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An alcohol acyl transferase from apple (cv. Royal Gala), MpAAT1, produces esters involved in apple fruit flavor

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Apple flavor is characterized by combinations of ester compounds, which increase markedly during fruit ripening. The final step in ester biosynthesis is catalyzed by alcohol acyl transferases (AATs) that use coenzyme A (CoA) donors together with alcohol acceptors as substrates. The gene *MpAAT1*, which produces a predicted protein containing features of other plant acyl transferases, was isolated from *Malus pumila* (cv. Royal Gala). The *MpAAT1* gene is expressed in leaves, flowers and fruit of apple. The recombinant enzyme can utilize a range of alcohol substrates from short to medium straight chain (C3–C10), branched chain, aromatic and terpene alcohols. The enzyme can also utilize a range of short to medium chain CoAs. The binding of the alcohol substrate is rate limiting compared with the binding of the CoA substrate. Among different alcohol substrates there is more variation in turnover compared with K_m values. MpAAT1 is capable of producing many esters found in Royal Gala fruit, including hexyl esters, butyl acetate and 2-methylbutyl acetate. Of these, MpAAT1 prefers to produce the hexyl esters of C3, C6 and C8 CoAs. For the acetate esters, however, MpAAT1 preference depends upon substrate concentration. At low concentrations of alcohol substrate the enzyme prefers utilizing the 2-methylbutanol over hexanol and butanol, while at high concentrations of substrate hexanol can be used at a greater rate than 2-methylbutanol and butanol. Such kinetic characteristics of AATs may therefore be another important factor in understanding how the distinct flavor profiles of different fruit are produced during ripening.

Apples have long been cultivated by humans for their fruit. They produce a complex mixture of over 200 volatile compounds [1], including alcohols, aldehydes, ketones, sesquiterpenes and esters. Esters are associated with 'fruity' attributes of fruit flavor and typically increase to high levels late in the ripening process [2]. In the commercial apple cultivar, *Malus pumila* cv. Royal Gala, over 30 esters have been identified [3,4]. These can be broadly separated into straight chain esters and branched chain esters. In apples, straight chain esters are thought to be biosynthesized from fatty acids via the lipoxygenase pathway [5]. In contrast branched chain esters are thought to be produced from the metabolism of branched chain amino acids such as isoleucine [6]. Of the esters produced by Royal Gala, butyl acetate, hexyl acetate, and 2-methylbutyl acetate dominate the flavor of ripe fruit, with the latter two being identified by analytical sensory panels as having the greatest impact on the attractiveness of the fruit [4].

Abbreviations
AAT, alcohol acyl transferase; coA, coenzyme A; IPTG, isopropyl thio-β-D-galactoside; MpAAT1, apple AAT1; SPME, solid phase microextraction.

The final step in ester biosynthesis is catalyzed by acyl transferases (EC 2.3.1.x), members of the BAHD superfamily [7]. These enzymes transfer an acyl group from a donor (often CoA) to the hydroxyl, amino, or thiol group of an acceptor molecule to yield an acyl ester derivative. Plants contain a large family of such acyl transferases with approximately 70 found in *Arabidopsis* [8]. Alcohol acyl transferase (AAT) activity is responsible for the production of volatile esters and has been observed in plant tissues such as the flowers and fruit [9–11]. A major question in the field has been to identify which enzymes in the biosynthetic pathway are critical for producing the distinct blends of esters characteristic of different fruit. To address this, genes encoding AATs have been isolated from fruit including melon [12] and strawberry [11], and their activity studied after expression in yeast and bacteria, respectively. These studies have found that AATs have the ability to utilize a broad range of substrates suggesting that substrate availability rather than AAT enzyme preferences are explanatory of different aromas of fruits. Here we describe the cloning and characterization of an AAT expressed in the fruit of Royal Gala and report on the kinetic characterization of the enzyme.

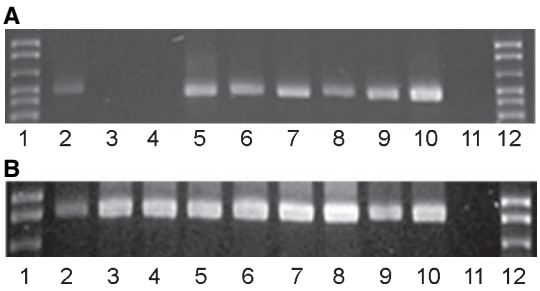

Fig. 1. *MpAAT1* gene expression in apple tissues. (A) RT-PCR using *MpAAT1*-specific primers amplifying a fragment of 443 bp. (B) Amplification products of 850 bp from actin-specific primers. Lanes: (1) 1 kb-plus DNA ladder; (2) leaf; (3) phloem; (4) xylem; (5) flower; (6) young fruit with seeds removed 59 days after full bloom (DAFB); (7) fruit cortex 87 DAFB; (8) fruit cortex 126 DAFB; (9) fruit core 126 DAFB; (10) fruit skin 150 DAFB; (11) water control; (12) 1 kb-plus DNA ladder.

Results

The MpAAT1 gene and its predicted protein

Gene mining identified 20 acyl transferase-encoding genes from the HortResearch apple EST database of which 13 contain full-length cDNAs. One of these (*MpAAT1*) was chosen for characterization since it includes EST accessions from fruit cDNA libraries, is closely related to acyl transferases that can utilize alcohol as an acceptor, and it is able to be expressed in *Escherichia coli* in a soluble form. The longest *MpAAT1* cDNA clone isolated is 1591 nucleotides in length, and contains an open reading frame that encodes a predicted protein of 455 amino acids (GenBank accession number AY707098) leaving a 5′-UTR of 24 and a 3′-UTR of 202 nucleotides. Transcripts of the *MpAAT1* gene were detected by RT-PCR in leaves, flowers and all stages of developing and ripening apple fruit (Fig. 1). The predicted MpAAT1 protein has a molecular mass of 50.9 kDa and pI of 7.9. MpAAT1 exhibits the features of other plant acyl transferases [7] including an active site motif, HXXXDG (amino acids 181–186, Fig. 2). In MpAAT1 the His and Asp of the active site are conserved but the Gly is substituted for the slightly larger Ala. Several other plant acyl transferases also have an Ala at this position. It is not known whether this amino acid substitution affects activity or substrate preference. A second region conserved amongst acyl transferases is the DFGWG motif. MpAAT1 contains a similar motif (amino acids 445–449), however, the sequence is slightly different, with Asn substituting for an Asp.

Comparison and phylogenetics of MpAAT1

MpAAT1 was aligned with 11 other plant acyl transferases of known function (Fig. 2). BEBT (*Clarkia breweri* [13]) was the most similar to MpAAT1 at 54% identity at the amino acid level. A phylogenetic tree constructed from this alignment contains two major groupings (Fig. 3). MpAAT1 clusters with the group of AATs involved in ester biosynthesis from melon and *Clarkia* (CM-AAT1 [12] and BEBT [13]). Basal to these is an AAT from banana, BanAAT [14], and more distant is an anthranilate acyl transferase from *Dianthus caryophyllus*, HCBT [15]. The second group also contains AATs including SALAT (*Papaver somniferum* [16]) and BEAT (*Clarkia breweri* [17]). Sister to these is another clade of AATs involved in strawberry ester biosynthesis (SAAT [11] and VAAT [14]) and two acyl transferases from *Catharanthus roseus*, DAT and MAT [18,19]. DAT and MAT are both involved in indole alkaloid biosynthesis and use more complex donor groups. The tree was rooted with an anthocyanin acyl transferase [20].

