosa and *F. vesca* (Table 5). The most abundant esters were acetates, followed by butanoates and hexanoates. Butyl acetate, ethyl acetate, ethyl butanoate, and ethyl hexanoate are the esters which contribute to the strawberry fruit aroma (Jetti *et al.*, 2007). The major esters detected in *F. vesca* were octyl acetate, hexyl acetate, and butyl butanoate. In contrast, the most abundant esters detected in *F.* × *ananassa* cv. Camarosa were methyl butanoate, methyl acetate, methyl hexanoate, and hexyl acetate. Some of the esters present in *F. vesca* (e.g. ethyl acetate, pentyl acetate, heptyl acetate, and myrtenyl acetate) did not appear in *F.* × *ananassa*, and other esters present in the commercial variety (e.g. methyl acetate, methyl propionate, and butyl acetate) were not detected in the wild variety. The concentration of many alcohols increased or remained constant during strawberry fruit ripening, correlating with increased levels of their respective esters. Thus, ethanol and octanol appeared in the red fruit stage of *F. vesca* whereas the hexanol concentration increased during ripening. The same holds for butanol, hexanol, and octanol in *F.* × *ananassa* cv. Camarosa. On the other hand, the level of alcohols such as heptanol and nonanol decreased during fruit development in *F. vesca*. These alkanols could not be detected in red fruits of *F.* × *ananassa* cv. Camarosa.

Silencing of FaAAT2 *by agroinfiltration*

The expression of *FaAAT2* in strawberry fruit underwent a transitory downregulation by RNAi. The analysed transgenic fruits showed an important reduction in *FaAAT2* expression while *SAAT* expression remained constant (Fig. 7). Moreover, the production of volatile esters such as ethyl acetate, methyl acetate, methyl butanoate, methyl 2-methylbutanoate, methyl 2-methylbutanoate, methyl ethyl hexanoate, butyl acetate, and hexyl acetate was reduced in *FaAAT2*-silenced fruits (Table 6).

Discussion

Since volatile esters play a fundamental role in developing the characteristic strawberry aroma during ripening, this study was focused on the novel strawberry alcohol acyl-CoA transferase 2 *FaAAT2*, a gene which encodes an important enzyme involved in fruit aroma biogenesis (Pérez *et al.*, 1993, 1996; Aharoni *et al.*, 2000; Olías *et al.*, 2002; Beekwilder *et al.*, 2004; González *et al.*, 2009). *FaAAT2* shares the characteristic motifs of the BAHD class III acyltransferases involved in the production of volatile ester in flowers and ripening fruits. The HXXXDG motif, corresponding to residues 174–179 (Supplementary Fig. S3), is shared by several other families of enzymes that use Co-A thioesters and seems to be involved in catalysis (St-Pierre and

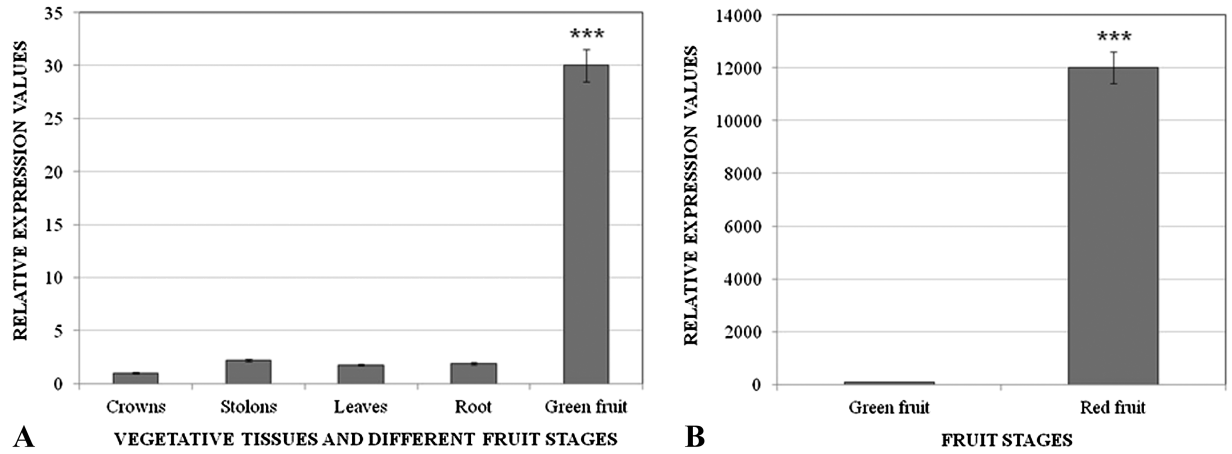

Fig. 4. Analysis by quantitative real-time PCR of strawberry *FaAAT2* expression in vegetative tissues and different fruit stages (A) and strawberry receptacles of mature (red) and immature (green) fruits (B). The results were obtained using *FaAAT2* specific primers. Quantification is based on C_t values as described in Materials and Methods. The increase in the mRNA values was relative to crown tissue, which had the lowest *FaAAT2* expression, and was assigned an arbitrary value equal to unity. Statistical significance with respect to the reference sample (crown tissue) was determined by the Student's *t*-test: *** $P < 0.001$.

