Production of esters and phenylpropenes are linked through *MdAAT1* in apple fruit

ROSS G. ATKINSON[1], Edwige J.F. Souleyre[1], Adam J. Matich[2], Ye Han[1,3], Xiuyin Chen[1], Mindy Y. Wang[1], Blue Plunkett[1], Nadeesha Ileperuma[1], Andrew P. Dare[1], Richard V. Espley[1], Sumathi Tomes[1], David Chagné[2] and Yar-Khing Yauk[1]

[1] The New Zealand Institute for Plant & Food Research Limited (PFR), Private Bag 92169, Auckland 1142, New Zealand

[2] PFR, Private Bag 11600, Palmerston North 4442, New Zealand

[3] Northwest Agricultural and Forestry University, Xi'an, Shaanxi, China

Abstract

Major QTLs (quantitative trait loci) for the production of two-character impact compounds in apple (*Malus x domestica*), namely 2-methylbutyl acetate (2-MBA, over ripe, fruity, sweet notes) and estragole (anise, licorice notes), both map to the *MdAAT1* (*alcohol acyl transferase 1*) locus in the apple genome. Biochemical analysis shows that *MdAAT1* is required for the production of volatile acetate esters such as 2-MBA, hexyl acetate and butyl acetate and for the production of *p*-hydroxycinnamyl acetates that are substrates for the production of phenylpropenes such as eugenol, chavicol and estragole in ripe apple fruit. The importance of the *MdAAT1* gene in ester and phenylpropene production was validated in transgenic 'Royal Gala' knockdown lines that produced significantly reduced 2-MBA and estragole levels in ripe fruit. Manipulation of flux through the phenylpropanoid pathway in apple using *MdCHS* (chalcone synthase) knockout and *MdMYB10* (transcription factor) over-expression lines increased phenylpropene production. Transient over-expression of alcohol acyl transferases (AATs) from ripe strawberry and tomato fruit showed these enzymes can also produce *p*-hydroxycinnamyl acetates, indicating that ripening-related AATs are likely to link volatile ester and phenylpropene production in many different fruit. These results significantly increase our understanding of volatile synthesis in fruit and provide the basis for breeding new apple varieties with improved flavour profiles by marker assisted selection.

Introduction

The characteristic taste and aroma of different fruit species and cultivars is derived from non-volatile components such as sugars (providing sweetness), acids (sourness, tartness) and tannins (astringency, bitterness), as well as volatile compounds such as esters, phenylpropenes, alcohols, aldehydes, terpenes and furans. Fruit such as apple, banana, kiwifruit, melon and pear produce high levels of volatile esters which contribute characteristic 'fruity' notes to the aroma. In 'Gala' apples, the major odour-active esters are hexyl acetate (fruity, green, apple notes), butyl acetate (ethereal, solvent, fruity) and 2-methylbutyl acetate (overripe, fruity, sweet) [1]. Volatile esters are synthesised via fatty acid degradation or from amino acid precursors with the final step being catalysed by alcohol acyl transferases (AATs). AATs catalyse the transfer of an acyl group from a CoA donor to an alcohol acceptor.

Phenylpropenes are typically found at low levels in fruit, and impart flavour notes associated with aromatic spices [2]. In apple, estragole (anise, licorice notes) is the most widely described odour-active phenylpropene; with eugenol (sweet, spicy, clove) and chavicol (clove, spicy) also being reported [2]. In tomato, eugenol and guaiacol content

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is correlated with a 'pharmaceutical' aroma [3], whilst in musk strawberry the high content of eugenol, methyleugenol, and methylisoeugenol has been associated with a cinnamon smell [4]. Phenylpropenes are produced as a side branch of the general phenylpropanoid pathway and the initial biosynthetic steps are shared with the lignin biosynthetic pathway up to the production of *p*-coumaryl alcohol and coniferyl alcohol. The first committed step in phenylpropene production involves the conversion of *p*-coumaryl and coniferyl alcohols to *p*-coumaryl and coniferyl acetates (*p*-hydroxycinnamyl acetates). The acetates are then reduced by NADPH-dependent phenylpropene reductases. Methoxylated phenylpropenes such as estragole and anethole are formed by *O*-methyltransferases (OMT) using S-adenosylmethionine (SAM) as the methyl donor.