E. coli expression of MpAAT1

Semi-purified protein from recombinant *E. coli* includes the predicted fusion protein that contains the expected MpAAT1 polypeptide. Two major proteins

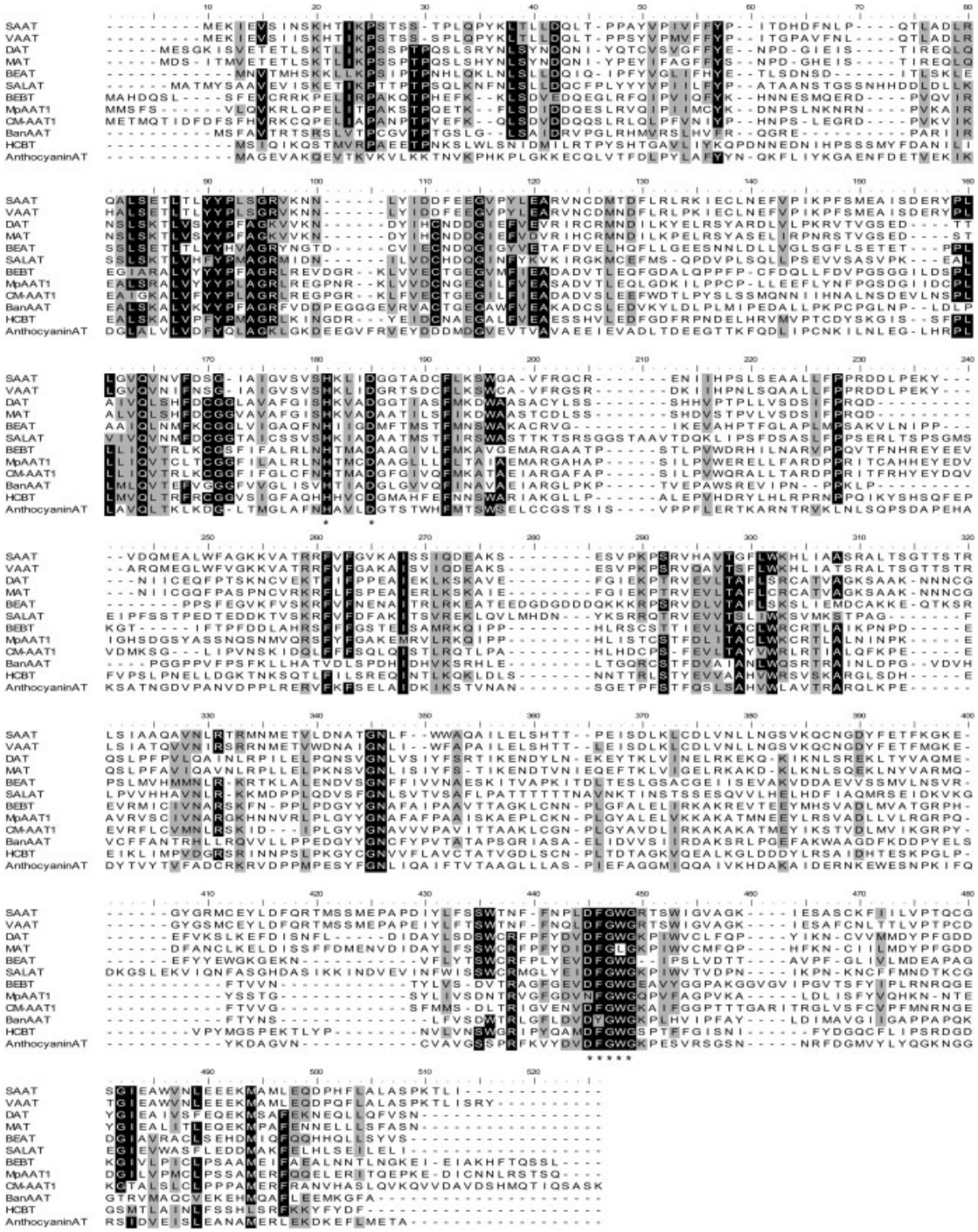

Fig. 2. Amino acid sequence alignment of MpAAT1 with other plant acyl transferases of known function. DAT, (*Catharanthus roseus* deacetylvindoline 4-*O*-acetyltransferase; AF053307 [19]); MAT (*Catharanthus roseus* minovincinine 19-hydroxy-*O*-acetyltransferase; AAO13736 [18]), BEAT (*Clarkia breweri* acetylCoA:benzylalcohol acetyltransferase; AF043464 [10]); SALAT (*Papaver somniferum* salutaridinol 7-*O*-acetyltransferase; AF339913 [16]); BEBT (*Clarkia breweri* benzoyl-CoA:benzyl alcohol benzoyl transferase; AF500200 [13]); MpAAT1 (*Malus pumila* alcohol acyltransferase; AY707098); CM-AAT1 (*Cucumis melo* alcohol acyltransferase; CAA94432 [12]); SAAT (strawberry alcohol acyltransferase; AAG13130 [11]); HCBT (*Dianthus caryophyllus* anthranilate *N*-hydroxycinnamoyl benzoyltransferase; Z84383 [15]); AnthocyaninAT (*Petunia frutescens,* anthocyanin acyl transferase; BAA93453 [20]); VAAT (*Fragaria vesca* alcohol acyl transferase; AX025504 [14]); BanAAT (Banana alcohol acyl transferase; AX025506 [14]). Black and grey boxes contain residues that are identical and similar, respectively. Asterisks indicate the positions of the conserved amino acids in active site regions of plant acyl transferases.

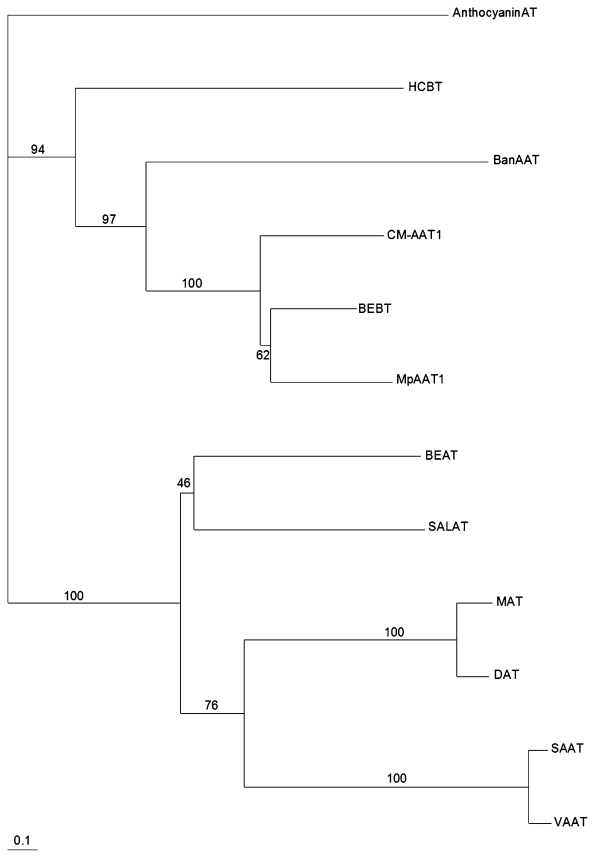

Fig. 3. Phylogram of plant acyl transferases of known function, including MpAAT1. Taxa codes are as for Fig. 2. Percentage bootstrap values (1000 bootstrap replicates) for groupings are given below each branch.

were eluted from the HiTrap™ chelating column, one of 58.1 kDa and a second of 57.2 kDa (Fig. 4A). A western blot using an anti-His antibody against the two proteins suggested only one contained a His_6-tag (Fig. 4B). Peptide electrospray MS-MS analysis of these proteins identified the larger protein (58.1 kDa) as containing the predicted MpAAT1 polypeptide with confirmed peptides accounting for 28% coverage over the protein sequence and the N-terminally fused thioredoxin (58% coverage). The smaller 57.2 kDa band was identified as the *E. coli* chaperon protein GroEL, which presumably remained bound to MpAAT1 during purification. The presence of *E. coli* GroEL may assist MpAAT1 to remain soluble throughout the purification procedure. It is also of interest to note that soluble MpAAT1 was only attained when C43 cells were used as host. All other BL21 derivatives tested did not yield soluble recombinant MpAAT1. Perhaps C43 cells contain a more highly expressed or inducible version of GroEL.

