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ARTICLE

Heritability and Genetic and Phenotypic Correlations of Apple (*Malus* × *domestica*) Fruit Volatiles in a Genetically Diverse Breeding Population

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Flavor is an important quality trait of fruit and a target for improvement through plant breeding. Eighty-nine flavor volatiles from 240 apple (*Malus domestica*) genotypes from a highly diverse breeding population were measured by headspace gas chromatography−mass spectrometry (GC-MS) over 2 years. Heritabilities and phenotypic and genetic correlations were calculated for 23 flavor volatiles. Genetic correlations showed coinheritance of five groups of volatiles, ethyl esters, alcohols and α-farnesene, propyl and butyl esters, propanoate and 2-methylbutanoate esters, and acetate esters, consistent with our knowledge of volatile biosynthesis in apple. This work demonstrates a genetic structure underlying the highly variable volatile profiles observed for apple fruit and the potential of GC-MS volatile profiling for the genetic analysis of aroma volatiles in genetically diverse populations.

KEYWORDS: Apple; *Malus domestica*; flavor volatiles; volatile profiling; heritability; genetic and phenotypic correlations

INTRODUCTION

Flavor is a key determinant of the consumer acceptability of fruit and is receiving increasing attention from plant breeders with the improvement of flavor now an objective in many apple breeding programs (*1*). Flavor is a complex trait involving contributions from the relative levels of sugars, acids, and flavor volatiles with some volatiles making greater contributions than others to particular flavors. Volatiles considered important for apple flavor include esters such as ethyl butanoate, ethyl 2-methylbutanoate and butyl, 2-methylbutyl, and hexyl acetate; however, the relative contribution of these volatiles to flavor is also dependent on the apple cultivar and its maturity (*2*−*5*). Production of volatile compounds by fruit is sensitive to developmental and environmental cues so that the level of flavor volatiles produced depends not only on the genotype but also on the extent to which nongenetic factors affect individual genotypes (*6*−*9*).

Knowledge of the genetic systems controlling the inheritance of desirable traits such as flavor, and of the genetic and environmental factors that influence their expression, is fundamental for a successful breeding program. Heritability estimates are useful for making predictions of genetic progress in the offspring when the parents are selected on the basis of their own performance and for choosing among selection strategies to improve breeding efficiency (*10*). Heritability information may also provide explanations for major changes in the amount and nature of genetic variability over generations (*11*). Phenotypic correlations show relationships between traits and are a combination of both genetic and environmental correlations. For traits with low heritability, the phenotypic correlation is determined mainly by the environmental correlation, whereas for characters with high heritability, the genetic correlation is more important (*12*). Additive genetic correlation occurs when a single gene affects two traits and is that proportion of the variance that two traits share that is due to additive genetic, as opposed to nonadditive genetic (e.g., epistasis) or nongenetic (environmental) causes. Genetic correlations of traits are independent of their heritability; hence, traits with low heritability can have high genetic correlations. Genetic correlations are useful for predicting the level of expression of a character without the need for measuring that character directly, for the construction of selection indices, and for a better understanding of gene action and responses to multitrait selection (*13*). The breeding value identifies the value of an individual genotype as a breeding parent for a trait and is that part of an individual's genotypic value that is due to additive and therefore transmittable gene effects (*12*, *14*). Estimation of breeding values enables the selection of superior parents.

While the biosynthesis and postharvest responses of apple volatiles have been extensively studied (*2*, *15*, *16*), little is known of the inheritance of flavor volatiles in apple or of the relative importance of environmental and genetic factors in determining

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the concentrations of particular volatiles (*6*). Measurement of fruit texture and sugars is facilitated by the use of the penetrometer to measure firmness and the refractometer for soluble solids; hence, estimates of genetic parameters for fruit quality traits in apple have focused mainly on texture, sweetness, and acidity (*17*−*21*). The assessment of volatile flavor requires expensive instrumentation such as gas chromatography−mass spectrometry (GC-MS) or the electronic nose (*2*) or the use of sensory analysis, which may describe the flavor but fail to identify or quantify the particular compounds responsible. The cost and relatively low sample throughput of GC-MS flavor analysis have made it difficult to incorporate the objective measurement of flavor volatiles into plant breeding programs. The genetic analysis of apple flavor volatiles is less advanced than for fruit such as tomato (*Lycopersicum esculentum*) where there is a comprehensive body of literature, including surveys of volatile concentrations in fruit of diverse genotypes (*22*, *23*) and the identification using GC-MS of quantitative trait loci (QTL), which influence flavor (*24*). Recently, QTL for apple flavor volatiles have been identified in a small 'Fiesta' × 'Discovery' F_1 population using proton transfer reaction-mass spectrometry (PTR-MS) (*25*) and in a 'Discovery' × 'Prima' population using headspace solid-phase microextraction GC (*26*). This latter study (*26*) identified putative QTL for 27 apple fruit volatiles distributed over 12 of the 17 apple chromosomes but mainly clustered on linkage groups 2, 3, and 9.