QTLs (quantitative trait loci) for volatile production have recently been described for aldehydes, esters, alcohols and phenylpropenes in apple (*Malus x domestica*). Forty-six QTLs for ester and alcohol production were reported in a cross between the highly aromatic cultivar 'Royal Gala' (RG) and the low aroma cultivar 'Granny Smith' (GS) [5]. The major QTL for the production of 2-methylbutyl acetate (2-MBA) and 34 other volatiles in this population was located on linkage group 2 (LG2) and co-located with the *Malus x domestica alcohol acyl transferase 1* (*MdAAT1*) gene. Two QTLs for production of the phenylpropene estragole were also identified in the same segregating population. The first QTL was located on LG1 and was responsible for 9.2% of the variation. The *MdoOMT1* (*O-methyltransferase 1*) gene was shown to co-locate with the LG1 QTL and biochemical and molecular analysis showed that this gene was required for estragole production in ripe RG fruit [6].

Results and discussion

QTL analysis

The major QTL for the production of estragole (accounting for 24% of the variance) in the segregating RG x GS population is located on LG2. The nearest marker to the maximum logarithm of the odds (LOD) peak for estragole production was identical to that previously identified as co-segregating with the production of volatile esters such as 2-MBA, butyl acetate and hexyl acetate in ripe apple fruit [7]. This result suggested that MdAAT1 might be the enzyme responsible for an acylation step in both the ester biosynthetic and phenylpropene biosynthetic pathways.

Biochemical characterisation

The enzymatic activity of MdAAT1 has previously been reported with respect to a wide range of alcohols and acyl CoAs involved in volatile ester production in ripe apple fruit [8]. In Yauk et al., 2017 [7], MdAAT1 was also shown to convert coniferyl and *p*-coumaryl alcohols to *p*-hydroxycinnamyl acetates that serve as substrates for phenylpropene production in apple. The relative activity of recombinant MdAAT1-RGa from RG towards alcohols such as butanol, 2-methylbutanol and hexanol used for volatile ester production was high (64–100%, Table 1). In contrast, relative activity towards *p*-coumaryl alcohol and coniferyl alcohol was much lower (< 2%, Table 1). Kinetic studies indicated that the affinity of MdAAT1-RGa towards *p*-coumaryl alcohol was comparable to that reported for hexanol and butanol, however the V_{max} was much lower [7]. Compared with MdAAT1-RGa, recombinant MdAAT1-GSa from GS showed weak activity (< 3%) towards alcohols used for volatile ester biosynthesis and barely detectable activity (0.11%) towards *p*-coumaryl alcohol (Table 1). This difference in kinetic properties likely explains the QTL in the segregating RG x GS population.

Table 1: Activity of recombinant MdAAT1 enzymes from 'Royal Gala' (RG) and 'Granny Smith' (GS). Activity assays contained MdAAT1-RGa (0.5 µg) or MdAAT1-GSa (31.2 µg), 10 mM alcohol, 1 mM CoA, in 50 mM Bis-Tris propane pH 8.0. Activity was set at 100% for acetyl-CoA and hexanol using MdAAT1-RGa. Data are presented as mean ± SE (n=3). Chavicol was synthesised by esterification of *p*-coumaric acid followed by DIBAL reduction to *p*-coumaryl alcohol. Radiolabelled acetyl-CoA was obtained from American Radiolabeled Chemicals and all other chemicals from Sigma-Aldrich. Data derived from Yauk et al., 2017 [7].