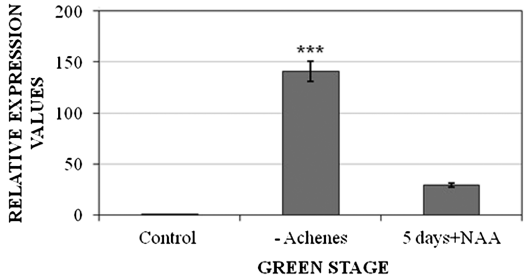

Fig. 5. Analysis of the effects of removing achenes from G2 developing fruits and their treatment with auxins on *FaAAT2* expression by quantitative real-time PCR. After auxin treatment, the increase in mRNA value was relative to G2 fruit (control), which was assigned an arbitrary value equal to unity. Control, middle-sized green fruit receptacle (G2 fruit); –Achenes, G2 fruit receptacle without achenes for 5 days; 5 days + NAA, G2 fruit receptacle without achenes plus 1-naphthalenacetic acid for 5 days (added at day 0). Statistical significance with respect to the control sample (G2 fruits) was determined by the Student's *t*-test: *** $P < 0.001$.

De Luca, 2000; D'Auria, 2006). However, although in FaAAT2 the histidine and aspartic acid residues are conserved, the glycine residue is substituted by alanine. This replacement occurs in other plant AAT such as CbBEBt from *Clarkia breweri* (D'Auria *et al.*, 2002), VpAAT1 from *V. pubescens* (Balbontín *et al.*, 2010), and CmAAT3 from *Cucumis melo* (El-Sharkawy *et al.*, 2005); while the glycine residue is substituted by methionine in BEAT from *Clarkia breweri*. The second conserved motif of the FaAAT2 protein is the DFGWG motif, corresponding to residues 422–426 (Supplementary Fig. S3), which is located near the C-terminus and seems to play a role in maintaining the enzyme structure (El-Sharkawy *et al.*, 2005; D'Auria, 2006). This motif shows the substitution of a tryptophan residue by phenylalanine. In addition, the FaAAT2 protein has a third consensus sequence, LXXYYPLAGR (residues 80–89), previously described in other AAT proteins such as SAAT and BEAT (Aharoni *et al.*, 2000), and VpAAT1 (Balbontín *et al.*, 2010). Although less conserved than the previous ones, this motif is located at the N-terminus and it is common among AAT involved in the synthesis of volatile compounds in fruits and flowers (Balbontín *et al.*, 2010). Nevertheless, its function could also be related with the use of acetyl-CoA as co-substrate during the enzymic reaction (Aharoni *et al.*, 2000).

Moreover, the majority of BAHD class III members accept a diverse range of alcohol substrates and utilize acetyl-CoA as the major acyl donor, a behaviour that matches with the strawberry FaAAT2 protein. Like BanAAT1 from banana, the FaAAT2 protein has preference for the formation of cinnamyl and geranyl acetate (Beekwilder *et al.*, 2004). However, it can also produce benzyl acetate as the CmAAT3 from *Cucumis melo* and the CbBEBT and CbBEAT (both from *Clarkia)*, all of which are included into subgroup III in the phylogenetic analysis (Dudareva *et al.*, 1998; D'Auria *et al.*, 2002). Contrary to what one may expect, other AAT proteins isolated from strawberry fruit, such as SAAT, VAAT, and FcAAT1 (Aharoni *et al.*, 2000; Beekwilder *et al.*, 2004; González *et al.*, 2009) were included in the subgroup II which is phylogenetically more distant, although SAAT and VAAT share with FaAAT2 the ability to produce geranyl acetate and other esters present in strawberry fruit, such as hexyl acetate and octyl acetate (Aharoni *et al.*, 2000; Beekwilder *et al.*, 2004).

SAAT and *FaAAT2* transcript levels increase dramatically during the late stages of fruit ripening in the receptacles (Fig. 2) and their expression is negatively regulated by auxins (Fig. 5, Aharoni *et al.*, 2000). This expression pattern is coincident with those reported previously for other strawberry ripening-related genes, many of them related with the organoleptic properties of the strawberry fruit (Medina-Escobar *et al.*, 1997; Moyano *et al.*, 1998; Trainotti *et al.*, 1999; Benítez-Burraco *et al.*, 2003; Blanco-Portales *et al.*, 2002, 2004; Raab *et al.*, 2006).