In vivo recombinant MpAAT1 volatile trapping experiments

Recombinant *E. coli* expressing MpAAT1 was able to produce a wide range of volatile esters when fed with alcohol substrates. For example, when supplied with the alcohols 1-methoxy propan-2-ol, 3-methylbut-3-enol (*E/Z*)-hex-3-enol, furfuryl alcohol and 2-phenylethanol, the esters 3-methylbut-3-enyl acetate (*E/Z*)-hex-3-enyl acetate, furfuryl acetate and 2-phenylethyl acetate were produced (Table 1). In this system not all acetate esters derived from the respective added alcohols were detected (e.g. the acetate ester of 1-methoxy propan-2-ol from above). MpAAT1 can use endogenous *E. coli* acetyl-CoA since no exogenous source was provided. Moreover longer endogenous CoAs can also serve as substrates. For

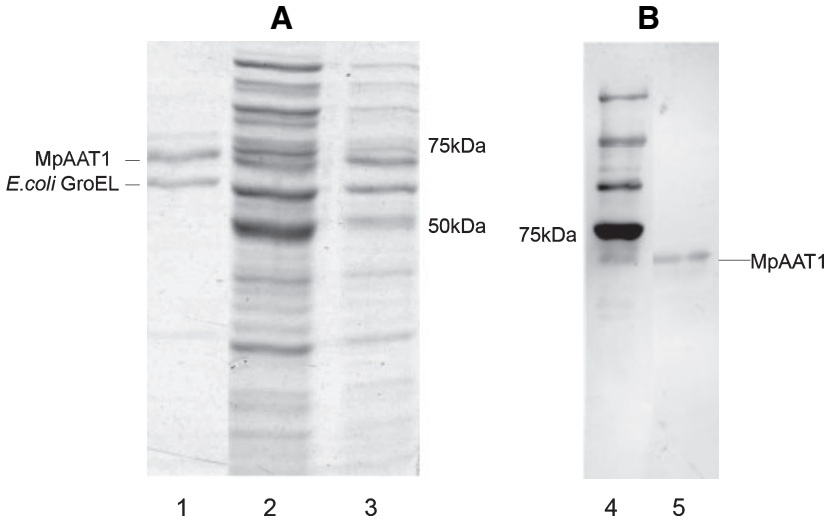

Fig. 4. Semi-purification of MpAAT1 produced in *E. coli*. (A) 1, semipurified recombinant MpAAT1; 2, IPTG-induced sample; 3, noninduced sample. (B) 4, prestained precision plus protein standards (Bio-Rad); 5, immunodetection of semipurified MpAAT1 using His-tag antibodies.

Table 1. Substrates utilized by MpAAT1 recombinant enzyme. [a]Dominant esters in Royal Gala fruit [4].

Alcohol added	Carbon number	Esters expected	Esters produced in *E. coli*	Esters produced in tobacco	Esters produced by semipurified MpAAT1	Reported from Royal Gala apple fruit [3,4]
Ethanol	C2:0	Ethyl acetate	–	–	–	–
Propanol	C3:0	Propyl acetate	+	–	+	+
		Propyl octanoate	+	–	ND	+
Butanol	C4:0	Butyl acetate	+	+	+	+[a]
		Butyl butanoate	+	–	ND	+
		Butyl hexanoate	+	–	ND	+
		Butyl octanoate	+	–	ND	+
Butan-1,3-diol	C4:0	3-Hydroxybutyl acetate	–	–	–	–
		1-Methyl-3-hydroxypropyl acetate	–	–	–	–
1-Methoxypropan-2-ol	C4:0	1-Methoxypropan-2-yl acetate	–	–	–	–
Pentanol	C5:0	Pentyl acetate	+	+	+	+
		Pentyl propanoate	+	–	ND	+
		Pentyl hexanoate	+	–	ND	+
Pent-3-en-2-ol	C5:1	Pent-3-en-2-yl acetate	–	–	–	–
Hexanol	C6:0	Hexyl acetate	+	+	+	+[a]
		Hexyl butanoate	+	–	ND	+
Hex-2-enol	C6:1	Hex2-enyl acetate	+	–	+	+
(*E/Z*)-Hex-3-enol	C6:1	(*E/Z*)-Hex-3-enyl acetate	+	+	+	+
		(*E/Z*)-Hex–3-enyl propanoate	+	–	ND	+
		(*E/Z*)-Hex3-enyl hexanoate	+	–	ND	+
		Hex-3-enyl octanoate	+	–	ND	+
		Hex-3-enyl formate	+	–	ND	–
Octanol	C8:0	Octyl acetate	+	+	+	+
Decanol	C10:0	Decyl acetate	+	–	+	–
2-Methylpropanol	C4:0	2-Methylpropyl acetate	–	–	–	–
3-Methylbut-3-enol	C5:1	3-Methylbut-3-enyl acetate	+	–	–	+
2/3-Methylbutanol (30/70)	C5:0	2/3-Methylbutyl acetate	+	+	+	+[a]
		3-Methylbutyl octanoate	+	–	ND	–
Terpinen-4-ol	C10:1	Terpinen-4-yl acetate	–	–	–	–
Geraniol	C10:2	Geranyl acetate	+	+	+	–
		Geranyl propanoate	+	–	ND	–
		Geranyl butanoate	+	–	ND	–
Linalool	C10:2	Linalyl acetate	–	–	–	–
Farnesol	C15:3	Farnesyl acetate	–	–	–	–
Furfuryl alcohol	C5:2	Furfuryl acetate	+	+	+	–
Benzyl alcohol	C7:3	Benzyl acetate	+	+	+	–
Orcinol	C7:3	Orcinyl acetate	–	–	–	–
Salicyl alcohol	C7:3	Salicyl acetate	–	–	–	–
2-Phenylethanol	C8:3	2-Phenylethyl acetate	+	+	+	–
		2-Phenylethyl propanoate	+	–	ND	–
		2-Phenylethyl butanoate	+	–	ND	–
Eugenol	C10:4	Eugenyl acetate	–	–	–	–

example, at substantially lower levels (*E/Z*)-hex-3-enyl propanoate, butyl hexanoate, ethyl octanoate, butyl octanoate (*E/Z*)-hex-3-enyl hexanoate, hex-3-enyl octanoate, 2-phenylethyl propanoate and 2-phenylethyl butanoate were also detected (Table 1). In total 25 alcohols were tested on the recombinant *E. coli*. MpAAT1 was able to produce esters using endogenous *E. coli* CoAs from 14 of these alcohols. In comparison, recombinant *E. coli* expressing the deleted acyl transferase produced no volatile esters revealing that MpAAT1 was involved in the biosynthesis of these compounds.

A plant transient expression system expressing MpAAT1 produced a smaller range of esters compared

with *E. coli* using the same set of alcohols as substrates (Table 1). All the same acetate esters were detected as in *E. coli* except for propyl acetate, hex-2-enyl acetate, 3-methylbut-3-enyl acetate and decyl acetate. Esters made from the added alcohols and CoAs longer than acetyl-CoA were not detected. However four esters were detected (methyl-3-methyl pentanoate, methyl-3-methyl butanoate, methyl octanoate, and methyl acetate) formed from endogenous tobacco alcohols and CoAs. None of these esters were detected in a P19 *Agrobacterium* infected tobacco leaves.