Esters			
Substrate 1	*Substrate 2*	*MdAAT1-RGa*	*MdAAT1-Gsa*
Hexanol	acetyl-CoA	100±1.4	2.94±0.16
Butanol		63.8±3.0	1.03±0.06
2-methylbutanol		77.6±3.3	0.26±0.02

Phenylpropenes			
Substrate 1	*Substrate 2*	*MdAAT1-RGa*	*MdAAT1-GSa*
p-coumaryl alcohol	acetyl-CoA	1.2±0.2	0.11±0.01
coniferyl alcohol		0.3±0.02	not detected

Analysis of MdAAT1 knockdown lines

To validate the importance of *MdAAT1* in phenylpropene production, transgenic RG lines containing an RNAi construct of *MdAAT1* were examined. Our hypothesis was that decreasing *MdAAT1* expression (Figure 1A) would reduce the production of *p*-hydroxycinnamyl acetates and the subsequent accumulation of phenylpropenes in ripe fruit. The results from solvent extraction and GC-MS analysis on ripe fruit samples from two transgenic lines and RG controls is presented in Figure 1B. Total ester production in the *MdAAT1* lines was reduced by > 90%, confirming the results previously reported in Souleyre et al., 2014 [5]. Production of the phenylpropenes chavicol, (*E*)-isochavicol, eugenol and estragole were also reduced in the *MdAAT1* knockdown lines.

Manipulation of flux in the phenylpropanoid pathway

Two additional sets of transgenic apple plants were investigated to determine what effect manipulating flux through the phenylpropanoid pathway would have on phenylpropene accumulation. The first set of transgenic lines were down-regulated for expression of the *MdCHS* (chalcone synthase) gene, a key biosynthetic gene in the phenylpropanoid pathway. *MdCHS* knockout lines do not accumulate anthocyanins or dihydrochalcones which are normally abundant in apple fruit [9]. Our hypothesis was that redirection of flux in these plants would lead to accumulation of higher levels of phenylpropenes (Figure 2A). The results from solvent extraction and GC-MS analysis on ripe fruit samples from three transgenic lines and RG controls is presented in Figure 2B. Production of the phenylpropenes chavicol and (*E*)-isochavicol increased in all three *MdCHS* lines compared to the control. Production of eugenol and estragole was elevated in lines A2 and A7 respectively. As expected, total ester production in the *MdCHS* knockout lines was similar to the RG control.

Analysis of the glycosides present in two of the *MdCHS* knockout lines compared to the RG control indicated that production of chavicol and eugenol glycosides was elevated in the transgenic lines (Figure 2B). Chavicol glycosides were found at 80–100 fold higher levels in the *MdCHS* lines compared to controls, whilst eugenol glycosides were found at 6–12 fold higher levels. Much of the increased flux towards phenylpropene production in the *MdCHS* knockout lines appeared to be directed towards glycoside sequestration. The total phenylpropene glycoside concentration in the *MdCHS* lines was 35,000–40,000 ng/g

(vs 700 ng/g in controls), whilst the total 'free' phenylpropene concentration was 1,400–4,000 ng/g in the *MdCHS* lines (vs 800 ng/g in controls).

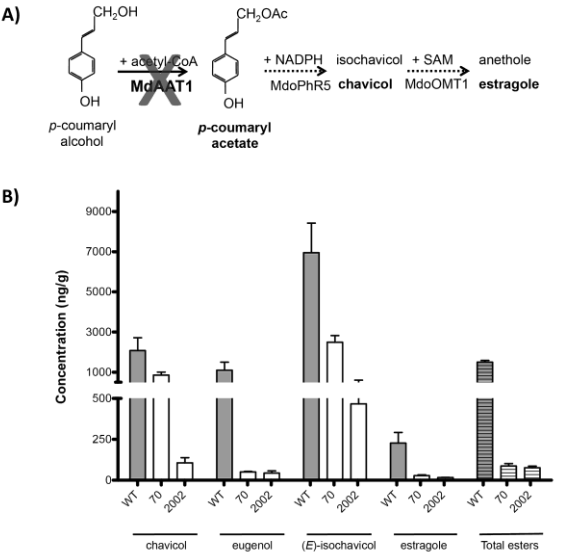

Figure 1: Analysis of *MdAAT1* knockdown lines (**A**) In *MdAAT1* knockdown lines the production of *p*-coumaryl acetate and phenylpropene derivatives should be reduced. *MdAAT1 = Malus domestica alcohol acyl transferase 1*, *MdoPhR5 = Malus domestica phenylpropene reductase 5*, *MdoOMT1 = Malus domestica O-methyltransferase 1*. (**B**) Volatile phenylpropene and ester production in 'Royal Gala' wildtype (WT) controls (grey bars) and *MdAAT1* knockdown lines (AS70 and AS2002, white bars). Volatiles were obtained from ripe fruit skin tissue extracted into diethyl ether. GC-MS analysis of solvent extracts was performed on an Agilent 6890N GC coupled to a Waters GCT time of flight-mass spectrometer [10]. Data are presented as mean ± SE and are derived from Yauk et al., 2017 [7].