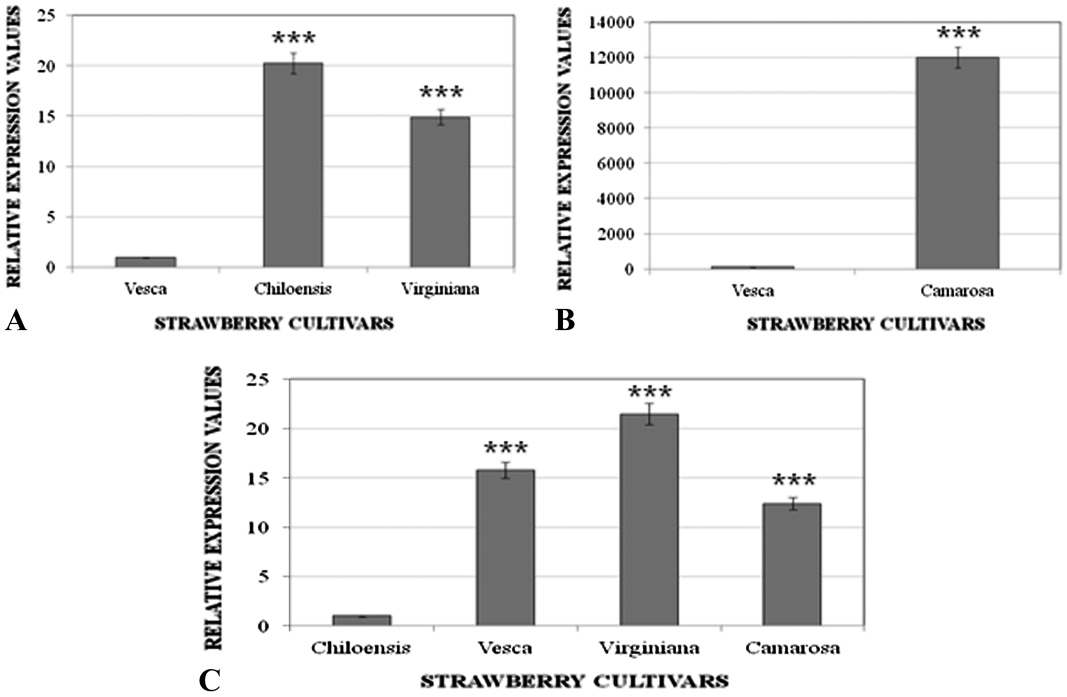

Fig. 6. Analysis by quantitative real-time PCR of *FaAAT2* (A and B) and *SAAT* (C) expression in different strawberry cultivars. The results were obtained using *FaAAT2* and *SAAT* specific primers for each gene. Quantification is based on C_t values as described in Materials and Methods. The increase in the mRNA value was relative to the mRNA level found in *Fragaria vesca*, which was assigned an arbitrary value of unity. Statistical significance with respect to reference sample (*F. vesca*) was determined by the Student's *t*-test: *** $P < 0.001$.

However, a comparative study of *FaAAT2* and *SAAT* expression in different strawberry cultivars throughout fruit ripening also showed significant quantitative differences (Fig. 2). Cultivar-dependent AAT activity was also demonstrated in four different varieties of strawberry (Pérez *et al.*, 1996). In these varieties, AAT activity clearly increased in the later fruit maturation stages, being much higher in the Chandler and Oso Grande varieties than in variety I-101 (Pérez *et al.*, 1996). The low AAT activity in the I-101 variety could be correlated with the poor flavour of its fruits (López-Aranda *et al.*, 1995), suggesting that AAT activity influences the aroma level of strawberry fruits.

Among the substrates found in strawberry fruits, the FaAAT2 protein has preference for C6–10 alcohols, being most active with hexanol followed by octanol and heptanol (Tables 2 and 4). The specificity and activity are lower with short-chain alcohols. This preference of FaAAT2 for substrates is quite similar to the preference of the partially purified AAT from the Chandler variety, which preferably uses hexanol and shows lower activity with short-chain alcohols (the activity with ethanol was 25% compared to hexanol) (Pérez *et al.*, 1993). Moreover, both enzymes show higher activity when using linear rather than branched-chain alcohols with the same number of carbons, like in the yeast AAT (Yoshioka and Hasimoto, 1981). In contrast, the SAAT protein (*F.* × *ananassa* cv. Elsanta) also accepts longer-chain alcohols as substrates and is active with heptanol and octanol but less with nonanol and decanol with respect to hexanol (Aharoni *et al.*, 2000). FaAAT2, like the SAAT protein, did not have significant activity with unsaturated C6 derivatives. The same behaviour was observed with the C3 isomer 2-propanol, the C4 isomer 2-butanol, and the acyclic monoterpene alcohol linalool.