In vitro recombinant MpAAT1 volatile trapping experiments

MpAAT1, semipurified through a HiTrap™ column, synthesized volatile esters *in vitro* when provided with certain alcohol and CoA substrates. After SPME trapping, overlaying each sample's total ion chromatogram with the boiled control traces clearly showed that esters had been synthesized. In total, 25 alcohols were tested with acetyl-CoA as a donor. MpAAT1 utilized many of these straight, branched and aromatic alcohols (Table 1). MpAAT1 can use C3-C10 straight chain alcohols as a substrate with acetyl-CoA as well as aromatic and branched chain alcohols (Table 1). Using (*E/Z*)-hex-3-enol as the acceptor, MpAAT1 shows a range of responses to different CoA donors. Esters of acetyl-CoA, propionyl-CoA, butyryl-CoA and hexanoyl-CoA could be detected while those derived from malonyl-CoA and palmitoyl-CoA were not. Also palmitoyl-CoA was inhibitory on enzyme activity with acetyl-CoA and (*E/Z*)-hex-3-enol (data not shown).

Effect of pH, temperature and ionic strength on MpAAT1 activity

Recombinant MpAAT1 is active using acetyl-CoA and (*E/Z*)-hex-3-enol as substrates from pH 5.0–10.0 with maximum activity between pH 7.0–9.0 (data not shown). The enzyme is active from 20 to 37 °C. However activity is dramatically reduced when the enzyme is incubated at 45 °C (Fig. 5). Some activity was lost after incubation for one hour (Fig. 5).

The effect of ionic strength on MpAAT1 activity was studied with different concentrations of metal ions (Table 2). Zinc has the most dramatic effect on activity, inhibiting the enzyme by 80–91% at concentrations 0.5–1 mM, respectively. Magnesium, cobalt, nickel, manganese and calcium all partially inhibit MpAAT1 activity while potassium only had an effect on activity at the highest concentration of 5 mM. The reducing agent dithiothreitol did not enhance MpAAT1 activity but instead was a strong inhibitor as was the sulfhydryl reagent, *p*-chloromercuribenzoic acid (74 and 98% inhibition, respectively, at 5 mM).

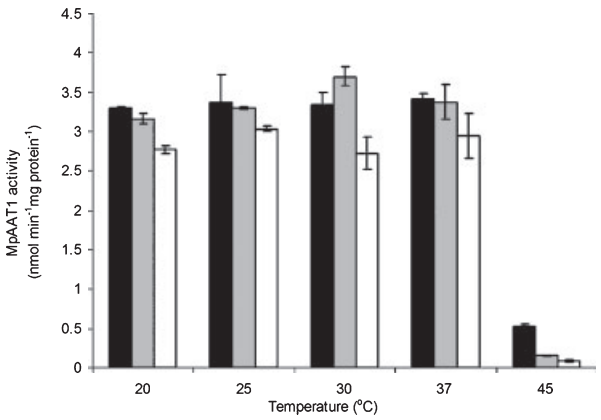

Fig. 5. Influence of temperature on recombinant semipurified MpAAT1 protein. Activities of MpAAT1 protein with octanol and acetyl-CoA were measured at different temperatures and at different incubation times [30 min (black bar), 1 h (grey bar) and 1 h 30 min (white bar)]. Data are means ± SD of three replicates.

Table 2. Effect of metal ions and reducing agents on the activity of semipurified recombinant MpAAT1. Data are means of minimum three replicates.

Metal ions and reducing agents	Concentration (mM)	Relative activity (%)
Potassium	0.5	100
	1	100
	5	95
Magnesium	0.5	96
	1	94
	5	80
Cobalt	0.5	98
	1	87
	5	85
Nickel	0.5	97
	1	99
	5	87
Manganese	0.5	84
	1	85
	5	73
Calcium	0.5	84
	1	93
	5	80
Zinc	0.5	20
	1	9
	5	2
Dithiothreitol	0.5	76
	1	43
	5	26
p-Chloromercuribenzoic acid (PCMB)	0.5	20
	1	21
	5	2

Activity of MpAAT1 with different substrates

Kinetic parameters were determined for MpAAT1 by using combinations of different CoAs and alcohols (Table 3). Butanol, hexanol and 2-methylbutanol were chosen as alcohol substrates as their respective acetate esters are dominant esters found in Royal Gala fruit [4]. When hexanol is used as the acceptor (Table 3), the affinity for acetyl-CoA ($K_m = 2.7$ mM) was not as high as when either butanol or 2-methylbutanol were used ($K_m = 110$ and 90 μM, respectively). However, the V_{max} value was much higher for hexanol (376.0 nmol·min^{-1} mg protein^{-1}) in comparison to V_{max} values for butanol and 2-methylbutanol (20.0 and 16.6 nmol·min^{-1}·mg protein^{-1}, respectively). Kinetic parameters were also determined for hexanol, butanol and 2-methylbutanol (Table 3) using different CoAs as donors. The K_m values for hexanol were higher for acetyl-CoA (7.4 mM) than for butyryl-CoA, hexanoyl-CoA and octanoyl-CoA (1.5, 2.6 and 3.1 mM, respectively) (Table 3). V_{max} values for hexanol were similar with hexanoyl-CoA giving the highest V_{max} (320.0 nmol·min^{-1}·mg protein^{-1}). When butanol was tested as a substrate (Table 3), the K_m was lower (2.7 mM) for acetyl-CoA than for octanoyl-CoA (12.4 mM). No activity could be detected and therefore no kinetics could be determined for butyryl-CoA and hexanoyl-CoA as donors (Table 3). The K_m values for 2-methylbutanol (Table 3) were similar for acetyl-CoA and butyryl-CoA (1.11 and 1.7 mM), however, K_m values were higher for hexanoyl-CoA (3.2 mM) and octanoyl-CoA (6.2 mM). V_{max} values for 2-methylbutanol using the different CoAs were very similar.

Discussion

Fruit produce a range of volatile compounds that make up their characteristic aromas and contribute to their flavor. An important class of these compounds is esters, which can be formed from acids and alcohols by AATs, members of the BAHD superfamily of proteins [7]. A cDNA was isolated from the apple cultivar Royal Gala that encoding a predicted protein (MpAAT1) that contains motifs found in other acyl transferases including an active site region containing a His and a conserved DFGWG motif [7].

We show that the *MpAAT1* gene is expressed in apple flowers and fruit, tissues that produce volatile esters. As the method that we used (RT-PCR) is not quantitative and since the number of amplification cycles used was likely to be saturating, we would not except to be able to detect varying levels of expression of the gene between tissues or during fruit ripening. MpAAT1 is identical at the amino acid level to another recently sequenced AAT from apple (AX025508 [14]) except for a His212Arg substitution, and also an AAT isolated from apple cv. Greensleeves [21]. The expression of the MpAAT1 Greensleeves homologue has been studied using a quantitative PCR method in ethylene treated fruit and revealed up-regulation of the transcript upon ethylene treatment [21]. Our own microarray studies in both developing Royal Gala fruit and ethylene treated fruit also show up-regulation of the *MpAAT1* transcript in both cases (data not shown).

MpAAT1 is most closely related to other AATs including those from melon [12] and *Clarkia* [13], both

Table 3. Kinetic properties of semipurified recombinant MpAAT1 protein. ND, no detectable activity.