The second set of transgenic apple plants in which phenylpropene flux was manipulated constitutively over-expressed a copy of the *MdMYB10* gene, a transcription factor that up-regulates flux through the phenylpropanoid pathway. *MYB10* over-expression lines have red foliage and red-fleshed fruit and accumulate much higher levels of anthocyanins, flavonols and total phenolics due to increased expression of several phenylpropanoid biosynthetic genes [12]. Our hypothesis was that the *MYB10* over-expression lines would also accumulate higher levels of phenylpropenes either as a result of higher flux through the phenylpropanoid pathway or that *MdMYB10* might transcriptionally activate genes involved in phenylpropene biosynthesis. The results of GC-MS analysis indicated that compared to controls, the *MYB10* lines accumulated higher levels of phenylpropenes at all four stages of fruit development tested, but particularly at the two latter time points [6]. No evidence for transcriptional activation of *MdAAT1*, *MdoPhR5 (phenylpropene reductase 5*) or *MdoOMT1* in *MYB10* over-expression lines was observed [7]. Together these results suggested that the increased phenylpropene levels in the *MYB10* fruit was due to some of the increased flux in the phenylpropanoid pathway being diverted into the phenylpropene biosynthetic pathway.

Do AAT genes link ester and phenylpropene biosynthesis in other fruit?

Our results in apple clearly demonstrated the importance of *MdAAT1* to ester and phenylpropene production in apple, but is this true for *AAT* genes from other species that accumulate both esters and phenylpropenes such as strawberry and tomato? To test this

hypothesis, AATs from ripe strawberry (*SAAT*) and tomato (*SlAAT1*) fruit were transiently expressed in *Nicotiana benthamiana* in coupled reactions with *MdoPhR5*.