Recently, other plant AAT proteins have been characterized. The VAAT (*F. vesca*) protein shows a preference for small aliphatic alcohols (C4–6) (Beekwilder *et al.*, 2004), in contrast to SAAT and FaAAT2. However, while VAAT and SAAT had only a modest activity with cinnamyl alcohol as substrate, this compound is the best substrate for BanAAT (Beekwilder *et al.*, 2004) and FaAAT2. These results suggest that SAAT, VAAT, and FaAAT2 can clearly produce esters such as hexyl acetate and octyl acetate present in both wild and cultivated strawberry varieties, and therefore may well be implicated in determining the aroma of strawberry fruits. Geraniol is the preferred alcohol substrate for RhAAT from hybrid rose (*Rosa* × *hybrida*) being the presence of geranyl acetate in the rose flowers associated with RhAAT activity (Shalit *et al.,* 2003). Although SAAT (Beekwilder *et al.*, 2004) and FaAAT2 showed high substrate specificity for geraniol (Tables 2 and 4), the strawberry fruit is devoid of geranyl acetate (Table 5). Other studies performed with partially purified AAT from different commercial strawberry varieties showed an activity pattern similar to FaAAT2, a higher affinity for the straight-chain alcohols (C2–7) and a lower activity with butanol (Olías *et al.*, 2002). Moreover, Olías *et al.* (2002) proved that hexanol was the preferred alcohol for American cultivars (Camarosa and Sweet Charlie), which is consistent with the present results for the FaAAT2 protein (Table 4). However, it is now obvious that strawberry has more than one AAT enzyme,

Table 1. Alcohol substrates used with acetyl-CoA in the FaAAT2 recombinant enzyme assay and esters produced by semi-purified FaAAT2

Enzyme assays were performed with the recombinant FaAAT2 protein before purification in *Escherichia coli* and after semi-purification. In all assays, the empty vector pGEX-T4 was used as control. The esters indicated were produced by the activity of the FaAAT2 recombinant enzyme and not by the internal alcohol acyl transferase from *E. coli*. nd, not determined. Esters produced from strawberry reported by Zabetakis and Holden (1997) and Pyysalo *et al*. (1979).

Alcohol added	Carbon structure	Esters expected	Esters produced			Esters produced from strawberry
			Before purification	After purification	Control	
Methanol	1:0	Methyl acetate	+	+	–	+
		Methyl propanoate	+	+	–	+
		Methyl butanoate	+	+	–	+
		Methyl hexanoate	+	+	–	+
Ethanol	2:0	Ethyl acetate	+	+	–	+
		Ethyl butanoate	+	+	–	+
		Ethyl hexanoate	+	+	–	+
		Ethyl heptanoate	+	nd	–	+
Propanol	3:0	Propyl acetate	+	+	–	–
		Propyl propanoate	–	–	–	–
		Propyl butanoate	–	–	–	–
		Propyl hexanoate	–	–	–	–
2-Propanol	3:0	2-Propyl acetate	–	–	–	+
Butanol	4:0	Butyl acetate	+	+	–	+
		Butyl propanoate	+	+	–	–
		Butyl butanoate	+	+	–	+
		Butyl hexanoate	+	+	–	+
		Butyl octanoate	+	nd	–	+
2-Butanol	4:0	2-Butyl acetate	–	–	–	+
3-Methylbutanol	5:0	3-Methylbutyl acetate	–	–	–	+
Pentanol	5:0	Pentyl acetate	+	+	–	+
		Pentyl propanoate	–	–	–	–
		Pentyl hexanoate	–	–	–	+
Hexanol	6:0	Hexyl acetate	+	+	–	+
		Hexyl propanoate	+	+	–	+
		Hexyl butanoate	+	+	–	+
		Hexyl hexanoate	+	+	–	+
		Hexyl octanoate	+	nd	–	+
Hex-3(Z)-enol	6:1	Hexen-3(Z)-enyl acetate	–	–	–	+
Hex-2(E)-enol	6:1	Hexen-2(E)-enyl acetate	–	–	–	+
Heptanol	7:0	Heptyl acetate	+	+	–	–
		Heptyl butanoate	+	+	–	–
Octanol	8:0	Octyl acetate	+	+	–	+
		Octyl propanoate	+	+	–	–
		Octyl butanoate	+	+	–	+
		Octyl hexanoate	+	+	–	+
Oct-1-en-3-ol	8:1	Oct-1-en-3-yl acetate	–	–	–	–
Nonanol	9:0	Nonyl acetate	–	–	–	–
Decanol	10:0	Decyl acetate	–	–	–	+
Geraniol	10:2	Geranyl acetate	+	+	–	–
Linalool	10:2	Linalyl acetate	–	–	–	–
Farnesol	15:3	Farnesyl acetate	–	–	–	–
Benzyl alcohol	7:3	Benzyl acetate	+	+	–	+
Nerol	10:2	Neryl acetate	–	–	–	–
Nerolidol	15:3	Nerolidyl acetate	–	–	–	–
Eugenol	10:4	Eugenyl acetate	+	+	–	–
Furfuryl alcohol	5:2	Furfuryl acetate	–	–	–	–
2-Phenylethanol	6:3	2-Phenylethyl acetate	+	+	–	+
		2-Phenylethyl propanoate	+	+	–	+
		2-Phenylethyl butanoate	+	+	–	–
Cinnamyl alcohol	9:4	Cinnamyl acetate	+	+	–	–