We wished to determine if GC-MS volatile analysis could be deployed as a useful tool to assist apple breeders in making informed breeding and management decisions. To this end, we undertook to determine the heritability and genetic and phenotypic correlations of apple volatiles in an especially widely based apple breeding population (*27*). This work demonstrates a genetic structure underlying the highly variable volatile profiles observed for apple fruit and the potential of GC-MS volatile profiling for the genetic analysis of aroma volatiles in genetically diverse populations. To our knowledge, this is the first study to use GC-MS volatile profiling to calculate genetic parameters for apple while sampling from an operational breeding program.

MATERIALS AND METHODS

Plant Material. Fruits were harvested from 166 and 101 genotypes (individual apple trees) in 2006 and 2007, respectively, from one site (Havelock North, New Zealand, 39°39′S 176°53′E) of Plant and Food Research's recurrent selection apple breeding program (*27*). The first generation of this program, established in the early 1990s, consisted of families derived from open pollinated seeds from about 400 old and modern cultivars and a hundred individual trees found growing wild in Kazakhstan, the probable center of origin of the domestic apple. For this study, 73 genotypes were chosen from the 310 first generation genotypes used as parents for the second generation, and 167 genotypes were chosen from the second generation progeny (**Table 1**). Most trees were harvested only once, but some were harvested twice in one year or in both years. Trees were chosen based on fruit availability and, in the second year, to increase the degree of relatedness within the study.

Fruits were harvested when judged mature by an experienced horticulturalist based upon the background color (cream or green/yellow), fruit drop (if any), and taste (starch/sugar balance) and stored at 1 °C for 15 days and then for 5 days at 20 °C (*28*−*30*) before measurement of headspace volatiles. Longer cold storage times resulted in damage to some fruit samples harvested early in the season. In 2006, the fruit surface area was calculated from the average diameter measured across the fruit using a hand-held micrometer. In 2007, fruit volume, and hence surface area, was calculated by measuring the mass of water displaced when fruits were submerged. In each case, fruits were assumed to be perfectly spherical.

Chemicals. Ethyl butanoate, butyl acetate, 2-methylbutanol, and ethyl hexanoate were obtained from Aldrich; ethyl 2-methylbutanoate was from Acros; butanol was from BDH; and hexanol was from Sigma. All other esters indicated in **Table 2**, except propyl 2-methylbutanoate, for which no standard was prepared, were obtained by reaction of the appropriate alcohols and acid anhydrides (*31*). (*E*,*E*)- and (*Z*,*E*)-α-Farnesene were obtained from apple skin (*32*).

Table 1. Numbers of Genotypes, Sampled Once or Twice, from Each Group during 2006 and 2007[a]

group	times sampled	2006	2007	total
parents	1	38	29	
	2	7	0	
	total	45	29	73
progeny	1	110	70	
	2	11	2	
	total	121	72	167
combined	1	148	99	
	2	18	2	
	total	166	101	240

[a] One parental genotype and 26 progeny genotypes were assessed in both years.

Volatile Analysis. Fruits (typically 3−10 depending on size) were placed in 1.5 L glass jars as previously described (*15*, *31*). Small-sized fruits of limited number were placed on a glass Petri dish supported near the top of the jar on an inverted glass beaker. Charcoal-filtered air was drawn into the bottom of each jar for 3 h at 40 mL min^{-1}, and volatiles were absorbed onto Tenax TA (350 mg) contained in glass tubes attached to the top of each jar. After volatile collection, the traps were eluted with diethyl ether (2 × 1 mL) containing tetradecane at 10 nL mL^{-1} into preweighed 4 mL glass vials at a flow rate of 2 mL min^{-1}. Samples were stored at −20 °C prior to GC-MS analysis.