Co-substrate S1 (variable concentration)	Co-substrate S2 (saturating concentration)	K_m (mM)	V_{max} (nmol·min^{-1}·mg protein^{-1})	V_{max}/K_m (10^{-6} L·min^{-1}·mg protein^{-1})
Acetyl-CoA	Butanol	0.11 ± 0.04	20.0 ± 2.5	181.8
Acetyl-CoA	Hexanol	2.7 ± 0.2	376.0 ± 16.1	139.3
Acetyl-CoA	2-Methylbutanol	0.09 ± 0.02	16.6 ± 1.2	184.4
Hexanol	Acetyl-CoA	7.4 ± 0.9	148.6 ± 6.4	20.1
Hexanol	Butyryl-CoA	1.5 ± 0.2	207.6 ± 6.2	138.4
Hexanol	Hexanoyl-CoA	2.6 ± 0.4	320.0 ± 15.5	123.1
Hexanol	Octanoyl-CoA	3.1 ± 0.6	252.3 ± 14.0	81.4
Butanol	Acetyl-CoA	2.7 ± 0.6	35.4 ± 1.8	13.1
Butanol	Butyryl-CoA	ND	ND	
Butanol	Hexanoyl-CoA	ND	ND	
Butanol	Octanoyl-CoA	12.4 ± 0.2	47.4 ± 2.4	3.8
2-Methylbutanol	Acetyl-CoA	1.1 ± 0.1	66.8 ± 2.3	60.7
2-Methylbutanol	Butyryl-CoA	1.7 ± 0.7	41.3 ± 4.4	24.3
2-Methylbutanol	Hexanoyl-CoA	3.2 ± 0.3	47.1 ± 1.2	14.7
2-Methylbutanol	Octanoyl-CoA	6.2 ± 0.7	53.7 ± 2.4	8.7

of which have been shown to produce esters. In our phylogenetic analysis another separate clade also contains AATs (BEAT [10], SALAT [16], SAAT and VAAT [14]), suggesting that the ability to use an alcohol acceptor may have evolved several times within the plant acyl transferase family. The evolution of two separate AAT families is also clearly resolved when all approximately 70 *Arabidopsis* acyl transferases are included in a phylogenetic analysis (data not shown).

The overexpression of MpAAT1 in C43 *E. coli* cells allowed the enzyme to be characterized in the cultures and using protein substantially purified from these bacteria. Transient expression in *Nicotiana benthamiana* also allowed rapid screening of potential substrates for MpAAT1. In these three cases, we employed the use of cocktails of potential alcohol substrates and analyzed headspace samples by GC-MS to identify which esters can be produced by the enzyme. This technique allowed many alcohol and CoA substrates to be screened. This screening revealed that MpAAT1 can use a large range of alcohols as substrates including C3 to C10 straight chain alcohols, as well as some branched, aromatic and terpene alcohols. The MpAAT1 enzyme can also use CoA donors of varying chain length (C2–C8). However, when the CoA chain length is longer than C8 no products were detected. It is likely that these longer CoAs are still able to bind to the CoA binding site as, for example, palmitoyl-CoA is able to inhibit activity of the enzyme when acetyl-CoA and (*E/Z*)-hex-3-enol are used as substrates, even though no palmitoyl-CoA derived products were detected.

We have been careful to avoid concluding that MpAAT1 does not produce esters that are undetectable. Unfavored substrates are likely to be out competed by more favored substrates in the same cocktail resulting in the products of unfavored alcohols being at low levels and difficult to detect. Moreover, not all products are equally detectable due to differences in vapor pressures of the esters and different affinities of these products to the specific absorptive SPME matrix used. Again this may result in some products being difficult to detect and not being identified in our analysis. However given these shortcomings, the use of mixtures allowed many potential substrates to be rapidly identified for further kinetic characterization.

We also note that there are differences between the bacterial and plant expression systems in terms of the ester products that were detected. Twice as many esters were identified in headspace above *E. coli* cultures compared with the transient plant expression system. For many of the added alcohols, esters were detected above cultures made from longer CoA donors whereas in the plant only acetyl esters were produced from added alcohols. However, in the *E. coli* system when longer alcohols were added, ester products using longer CoA substrates (e.g. octanoate) were not detected. The alcohol substrates were incubated with recombinant enzymes for a longer period of time in the *E. coli* cultures (20 h) than in leaves (1 h), potentially allowing more minor products to be produced. This may explain the differences in product profiles. However, it is also reasonable to expect some differences since the biosynthetic environments are quite different. For example in the plant system, MpAAT1 is also producing esters from endogenous alcohols (e.g. methanol) to further compete with its ability to produce esters from added alcohols.

MpAAT1 shows comparable enzyme characteristics with other fruit AATs. Like semipurified protein from strawberry [22], MpAAT1 exhibits a broad range of activity across the pH range 5.0–10.0 with a preferred temperature range of 20–37 °C at pH 8.0 and decreased activity above 45 °C. As found with the anthocyanin 3-aromatic acyltransferase from *Perilla frutescens*, zinc is a strong inhibitor of MpAAT1 [23] and MpAAT1 is also inhibited by the sulfhydryl reactive compound, *p*-chloromercuribenzoic acid, and dithiothreitol. These inhibitor results may reflect the proximity of cysteine residues in the substrate binding pockets and/or catalytic region since zinc ions often use cysteine residues as coordinating ligands. In contrast to these results, many members of the BAHD superfamily are activated by dithiothreitol [7].

Acyl transferases are all thought to share a common fold and use a simple two-step reaction mechanism [7,24,25] and we presume MpAAT1 is not different. The active site is embedded in the middle of a solvent accessible tunnel that passes through the globular enzyme. On one side of the active site is a binding site for the CoA while on the other is an alcohol binding site [24,25]. The active site histidine (His181 in MpAAT1) is thought to deprotonate the hydroxyl group on the alcohol allowing the oxygen to conduct a nucleophilic attack of the carbonyl carbon of the CoA acid forming a tetrahedral intermediate that contains both the carboxyl and thiol ester groups. Finally the thiol ester breaks down with the addition of the same proton from the active site histidine and free enzyme is liberated together with ester and free CoA as products.

In the first step of this reaction acetyl-CoA is bound much more rapidly than the alcohol. Estimates of K_m for CoAs when alcohols are saturating are generally lower than K_m estimates for alcohols when CoAs are saturating. However the ability of the CoA to bind depends on which alcohol is already in the alcohol

binding pocket. For MpAAT1 the K_m for acetyl-CoA when hexanol is saturating is 2.7 mM. This is 25 times higher than for butanol (0.11 mM) and 2-methylbutanol (0.09 mM). The longer hexanol may be interacting with the enzyme to alter its conformation making it less able to bind acetyl-CoA or slowing its progression to the transition state. For both MpAAT1 and CM-AAT1 from melon [12], while there is variation in their K_m values for acetyl-CoA with alcohols at saturating concentrations, K_m values are generally in the micromolar range. In contrast, K_m values for alcohols with acetyl-CoA saturating are in the mM range. Overall this will mean that the K_m for the alcohol will have more impact on the kinetic ability of the enzyme since its binding is rate limiting compared with the ability of the CoA to bind.