Table 2. Specificity constants of semi-purified recombinant FaAAT2 for different alcohols in combination with different acyl-CoA molecules

Values are K_{cat}/K_m (s^{-1} μM^{-1}) Alcohols were used at saturated concentration (20 mM) while the acyl-CoAs were used at variable concentrations (0.01, 0.1, 0.25, 1, and 2.5 mM). nd, ester not detected.

Alcohol	Acetyl-CoA	Propionyl-CoA	Butyryl-CoA	Hexanoyl-CoA
Methanol	1.05	0.11	0.53	0.17
Ethanol	0.16	0.14	0.03	0.27
Propanol	3.41	nd	nd	nd
Butanol	7.51	0.02	0.04	0.18
Pentanol	10.49	nd	nd	nd
Hexanol	42.95	5.85	0.515	0.215
Heptanol	9.98	2.22	nd	nd
Octanol	18.92	6.14	0.05	0.06
Geraniol	33.41	3.25	5.07	5.50
Benzyl alcohol	0.25	1.85	0.09	0.39
Eugenol	12.1	33.41	0.10	nd
2-Phenylethanol	0.43	0.37	0.23	0.13
Cinnamyl alcohol	50.53	5.00	1.58	0.68

Table 3. Kinetic parameters of semi-purified recombinant FaAAT2 for different alcohols in combination with acetyl-CoA at variable concentrations

Alcohols were used at saturated concentration (20 mM) while the co-substrate 1 acetyl-CoA was used at variable concentrations (0.01, 0.1, 0.25, 1, and 2.5 mM).

Alcohol	K_m (μM)	V_{max} (nmol s^{-1} (mg prot)$^{-1}$)	K_{cat} (V_{max}/conc. enzyme)
Methanol	4.0 ± 1.4	0.08 ± 0.01	4.2
Ethanol	8.0 ± 0.7	0.02 ± 0.01	1.2
Propanol	28.0 ± 0.8	0.2 ± 0.0	57.3
Butanol	29.0 ± 0.2	0.4 ± 0.1	217
Pentanol	26.0 ± 0.4	0.5 ± 0.0	272
Hexanol	5.4 ± 0.3	0.4 ± 0.1	231
Heptanol	38.0 ± 0.1	0.7 ± 0.1	375
Octanol	36.0 ± 0.9	1.3 ± 0.7	681
Geraniol	49.0 ± 0.7	0.8 ± 0.2	391
Benzyl alcohol	26.0 ± 1.4	0.1 ± 0.01	6.5
Eugenol	31.0 ± 0.2	0.7 ± 0.1	376
2-Phenyl ethanol	53.0 ± 0.9	0.04 ± 0.02	23
Cinnamyl alcohol	77.0 ± 0.1	7.8 ± 0.9	3890

Table 4. Kinetic parameters of semi-purified recombinant FaAAT2 for different alcohols at variable concentrations in combination with acetyl-CoA

Alcohols were used at variable concentrations (1, 5, 10, 20, and 60 mM) while the acetyl-CoA was used at a fixed concentration (0.1 mM).

Alcohol	K_m (mM)	V_{max} (nmol s^{-1} (mg prot)$^{-1}$)	K_{cat} (V_{max}/conc. enzyme)	K_{cat}/K_m (s^{-1} mM^{-1})
Methanol	4.05 ± 0.07	0.03 ± 0.01	18.5	4.5
Ethanol	4.35 ± 0.05	0.01 ± 0.00	7.5	1.7
Propanol	2.40 ± 0.27	0.03 ± 0.01	17.5	7.2
Butanol	2.34 ± 0.31	0.04 ± 0.02	21.5	9.1
Pentanol	5.78 ± 0.87	0.81 ± 0.24	407	71.0
Hexanol	1.53 ± 0.02	0.96 ± 0.45	483	314
Heptanol	2.71 ± 0.08	0.34 ± 0.12	170	62
Octanol	2.23 ± 0.02	0.93 ± 0.05	466	208
Benzyl alcohol	4.57 ± 0.89	0.04 ± 0.01	23.5	5.14
2-Phenyl ethanol	4.32 ± 0.76	0.09 ± 0.01	49.6	11.4
Eugenol	3.78 ± 0.45	1.09 ± 0.04	548.5	150
Geraniol	2.04 ± 0.32	0.74 ± 0.01	370.5	180
Cinnamyl alcohol	2.03 ± 0.78	2.81 ± 0.54	1480	727

therefore the results of Olías *et al.* (2002) could be due to a mixture of AAT enzymes rather than to one specific AAT. Thus, these coincidences must be carefully considered.