GC-MS separations were carried out using an Agilent 6890N Gas Chromatograph coupled to a Waters GCT Time of Flight mass spectrometer using a 20 m × 0.18 mm i.d. × 0.18 μm film thickness Agilent DB-Wax capillary column and a 20 s splitless injection. The helium flow rate was 1 mL min^{-1}, the injection temperature was 220 °C, and the oven temperature was 35 °C (1 min), 2.9 °C min^{-1} to 100 °C, and 8 °C min^{-1} to 200 °C (5 min). Volatiles were identified from their retention index and by comparison with commercial mass spectral databases and authentic compounds. Volatiles reported and the ions used for quantitation are listed in **Table 2**. Generally, base peak intensities were used to aid automated peak identification and integration using Waters QuanLynx software. Volatiles are reported as ng tetradecane equivalents released cm^{-2} fruit surface area h^{-1}. A reference standard of ethyl 2-methylbutanoate, 2-methylbutanol, tetradecane, hexyl butanoate, and hexanol was injected after every ten samples to monitor instrument performance. The variability between recoveries from individual Tenax traps was assessed by carefully pipetting a solution of octyl acetate (8.7 μg) in diethyl ether (10 μL) onto a circle of filter paper folded into the neck of each headspace jar. The relative standard deviation of the peak area of octyl acetate measured by GC-MS was 19.7% (n=24). This variability was considerably less than that for duplicate apple samples, collected in a separate experiment, where the mean percent difference in volatile concentrations was 40.9 % (±6.9 SD) for 48 volatiles in cases where each volatile was detected in both duplicate samples (*30*) and also much less than the variability found between trees of different genotypes.

Statistical Analysis. Concentrations for the 89 volatiles measured were $\log_{10}$ transformed, after adding half the minimum value for each volatile to that volatile's values. Mixed linear modeling was undertaken for each volatile (excluding four volatiles which were rarely present) with year (2006 or 2007) and genetic groups (parent or progeny) set as fixed effects and genotypes as random effects. The numerator relationship matrix A (i.e., double the coancestry matrix) was incorporated in the model according to Lynch and Walsh (*14*). Thus

$$y = X\beta + Zu + e$$

where y is the vector of phenotypic values, X and Z are incidence matrices for the fixed and random effects, respectively, β is the vector of fixed effects (i.e., year and genetic group), u is the vector of random effects

Table 2. Retention Times (Minutes), Mass Spectral Quantitation Ions, Frequency of Occurrence, and Concentrations as ng Tetradecane Equivalents Released cm^{-2} Surface Area h^{-1} for 23 Apple Volatiles Measured by GC-MS for Which Satisfactory Heritabilities Were Calculated

year			2006				2007			
volatile	RI[a]	MS ion (*m*/*z*)	% found[b]	mean	median	maximum value	% found	mean	median	maximum value
methyl 2-methylbutanoate[c]	1001	88.1	82	1.1	0.26	34	89	3.2	0.29	61
ethyl butanoate[c]	1023	88.1	89	216	0.53	3534	100	227	3.2	4078
1-methylethyl butanoate[c]	1028	43.1	86	0.80	0.28	7.5	97	1.9	0.5	39
ethyl 2-methylbutanoate[c]	1038	57.1	89	39	0.31	1122	99	137	1.1	2552
butyl acetate[c]	1059	43.0	91	208	6.9	2437	100	116	2.6	854
2-methylbutyl acetate[c]	1109	70.1	59	29	5.8	152	93	70	1.7	365
propyl butanoate[c]	1110	43.0	57	4.4	0.88	68	87	19	3.7	177
ethyl pentanoate[c]	1122	115.1	79	0.6	0.26	12	100	1.6	0.20	32
propyl 2-methylbutanoate[d]	1125	57.1	30	2.3	0.73	10	76	11	1.3	170
butyl propanoate[c]	1127	57.0	66	9.0	1.9	82	88	9.7	1.8	62
butanol[c]	1135	56.1	93	35	5.7	518	99	32	16	293
pentyl acetate[c]	1159	70.1	88	3.6	0.45	42	99	2.8	0.35	21
2-methylbutanol[c]	1199	57.1	94	3.4	0.60	85	99	10	2.2	145
butyl butanoate[c]	1206	71.1	97	20	2.9	236	100	34	13	280
butyl 2-methylbutanoate[c]	1219	103.1	79	5.9	0.46	75	96	16	1.8	188
ethyl hexanoate[c]	1221	99.1	76	74	0.56	2305	93	77	0.27	1262
hexyl acetate[c]	1259	43.0	96	118	3.1	1535	100	64	1.4	613
hexyl propanoate[c]	1327	84.1	57	3.5	1.0	34	85	3.5	1.6	50
hexanol[c]	1348	56.1	95	11	1.4	228	99	6.2	1.3	65
butyl hexanoate[c]	1403	99.1	79	9.0	3.1	88	97	45	7.0	386
hexyl 2-methylbutanoate[c]	1418	57.1	94	19	2.4	298	100	34	8.7	340
ethyl octanoate[c]	1425	88.1	85	1.0	0.25	14	99	5.5	0.28	135
E,*E*-α-farnesene[c]	1738	135.1	100	108	33	1095	100	139	186	1988

[a] Retention index. [b] Percent of samples where volatile was detect. [c] Identified by GC-MS comparison with authentic material. [d] Tentatively identified from the mass spectrum and retention indices.