Alcohol acyl transferases show a wide range of substrate specificities for different alcohol acceptors [11,12,14]. Using kinetics we have been able to dissect where the differences in specificity occur within the reaction. For MpAAT1, K_m estimates for various alcohol substrates when acetyl-CoA is saturating are similar, ranging from 1.1 to 7.4 mM. Similarly for CM-AAT1, the K_m for two straight chain alcohols (C3, C6) was 8.0 mM and 1.4 mM, respectively. CoA chain length also has little effect on the ability of various alcohols to bind. When hexanol is bound in the alcohol binding site, K_m values are similar for the different CoAs, ranging from 1.5 to 7.4 mM (C2–C8). Where large differences are seen between different alcohols is in their rate of hydrolysis as estimated by V_{max}. Whenever hexanol is the alcohol acceptor, when it is saturating or not, the V_{max} for hexanol is always approximately 10-fold higher compared with the other alcohols tested. Similarly a threefold difference in V_{max} was observed in CM-AAT1 between butanol and hexanol. Together this suggests that the second step of the reaction mechanism proceeds more rapidly for some alcohols compared with the others. In these cases hexanol is a preferred alcohol. Perhaps for its product with acetyl-CoA, hexyl acetate, the transition state and His181 are more ideally positioned to generate the end products of the reaction.

Royal Gala apple fruit produce at least 34 esters upon ripening [3]. Of these, MpAAT1 prefers to produce hexyl esters from mid length CoAs at both low and high concentrations of alcohol substrate. For the substrates used in the kinetic analysis both the V_{max}/K_m and V_{max} values for hexyl butanoate, hexyl hexanoate and hexyl octanoate are the highest when CoAs are saturating. These compounds are all found in the headspace of Royal Gala fruit with hexyl hexanoate being the most abundant of all esters from a recent report [3]. It is not always the case that same esters are preferred by MpAAT1 at different concentrations of substrate. For the three important esters of acetyl-CoA [4], 2-methylbutyl acetate, butyl acetate and hexyl acetate the role of MpAAT1 in their biosynthesis varies depending on the availability of alcohol and CoA substrates. Both these substrates types are likely to vary in the fruit during the different phases of fruit ripening. For example, early in development when acetyl-CoA and alcohols are more limited, MpAAT1 will more likely contribute to producing 2-methylbutyl acetate, as the V_{max}/K_m for this alcohol (60.7 10^{-6} l min^{-1}·mg $protein^{-1}$) is three times greater than for butyl acetate (13.1×10^{-6} L·min^{-1}·mg $protein^{-1}$) and hexyl acetate (20.1×10^{-6} L·min^{-1}·mg $protein^{-1}$). However later in ripe fruit when the concentration of both acetyl-CoA and alcohols are higher, more hexyl acetate will be produced by the enzyme as the concentration of hexanol will not be rate limiting and the Vmax for this alcohol (148.6 nmol·min^{-1}·mg $protein^{-1}$) is greater than for butanol (35.4 nmol·min^{-1}·mg $protein^{-1}$) and 2-methylbutanol (66.8 nmol·min^{-1}·mg $protein^{-1}$). This varying preference by flavor biosynthetic enzymes at different concentrations of substrates may be important in explaining the changing profile of compounds produced by fruit as they develop and ripen.

MpAAT1 is also capable of producing many esters that are not found in apple cultivars such as terpene and aromatic esters. Thus the broad substrate preferences of the enzyme are not totally explanatory of the range of esters found in this fruit. In contrast, the pool of available substrates in apple is also likely to dictate what ester compounds are produced. This parallels the situation found in strawberry and melon where the AATs characterized from these fruit are also capable of making a broad range of esters, more than are found in each fruit [11,12,14]. Further complicating the situation, there are other AATs in fruit that might be contributing to ester biosynthesis. From our EST sequencing of Royal Gala apple fruit we have identified at least a further 12 acyl transferases from apple, seven of which have been identified from fruit libraries (data not shown). It is likely that some, if not all of these seven enzymes are also contributing to volatile ester biosynthesis. These enzymes may have different substrate preferences and thus contribute to different groups of esters being produced.

In conclusion, there are many factors that contribute to the ability of a fruit to synthesize its distinctive aroma. These include substrate availability, the number of AATs, their regulation and the different kinetic characteristics of these enzymes under different substrate concentrations.

Experimental procedures

Bioinformatics and molecular biology

Previously published plant alcohol acyl transferase (AAT) genes from GenBank were used to mine AAT genes from an apple EST database (HortResearch, unpublished work) using BLAST searches with an expect value of $< \exp^{-05}$ [26]. Amino acid alignments of predicted proteins were constructed using CLUSTAL X [27]. Criteria such as the presence of an active site histidine residue embedded in the HXXXDG motif were checked in alignments [7]. Phylogenetic analysis was carried out using the PHYLIP suite of programmes [28]. Distances were calculated using PROTDIST, and the FITCH method was used to construct a tree. Bootstrap analysis was conducted using 1000 bootstrap replicates implemented in SEQBOOT [28]. TREEVIEW (v.1.6.6) was used to display resulting trees [29].

Reverse transcriptase PCR (RT-PCR) was performed on 1 μg of total RNA extracted from aerial tissues of Royal Gala apples trees using a method developed for woody plants [30]. cDNA synthesis was performed using oligo dT as a primer and SuperScript III (Invitrogen) as per the manufacturer's conditions. Resulting cDNA was used as a template in 50 μL PCR reactions that contained 10 pmol of each primer, 1.5 mM $MgCl_2$, 20 mM Tris/HCl (pH 8.4), 50 mM KCl, 2.5 U recombinant *Taq* polymerase (Invitrogen) and 200 mM dNTPs. MpAAT1RTF (5′-CTCAGATATTGACGACCAA GAAA-3′) and MpAAT1RTR (5′-CGGTCAGGAACAA GAGCAAT-3′) primers were used to detect *MpAAT1* transcript. The presence of mRNA was confirmed using actin primers ApAct1 (5′-GAGCATGGTATTGTGAGCAA-3′) and ApAct2 (5′-CGCAATCCACATCTGCTGGA-3′). PCR conditions for *MpAAT1* RT-PCR were 94 °C 2 min then 35 cycles of 94 °C 10 s, 50 °C 30 s, 72 °C 30 s with a final elongation step of 72 °C 10 min. PCR for actin was performed using the same conditions. Five microliters of PCR sample was resolved on 1% agarose gels stained with ethidium bromide.

MpAAT1 overexpression construct

A full-length cDNA clone of *MpAAT1* was subcloned into the *E. coli* expression vector pET32Xa/LIC using the pET Ligation Independent Cloning System (Novagen) resulting in the clone pET32Xa/LIC-MpAAT1. PCR amplification was conducted using *MpAAT1* cDNA as template with FMpAAT1 (5′-GGTATTGAGGGTCGCATGATGTCATT CTCAGTACTTCA-3′) and RMpAAT1 (5′-AGAGGAG AGTTAGAGCCTCATTGACTAGTTGATCTAAGG-3′) primers to generate the insert. PCR amplification, T4 DNA polymerase treatment, vector annealing and *E. coli* transformation were carried out as recommended by the manufacturer for directional cloning of PCR products. A construct was made for use as a negative control that encoded a truncated version of an acyl transferase missing the active site region of the enzyme (pET32Xa/LIC-deletion). All constructs were verified by restriction enzyme analysis and DNA sequencing, and transformed into C43 (DE3) cells [31].