The preference of FaAAT2 for shorter acyl-CoA molecules as substrate in combination with aliphatic C6–8 alcohols (Table 2) is similar to the partially purified AAT activity from the Chandler cultivar, which uses acetyl-CoA as preferred substrate (Pérez *et al.*, 1993), while the SAAT activity prefers long acyl-CoAs combined with aliphatic C4–9 alcohols as substrates (Aharoni *et al.*, 2000). Similarly, the AAT activity characterized from the Oso Grande cultivar shows increasing preference for acyl-CoA molecules with increasing numbers of carbons, reaching its highest activity with hexanoyl-CoA (Olías *et al.*, 2002).

The specificity constant (K_{cat}/K_m) of FaAAT2 for acetyl-CoA was higher with cinnamyl alcohol and hexanol as co-substrates than with octanol (Table 2), despite the fact that both hexanol and octanol are used in the formation of volatiles in strawberry fruits. Thus, the FaAAT2 enzyme seems to be more efficient in the production of cinnamyl acetate and hexyl acetate than of octyl acetate. Comparison of the kinetic parameters of FaAAT2 with those of other proteins reported from strawberry (Pérez *et al.*, 1996; Aharoni *et al.*, 2000; Olías *et al.*, 2002) clearly show that FaAAT2 differs from AAT proteins reported from other varieties of strawberry until now.

In general, the wide range of acyl-CoAs and alcohol substrates available in strawberry is related to the great variety of esters found in this fruit (Zabetakis and Holden, 1997). However, this cannot fully explain the differences in composition of esters between the different developing stages and the different cultivars of strawberry (Aharoni *et al.*, 2000). In this sense, the availability of substrates depending on the activity of several catabolic pathways, such as lipid breakdown, could determine the ester composition in the strawberry fruits (Ueda and Ogata, 1976).

Despite the fact that the maxima of AAT activity and gene expression usually appear in the later maturation stages (Aharoni *et al.*, 2000; González *et al.*, 2009), AAT activity has also been detected in white fruits in the Chandler cultivar (Pérez *et al.*, 1996). Indeed, some esters, like hex-3-enyl acetate which are present in green-stage and white-stage fruits of the Camarosa cultivar have been detected in early developmental stages (Table 5).

Table 5. GC-MS analysis of alcohols and volatile esters present in red ripe strawberry fruit from *Fragaria* × *ananassa* cv. Camarosa and *Fragaria vesca* varieties

Values are given in pmol l^{-1}. G, green strawberry fruits in different development stages; R, red strawberry fruits; W, white strawberry fruits.