(i.e., genotype), $u \sim (0, G) = (0, \sigma_A^2 A)$ where σ_A^2 is the additive genetic variance, and $e \sim (0, R) = (0, \sigma^2 I)$.

Breeding values (best linear unbiased predictors of the genotype effect) and variance components were estimated for the 23 volatiles with residual plots that approximated a normal distribution. Narrow sense heritabilities were computed using the equation:

$$h^2 = \frac{\sigma_A^2}{\sigma_A^2 + \sigma^2}$$

and genetic correlations between volatiles were estimated as:

$$r_G = \frac{\mathrm{Cov}_{ab}}{\sqrt{\sigma_{Aa}^2 \times \sigma_{Ab}^2}}$$

where σ_{Aa}^2 and σ_{Ab}^2 are the additive genetic variances of volatiles *a* and *b* and Cov_{ab} is the additive genetic covariance between these two volatiles. This additive genetic covariance was derived from bivariate analysis of each pair of volatiles. Thus, the additive genetic correlation is that proportion of the variance that two traits share that is due to additive genetic as opposed to nonadditive genetic (e.g., epistasis) or nongenetic (environmental) causes. Standard errors for these estimates were derived according to the general formula of Ku (*33*). These computations were undertaken on the entire data set and also a data set restricted to those genotypes with at least five relatives.

Hierarchical cluster analysis of the volatiles was undertaken for both phenotypic and genetic correlations by setting the distance between any pair of volatiles as one minus the correlation between them ($1 - r$, genetic correlations with estimates greater than one were set to one). A heat map was constructed using the matrix of empirical breeding values (*14*) of the 240 genotypes for each of the 23 volatiles. The use of empirical breeding values was considered the best way to adjust for the differences between years. All analyses were undertaken in R 2.7.2 (*34*). Mixed modeling used the asreml() package (*35*).

RESULTS AND DISCUSSION

Initially, 89 volatiles were measured by GC-MS analysis of the headspace of the various apple fruit samples; however, some volatiles were detected only infrequently (germacrene D, 2-heptanone, 4-hexenol, and *Z*-3-hexenyl butanoate), while a few others could not be unambiguously identified. Volatiles that were detected infrequently tended to have non-normal residual distributions. After log transformation, 23 volatiles (**Table 2**) had residuals that were approximately normally distributed. These included 17 branched and straight chain esters, three alcohols (butanol, 2-methylbutanol, and hexanol), and α-farnesene representing the major flavor volatiles reported in apple. Twelve of these volatiles, methyl, ethyl, propyl, butyl, and hexyl 2-methylbutanoates, ethyl butanoate, butyl, 2-methylbutyl, and hexyl acetates, and butanol and 2-methylbutanol, have been reported as important contributors to apple aroma (*2*−*5*).

The genetic relationship between genotypes (genetically different apple trees) was quantified as a coancestry; that is, the probability that an allele in one genotype was identical by descent to an allele at the same locus in a second genotype. For the genotypes under study, the coancestry matrix was sparsely populated with 95% of the coancestries being zero, about 1.1% being half-siblings (coancestry 0.125), and 1% being full siblings (coancestry, 0.25). None of the genotypes were inbred (*27*). There were approximately 36 kinship groups of related genotypes (most full-sibs or parent/offspring but some half-sibs) with 2−12 genotypes per group. Twenty of these kinship groups contained 2−4 genotypes, but the number of genotypes in the other kinship groups was spread fairly evenly from 5 to 12. The volatile profiles of fruit from most (82%) of the genotypes were measured only once, but volatile profiles from 17% of the genotypes were measured twice, and three genotypes (1%) were measured three times over two seasons (**Table 1**). Thus, while there were a good number of kinship groups, the numbers of genotypes per group were less than ideal.