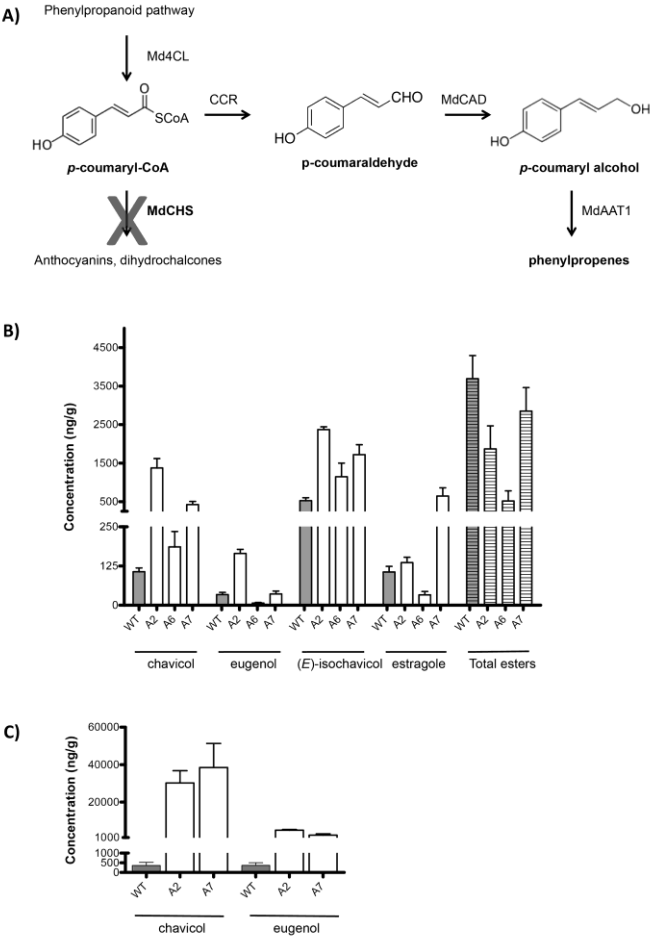

Figure 2: Analysis of *MdCHS* knockout lines (**A**) In *MdCHS* knockout lines the phenylpropanoid pathway is blocked leading to the accumulation of *p*-coumaryl-CoA which is then metabolised via *p*-coumaraldehyde to *p*-coumaryl alcohol. *p*-Coumaryl alcohol is the substrate for *MdAAT1* and the entry point for production of phenylpropenes. *Md4CL = Malus domestica 4-coumarate CoA-ligase*, *MdCHS = Malus domestica chalcone* synthase, *MdCCR = Malus domestica cinnamoyl-CoA reductase*, *MdCAD = cinnamyl alcohol dehydrogenase*, *MdAAT1 = Malus domestica alcohol acyl transferase 1*. (**B**) Volatile phenylpropene and ester production in 'Royal Gala' wildtype (WT) controls (grey bars) and *MdCHS* knockout lines (A2, A6 and A7, white bars). Volatiles were extracted from ripe fruit skin tissue into diethyl ether. GC-MS analysis of solvent extracts was performed on an Agilent 6890N GC coupled to a Waters GCT time of flight-mass spectrometer [10]. Data are presented as mean ± SE and are derived from Yauk et al., 2017 [7]. (**C**) Phenylpropene glycoside production in 'Royal Gala' WT controls (grey bars) and *MdCHS* knockout lines (white bars, A2 and A7). Glycosides were prepared using Amberlite XAD-2 columns as described in Yauk et al., 2014 [11] from ~ 3 g of ripe apple fruit skin tissue. Glycosides were digested with Rapidase AR2000 for 16 h at 37ºC in reactions overlaid with 100 µL diethyl ether/pentane. GC-MS analysis was performed as described in Nieuwenhuizen et al., 2013 [10]. Data are presented as mean ± SE.

Coupled reactions were used as the phenylpropene products are stable and readily detected by GC-MS analysis [7]. The results presented in Figure 3 show that chavicol

was produced by both *SAAT* and *SlAAT1* when leaves were infiltrated with *p*-coumaryl alcohol. Eugenol was also produced by both *SAAT* and *SlAAT1* when leaves were infiltrated with coniferyl alcohol, but at much higher levels with *SAAT* from strawberry. These results suggest that ripening-related AATs may link volatile ester and phenylpropene production in many different fruit and provide a rational basis for breeding new varieties with improved flavour profiles by marker assisted selection or metabolic engineering.

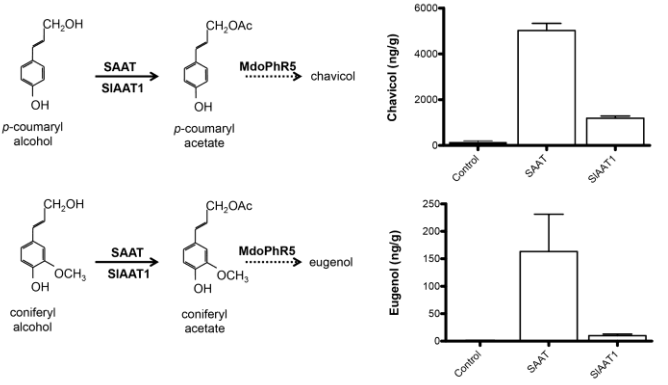

Figure 3: Functional characterisation of AATs from ripe strawberry and tomato fruit. The activities of strawberry AAT (*SAAT*) and tomato AAT *(SlAAT1*) were determined by transient expression analysis in *Nicotiana benthamiana*. Leaves were initially infiltrated with *SAAT* or *SlAAT1* coupled with *MdoPhR5* (*Malus domestica phenylpropene reductase 5*). GUS + *MdoPhR5* was used as the control. After seven days, leaves were infiltrated with either *p*-coumaryl or coniferyl alcohol. GC-MS analysis of solvent extracts was performed on an Agilent 6890N GC coupled to a Waters GCT time of flight-mass spectrometer [10]. Data are presented as mean ± SE and are derived from Yauk et al., 2017 [7].

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