	F. vesca			***F.* × *ananassa* cv. Camarosa**				
	G	**W**	**R**	**G1**	**G2**	**G3**	**W**	**R**
Ethyl acetate			3.2 ± 0.3			15.9 ± 4.6	18.5 ± 3.7	
Ethanol			5.8 ± 1.2	9.1 ± 2.0	6.7 ± 1.3	6.8 ± 1.9		
Methyl acetate								16 ± 5
Methyl propanoate								0.7 ± 0.1
Methyl butanoate			4.7 ± 0.3					78.3 ± 4.1
Methyl 3-methylbutanoate								3.5 ± 0.3
Ethyl butanoate			60 ± 7					2.1 ± 0.5
Butanol								0.3 ± 0.1
2-Butanol				0.5 ± 0.1				
1-Methylethyl butanoate								9.9 ± 1.7
Butyl butanoate			30.1 ± 9.2					1.2 ± 0.2
Butyl acetate								2.2 ± 0.9
2-Methylbutyl acetate				2.3 ± 1.2				1.4 ± 0.9
3-Methylbutyl acetate								1.7 ± 0.7
3-Methylbutyl butanoate								0.3 ± 0.0
Propyl butanoate			0.7 ± 0.3					
Methyl 4-methyl pentanoate								0.2 ± 0.0
Pent-1-en-3-ol	6.3 ± 1.3	1.7 ± 0.8		3.4 ± 0.3	3.8 ± 2.5	3.1 ± 2.0	1.8 ± 0.1	0.3 ± 0.0
Pent-2(Z)-enol	3.4 ± 1.2	1.6 ± 0.5			2.1 ± 0.8	1.5 ± 0.6	0.9 ± 0.0	0.4 ± 0.0
Methyl hexanoate			18.0 ± 5.1					14.0 ± 3.1
Ethyl hexanoate			61.0 ± 8.5					1.9 ± 0.9
3-Methylbut-2-enyl acetate			3.0 ± 0.4					
3-Methylbut-3-enyl acetate	1.8 ± 0.7							
Pentanol	8.3 ± 1.3	2.8 ± 0.9	3.4 ± 0.1	2.3 ± 0.0	2.0 ± 1.0	3.7 ± 0.7	8.0 ± 0.3	7.6 ± 0.3
2-Pentanol								
Pentyl acetate			1.7 ± 0.3	3.3 ± 1.1				
2-Heptanyl acetate			13 ± 2					
Hexyl acetate			97 ± 10					4.2 ± 1.7
Hex-2(E)-enyl acetate			45.0 ± 4.6					1.4 ± 0.0
Hex-3(Z)-enyl acetate			23 ± 7	3.4 ± 1.0		0.2 ± 0.0	0.5 ± 0.1	0.3 ± 0.0
Heptanol	2.9 ± 0.9							
3-Ethyl-4-methyl pentanol	3.3 ± 0.1							
Heptyl butanoate			0.9 ± 0.1					
Heptyl acetate			0.4 ± 0.0					
Hexanol	4.0 ± 0.8	4.5 ± 0.5	13 ± 2	1.7 ± 0.4	1.4 ± 0.2	1.5 ± 0.0	0.8 ± 0.0	1.1 ± 0.0
Hex-3(Z)-enol	8.9 ± 1.8	6.8 ± 1.7	2.8 ± 0.6	1.3 ± 0.0	10.1 ± 2.0	9.7 ± 0.3	2.0 ± 0.6	0.6 ± 0.0
Hex-2(E)-enol			14 ± 2	1.5 ± 0.2	1.4 ± 0.0	1 ± 0.1	0.9 ± 0.0	
Hex-3(Z)-enylbutanoate			0.3 ± 0.1					
Butyl hexanoate			4.5 ± 1.0					0.1 ± 0.0
Hexyl butanoate			1.5 ± 0.5					0.3 ± 0.6
3-Methyl butyl butanoate								0.2 ± 0.1
Ethyl octanoate			4.4 ± 0.2					
Hex-2(E)-enyl acetate								3.7 ± 0.7
Non-2-enol				2.3 ± 0.2			0.1 ± 0.0	2.7± 0.1
Octyl acetate			273 ± 15					0.2 ± 0.0
Hex-2(E)-enyl butanoate			4.1 ± 0.7					
Nonanol	2.2 ± 0.8	1.1 ± 0.1	0.5 ± 0.1					
2-Nonanol			96 ± 16					
Linalool	5.4 ± 1.2	1.1 ± 0.8	1.3 ± 0.3	9.0 ± 0.3	7.1 ± 0.6	2.2 ± 0.1	0.4 ± 0.1	7.5 ± 1.2
Methyl decanoate			1.3 ± 0.7					
Octanol			72 ± 7				0.1 ± 0.0	1.3 ± 0.7
Oct-1-en-3-ol	4.11 ±0.2	9.27 ± 2.1		1.2 ± 0.3	1.8 ± 0.7	2.6 ± 0.4	3.2 ± 1.1	3.2 ± 0.6
Octyl butanoate			18 ± 4					0.3 ± 0.0

Table 5. *Continued*

	F. vesca			***F.* × *ananassa* cv. Camarosa**				
	G	**W**	**R**	**G1**	**G2**	**G3**	**W**	**R**
Phenyl ethanol	3.9 ± 0.6			0.2 ± 0.0	0.1 ± 0.0			
Benzyl acetate								0.3 ± 0.0
Benzyl decanoate			5.4 ± 1.2					0.7 ± 0.1
Benzyl alcohol	5.2 ± 0.3	1.6 ± 0.5		3.9 ± 1.0	1.5 ± 0.8	1.1 ± 0.0	0.4 ± 0.0	
Myrtenol	2.2 ± 0.4	2.1 ± 0.6	5.9 ± 1.9	1.5 ± 0.7	0.3 ± 0.1	0.2 ± 0.0		
Myrtenyl acetate			28 ± 6					

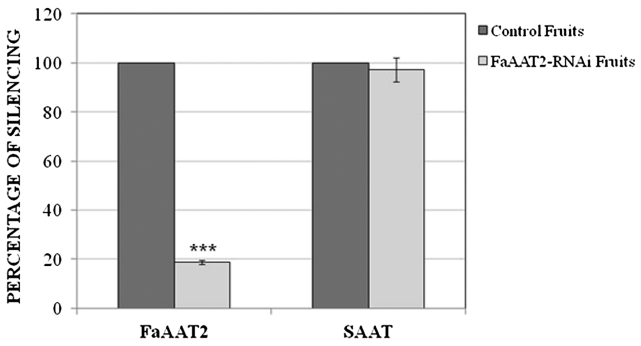

Fig. 7. Analysis by quantitative real-time PCR of *FaAAT2* and *SAAT* expression in transgenic strawberry fruits agroinfiltrated with the pFRN-*FaAAT2* construct and in control fruits only agroinfiltrated with the empty pFRN vector. The silencing level is expressed as a percentage. Statistical significance was determined by the Student's *t*-test: *** $P < 0.001$.