Expression of MpAAT1 recombinant protein in *E. coli*

For recombinant expression of protein, *E. coli* was grown in 500 mL 2YT broth in 3 L flasks inoculated with 500 μL overnight liquid cultures. Resulting cultures were incubated at 37 °C with continuous agitation (250 r.p.m.) until $D_{600} = 0.6$, then equilibrated to 20 °C and induced with 0.4 mM IPTG. The cells were further incubated at 20 °C for 20 h and then harvested by centrifugation at 10 000 ***g***. Cell pellets were resuspended in 20 mL of a cold buffer of 20 mM Tris/HCl (pH 7.9) containing 0.5 M NaCl, 5 mM imidazole and protease inhibitor cocktail tablets (EDTA-free, Roche). The cells were disrupted using an Emulsi-Flex®-C5 high pressure homogenizer (AVESTIN Inc.) with a pressure setting between 15 and 20 kpsi. The resulting cell debris was centrifuged at 10 000 ***g*** for 15 min at 4 °C. Protein purification was performed on the supernatant using a 5 mL HiTrap™ chelating HP column (Amersham Biosciences) according to the manufacturer's instructions. The soluble lysate and the eluate fractions (0.3 M imidazole, 30 μL) from the HiTrap™ columns were analyzed on 10% SDS/PAGE gels stained with colloidal Coomassie G-250 [32]. Proteins were transferred from SDS/PAGE gel to a nitrocellulose membrane using semidry electrophoresis (Trans-Blot Semi-Dry Cell, Bio-Rad Laboratories)). To detect the His_6 motif, the blots were incubated with anti-His_6 monoclonal antibody (Roche, dilution 1 : 1000), followed with anti-mouse IgG alkaline phosphatase conjugated antibodies (Stressgen, dilution 1 : 2000). Proteins were visualized using a 1-STEP™ NBT/BCIP alkaline phosphatase detection reagent according to the manufacturer's instructions (Pierce).

LC-MS analysis of proteins

Colloidal Coomassie-stained gel bands were excised from 1-D SDS/PAGE gels. Proteins were digested using trypsin and subjected to nanospray mass spectrometry using an LCQ Deca ion trap mass spectrometer fitted with a nano-electrospray interface (ThermoQuest, Finnigan) coupled to a Surveyor™ HPLC. The mass spectrometer was operated in positive ion mode and the mass range acquired was m/z 300–2000.

MS/MS data were analyzed using TurboSEQUEST™ (ThermoFinnigan) [33,34] with the spectra being pattern-matched against virtual digested translated apple EST sequences (HortResearch, unpublished work) and the

E. coli predicted protein set from GenBank. The criteria used for positive peptide identification for a doubly charged peptide were a correlation factor (XCorr) greater than 2.0, a delta cross-correlation factor (dCn) greater than 0.1 and a high preliminary scoring (Sp) value. For triply charged peptides the correlation factor threshold was set at 2.5. All matched peptides were confirmed by examination of the spectra.

Headspace analysis of recombinant MpAAT1 activity

One hour after induction with IPTG, cocktails of up to eight alcohols (10 μM of each alcohol) were added to *E. coli* cultures expressing pET32Xa/LIC-MpAAT1 or pET32Xa/LIC-deletion. Alcohols tested included straight chain alcohols [ethanol (BDH), propanol, butanol (BDH), butan-1,3-diol, 1-methoxypropan-2-ol, pentanol, pent-3-en-2-ol, hexanol, hex-2-enol (*E/Z*)-hex-3-enol, octanol, decanol], branched chain alcohols [2-methylpropanol, 3-methylbut-3-enol, 2/3-methylbutanol (30 : 70)], terpene alcohols (terpinen-4-ol, geraniol, linalool, farnesol) and aromatic alcohols [furfuryl alcohol, benzyl alcohol (BDH), orcinol, salicyl alcohol, 2-phenylethanol, eugenol (BDH)]. Unless otherwise stated alcohols were from Sigma-Aldrich. Each cocktail included 10 μM of (*E/Z*)-hex-3-enol as a standard. Incubation continued at 120 r.p.m. for a further 18 h at 20 °C. The headspace volatiles were collected using SPME fibers (75 μm Carboxen™/polydimethylsiloxane, Supelco). The fibers were checked for background contamination using GC-FID prior to use. All experiments were conducted at least twice.

The trapped headspace material was thermally desorbed at 260 °C onto a GC column [J & W DB Wax (30 m × 0.25 mm i.d., 0.5 μm film)] in an HP 5890 gas chromatograph coupled to a VG-70SE mass spectrometer via a heated (210 °C) capillary interface. The GC oven was temperature programmed at 30 °C for 6 min, 3 °C·min^{-1} to 102 °C, 5 °C·min^{-1} to 210 °C, and held for 5 min. The carrier gas was helium at 30 cm·s^{-1} and the MS electron impact ionization was at 70 eV with a scan range 30–320 atomic mass units. Component identification was assisted by reference to mass spectra of authentic standards (Wiley, NIST and in-house libraries) and GC retention indices.

To identify if MpAAT1 produces esters *in planta*, an enhanced transient expression system in *Nicotiana benthamiana* was used [35]. An *MpAAT1* cDNA clone was transferred into the pHEX2 vector [36], transformed into the *Agrobacterium* strain GV3101 (MP90) and selected by growth on Lennox plates containing spectinomycin, rifamycin, gentamycin (100, 25 and 10 μg·mL^{-1}, respectively). Transformed *Agrobacterium* strains containing MpAAT1 and a viral suppressor of silencing, P19, or P19 construct only were prepared for *Agrobacterium* infiltration [35]. Maturing leaves were infiltrated with the constructs [35] and after 14 days were infiltrated again, this time with the alcohol cocktails described above. After 1 h the treated leaves were removed and placed in 50 mL tubes with Chromosorb traps attached (100 mg Chromosorb® 105). The volatiles were trapped for 18 h using purified dry air purging at a rate of 50 mL·min^{-1}. Prior to analysis traps were brought to ambient temperature and dried with a stream of nitrogen (35 °C, 10 psig) for 15 min. Traps were then thermally desorbed at 150 °C with cryo-trapping onto a column ready for GC-MS analysis as described above.

Enzymatic characterization of the recombinant MpAAT1

The utilization of different alcohols and different acyl-CoA substrates were determined for MpAAT1 using the first 0.3 M imidazole eluate fraction (9 mL) from the HiTrap™ column. Enzyme assays were conducted at 20 °C as a modification of Aharoni *et al.* [11]. Control samples using boiled protein were also performed. The headspace volatiles were trapped with SPME fibers for 18 h at 20 °C and analyzed by GC-MS as described above. Experiments were performed at least twice.

The activity of MpAAT1 was also determined using ^{14}C-labeled acetyl-CoA, butyryl-CoA, hexanoyl-CoA or octanoyl-CoA (American Radiolabeled Chemical) and various alcohols following [11]. Protein was concentrated using Vivaspin columns (Vivascience) and concentration determined by absorbance at 280 nm. Reactions (1 mL) were conducted in triplicate and contained 3.6 μg of semipurified protein, 10 mM of each alcohol, 1 mM CoA in 50 mM Bis-Tris propane pH 8.0. Resulting esters were extracted with hexane and counted in a 1214 Rackbeta liquid scintillation counter (Wallac). Preferred conditions for MpAAT1 activity were assessed using octanol and acetyl-CoA as substrates for pH (5.0–10.0), temperature (20–45 °C) and for various ionic strengths (0.5, 1 and 5 mM) of a range of mono and divalent ions, the reducing agent, dithiothreitol, and the sulfhydryl reagent, *p*-chloromercuribenzoic acid.

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References

1 Dimick P & Hoskin J (1983) Review of apple flavor-state of the art. *Crit Rev Food Sci Nutr* **18**, 387–409.

2 Bengtsson M, Bäckman AC, Liblikas I, Ramirez MI, Borg-Karlson AK, Ansebo L, Anderson P, Löfqvist J & Witzgall P (2001) Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. *J Agric Food Chem* **49**, 3736–3741.

3 Young JC, Chu CLG, Lu X & Zhu H (2004) Ester variability in apple varieties as determined by solid-phase microextraction and gas chromatography mass spectrometry. *J Agric Food Chem* **52**, 8086–8093.