Nevertheless, the main increase of fruit esters occurs in the red stage where especially acetates, such as hexyl acetate and octyl acetate, have been detected. Although both acetates appear in commercial and wild varieties, the amount of hexyl acetate synthesized in *F.* × *ananassa* cv. Camarosa is greater than the amount of octyl acetate, probably due to the specificity of the FaAAT2 enzyme for hexanol and acetyl-CoA substrates and the high levels of expression of *FaAAT2* in this variety (Fig. 6). By contrast, the amount of octyl acetate is higher than hexyl acetate in *F. vesca*, suggesting the involvement of another AAT such as SAAT in the synthesis of esters. In general, these data seem to indicate that the amount of esters present in the fruits depends on the availability of substrates and specificity of the AAT enzyme(s). On the other hand and according to the expression patterns, the absence of esters in immature stages of the strawberry fruit could be associated with the absence of precursors and the low activity of alcohol acyl transferase activity (Yamashita *et al.*, 1977).

Volatile ester production was significantly reduced in strawberry fruits after transient downregulation of *FaAAT2* expression (Table 6). This reduction of volatile esters is probably only caused by the decrease of the alcohol acyl transferase activity due to silencing of *FaAAT2* as *SAAT* expression remained constant (Fig. 7). This suggests that FaAAT2 is involved in the formation of esters, such as ethyl acetate, methyl acetate, methyl butanoate, methyl-2/3-methyl butanoate, ethyl butanoate, and ethyl hexanoate, implicated in the aroma of strawberry fruit. On the other hand, the levels of ethanol and hexanol also decreased drastically in transiently silenced fruits (Table 6). Enhanced metabolism and/or decreased production of alcohols could be a consequence of the silencing process. However, the underlying mechanism remains elusive.

Table 6. GC-MS quantification of alcohols and volatile esters in strawberry red fruit cv. Elsanta with *FaAAT2* expression silenced by agroinfiltration

Values are given in ppm. Three biological replicates were used.
* $P < 0.05$; ** $P < 0.01$.

	***FaAAT2* silenced**	**Control**
Ethyl acetate	0.50 ± 0.03*	1.04 ± 0.27
Methyl acetate	0.60 ± 0.36**	3.26 ± 1.01
Methyl butanoate	0.54 ± 0.30*	1.35 ± 0.09
Methyl-2/3-methyl butanoate[a]	0.87 ± 0.58*	3.01 ± 1.15
Ethyl butanoate	0.05 ± 4.47*	0.89 ± 0.54
Methyl hexanoate	2.65 ± 1.06	4.57 ± 1.81
Ethyl hexanoate	0.78 ± 0.45*	3.33 ± 1.75
Butyl acetate	0.02 ± 0.00	0.11 ± 0.07
Hexyl acetate	0.13 ± 0.33	0.70 ± 0.17
Ethanol	4.25 ± 1.22**	47.53 ± 5.85
Hexanol	0.66 ± 0.08	2.61 ± 0.77

[a] Methyl-2/3-methyl butanoate stands for the co-elution of methyl 2-methyl butanoate and methyl 3-methyl butanoate quantified with the internal standard method assuming a response factor of one.

In conclusion, this study suggests that *FaAAT2* is involved in the synthesis of strawberry fruit volatiles. This theory is supported by its maximum gene expression during ripe stages and the increase in its activity throughout the ripening process in parallel with the production of esters. Factors such as substrate availability, participation of other AAT proteins, and mechanisms of gene regulation are other fundamentals that determine the characteristic aroma of strawberry fruits.

Supplementary material

Supplementary data are available at *JXB* online.

Supplementary Fig. S1. Alignment of the partial cDNA sequences of *FaAAT2* and *SAAT* corresponding to the RNAi fragment used for the transitory *FaAAT2* silencing

Supplementary Fig. S2. Alignment of the cDNA nucleotide sequences (A) and protein sequences (B) of *FaAAT2* (*F.* × *ananassa* cv. Camarosa) and *FvAAT2* (*F. Vesca*)

Supplementary Fig. S3. Alignment of the deduced FaAAT2 amino acid sequence with other members of the BADH family acyltransferases of known function from different floral and fruit species

Supplementary Fig. S4. Purification of the FaAAT2 protein from induced *E. coli* culture

Supplementary Table S1. Properties of qRT-PCR amplicons used for relative quantification of mRNA

Supplementary Table S2. Gene expression values of different housekeeping genes obtained by qRT-PCR analysis

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