4 Young H, Gilbert JM, Murray SH & Ball RD (1996) Causal effects of aroma compounds on Royal Gala apple flavours. *J Sci Food Agric* **71**, 329–336.

5 Rowan DD, Allen JM, Fielder S & Hunt BM (1999) Biosynthesis of straight-chain ester volatiles in red delicious and granny smith apples using deuterium-labeled precursors. *J Agric Food Chem* **47**, 2553–2562.

6 Rowan DD, Lane HP, Allen JM, Fielder S & Hunt BM (1996) Biosynthesis of 2-methylbutyl, 2-methyl-2-butenyl and 2-methylbutanoate esters in red delicious and granny smith apples using deuterium-labeled substrates. *J Agric Food Chem* **44**, 3276–3285.

7 St-Pierre B & De Luca V (2000) Evolution of acyltransferase genes: origin and diversification of the BAHD superfamily of acyltransferases involved in secondary metabolism. In *Evolution of Metabolic Pathways*, (Ibrahim, R, Varin, L, De Luca, V & Romeo, JT, eds), pp. 285–315. Elsevier Press, New York.

8 Huala E, Dickerman AW, Garcia-Hernandez M, Weems D, Reiser L, Lafond F, Hanley D, Kiphart D, Zhuang M, Huang W et al. (2001) The Arabidopsis Information Resource (TAIR): a comprehensive database and web-based information retrieval, analysis, and visualization system for a model plant. *Nucleic Acids Res* **29**, 102–105.

9 Ueda Y, Fujishita N & Chachin K (1997) Presence of alcohol acetyltransferase in melons (*Cucumis melo* L.). *Postharvest Biol Technol* **10**, 121–126.

10 Dudareva N, D'Auria JC, Nam KH, Raguso RA & Pichersky E (1998) Acetyl-CoA: benzylalcohol acetyltransferase – an enzyme involved in floral scent production in *Clarkia breweri*. *Plant J* **14**, 297–304.

11 Aharoni A, Keizer LCP, Bouwmeester HJ, Sun Z, Alvarez-Huerta M, Verhoeven HA, Blaas J, van Houwelingen AM, De Vos RC, van der Voet H *et al.* (2000) Identification of the SAAT gene involved in strawberry flavor biogenesis by use of DNA microarrays. *Plant Cell* **12**, 647–662.

12 Yahyaoui FEL, Wong-Aree C, Latche A, Hackett R, Grierson D & Pech J-C (2002) Molecular and biochemical characteristics of a gene encoding an alcohol acyltransferase involved in the generation of aroma volatile esters during melon ripening. *Eur J Biochem* **269**, 2359–2366.

13 D'Auria JC, Chen F & Pichersky E (2002) Characterization of an acyltransferase capable of synthesizing benzylbenzoate and other volatile esters in flowers and damaged leaves of *Clarkia breweri*. *Plant Physiol* **130**, 466–476.

14 Beekwilder J, Alvarez-Huerta M, Neef E, Verstappen FWA, Bouwmeester HJ & Aharoni A (2004) Functional characterization of enzymes forming volatile esters from strawberry and banana. *Plant Physiol* **135**, 1865–1878.

15 Yang Q, Reinhard K, Schiltz E & Matern U (1997) Charaterisation and heterologous expression of hydroxycinnamoyl/benzoyl-CoA: anthranilate *N*-hydroxycinnamoyl/benzoyl transferase from elicited cell cultures of carnation, *Dianthus caryophyllus* L. *Plant Mol Biol* **35**, 777–789.

16 Grothe T, Lenz R & Kutchan TM (2001) Molecular characterization of the salutaridinol 7-*O*-acetyltransferase involved in morphine biosynthesis in opium poppy *Papaver somniferum*. *J Biol Chem* **276**, 30717–30723.

17 Dudareva N, Raguso RA, Wang J, Ross JR & Pichersky E (1998) Floral scent production in *Clarkia breweri* – III. Enzymatic synthesis and emission of benzenoid esters. *Plant Physiol* **116**, 599–604.

18 Laflamme P, St-Pierre B & De Luca V (2001) Molecular and biochemical analysis of a Madagascar periwinkle root-specific minovincinine19-hydroxy-*O*-acetyl transferase. *Plant Physiol* **125**, 189–198.

19 St-Pierre B, Laflamme P, Alarco AM & De Luca V (1998) The terminal *O*-acetyltransferase involved in vindoline biosynthesis defines a new class of proteins responsible for coenzyme A-dependent acyl transfer. *Plant J* **14**, 703–713.

20 Fukuchi-Mizutani M, Okuhara H, Fukui Y, Nakao M, Katsumoto Y, Yonekura-Sakakibara K, Kusumi T, Hase T & Tanaka Y (2003) Biochemical and molecular characterization of a novel UDP-glucose: anthocyanin 3′-*O*-glucosyltransferase, a key enzyme for blue anthocyanin biosynthesis, from *Gentian*. *Plant Physiol* **132**, 1652–1663.

21 Defilippi BG, Kader AA & Dandekar AM (2005) Apple aroma: alcohol acyltransferase, a rate limiting step for ester biosynthesis, is regulated by ethylene. *Plant Sci* **168**, 1199–1210.

22 Pérez AG, Sanz C & Olias JM (1993) Partial purification and some properties of alcohol acyltransferase from strawberry fruits. *J Agric Food Chem* **41**, 1462–1466.

23 Fujiwara H, Tanaka Y, Fukui Y, Ashikari T, Yamaguchi M & Kusumi T (1998) Purification and characterization of anthocyanin 3-aromatic acyltransferase from *Perilla frutescens*. *Plant Sci* **137**, 87–94.

24 Ramsay RR & Naismith JH (2003) A snapshot of carnitine acetyltransferase. *Trends Biochem Sci* **28**, 343–346.
25 Ma X, Koepke J, Panjikar S, Fritzsch G & Stöckigt J (2005) Crystal structure of vinorine synthase, the first representative of the BAHD superfamily. *J Biol Chem* **280**, 13576–13583.
26 Altschul S & Lipman D (1990) Protein database searches for multiple alignments. *Proc Natl Acad Sci USA* **87**, 5509–5513.
27 Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins DG (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**, 4876–4882.
28 Felsenstein J (1993) Phylip (Phylogeny Inference Package), Version 3.6a2.
29 Page RDM (1996) Treeview: an application to display phylogenetic trees on personal computers. *Computer Applications Biosciences* **12**, 357–358.
30 Chang S, Puryear J & Cairney J (1993) A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol Reporter* **11**, 113–116.
31 Miroux B & Walker JE (1996) Over-production of proteins in *Escherichia coli*: mutant hosts that allow synthesis of some membrane proteins and globular proteins at high levels. *J Mol Biol* **260**, 289–298.
32 Neuhoff V, Arold N, Taube D & Ehrhardt W (1988) Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie brilliant blue G-250 and R-250. *Electrophoresis* **9**, 255–262.
33 Yates JR, Eng JK & McCormack AL (1995) Mining genomes: correlating tandem mass spectra of modified and unmodified peptides to sequences in nucleotide databases. *Anal Chem* **67**, 3202–3210.
34 Eng JK, McCormack AL & Yates JR (1994) An approach to correlate tandem mass spectral data of peptides with amino acid sequences in a protein database. *J Am Soc Mass Spectrom* **5**, 976–989.
35 Voinnet O, Rivas S, Mestre P & Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. *Plant J* **33**, 949–956.
36 Gleave AP (1992) A versatile binary vector system with a T-DNA organisational structure conducive to efficient integration of cloned DNA into the plant genome. *Plant Mol Biol* **20**, 1203–1207.