For the 23 volatiles with satisfactory residual plots, the variance due to genotype (genetic effect) was higher than or similar to the residual variance except in the case of methyl and ethyl 2-methylbutanoate, 1-methylethyl butanoate, and ethyl

Table 3. Best Linear Unbiased Estimates for the Fixed Effects and Variance Components for the Random Effects (σ_A^2 and σ^2) from the Mixed Modelling for 23 Volatiles (Log_{10} Transformed) Based on 240 Apple Genotypes[a]

	fixed effects (best linear unbiased estimates)				random effects		
	group		year		variance components		
volatile	progeny	parents	2006	2007	genotype	residual	h^2
methyl 2-methylbutanoate	−0.69 (0.081)	−0.98 (0.107)	−1.07 (0.078)	−0.59 (0.102)	0.14 (0.098)	0.75 (0.103)	0.15 (0.095)
ethyl butanoate	0.74 (0.168)	0.57 (0.177)	0.24 (0.142)	1.08 (0.175)	1.62 (0.282)	0.82 (0.152)	0.66 (0.057)
1-methylethyl butanoate	−0.53 (0.085)	−0.40 (0.100)	−0.71 (0.077)	−0.22 (0.099)	0.27 (0.097)	0.50 (0.080)	0.35 (0.090)
ethyl 2-methylbutanoate	0.04 (0.154)	0.05 (0.178)	−0.31 (0.137)	0.39 (0.176)	0.94 (0.314)	1.49 (0.249)	0.39 (0.089)
butyl acetate	0.94 (0.171)	1.11 (0.177)	0.99 (0.145)	1.06 (0.173)	1.87 (0.287)	0.60 (0.126)	0.76 (0.048)
2-methylbutyl acetate	0.13 (0.185)	0.31 (0.189)	−0.15 (0.155)	0.58 (0.182)	2.32 (0.297)	0.52 (0.104)	0.82 (0.036)
propyl butanoate	−0.21 (0.129)	−0.13 (0.141)	−0.69 (0.111)	0.34 (0.140)	0.83 (0.175)	0.71 (0.117)	0.54 (0.067)
ethyl pentanoate	−0.75 (0.074)	−0.72 (0.094)	−0.93 (0.070)	−0.54 (0.091)	0.14 (0.081)	0.54 (0.080)	0.20 (0.097)
propyl 2-methylbutanoate	−0.89 (0.128)	−0.71 (0.143)	−1.35 (0.111)	−0.24 (0.142)	0.75 (0.180)	0.81 (0.130)	0.48 (0.072)
butyl propanoate	−0.12 (0.142)	0.05 (0.156)	−0.30 (0.123)	0.23 (0.155)	0.99 (0.223)	0.87 (0.149)	0.53 (0.070)
butanol	0.91 (0.119)	0.83 (0.128)	0.69 (0.102)	1.04 (0.127)	0.77 (0.15)	0.49 (0.089)	0.61 (0.063)
pentyl acetate	−0.33 (0.112)	−0.12 (0.119)	−0.21 (0.096)	−0.24 (0.118)	0.70 (0.142)	0.40 (0.081)	0.64 (0.066)
2-methyl butanol	0.05 (0.091)	0.16 (0.104)	−0.10 (0.080)	0.30 (0.103)	0.35 (0.102)	0.48 (0.078)	0.42 (0.082)
butyl butanoate	0.87 (0.112)	0.79 (0.118)	0.56 (0.095)	1.10 (0.116)	0.75 (0.063)	0.33 (0.063)	0.70 (0.054)
butyl 2-methylbutanoate	−0.07 (0.125)	−0.09 (0.132)	−0.31 (0.106)	0.14 (0.130)	0.93 (0.151)	0.42 (0.077)	0.69 (0.052)
ethyl hexanoate	0.04 (0.162)	−0.13 (0.173)	−0.29 (0.138)	0.20 (0.172)	1.42 (0.286)	0.90 (0.169)	0.61 (0.065)
hexyl acetate	0.91 (0.164)	0.83 (0.167)	0.92 (0.138)	0.81 (0.159)	1.90 (0.238)	0.34 (0.073)	0.85 (0.032)
hexyl propanoate	−0.36 (0.115)	−0.30 (0.126)	−0.57 (0.099)	−0.09 (0.125)	0.64 (0.147)	0.57 (0.099)	0.53 (0.071)
hexanol	0.36 (0.091)	0.41 (0.099)	0.34 (0.078)	0.43 (0.099)	0.41 (0.094)	0.34 (0.062)	0.55 (0.072)
butyl hexanoate	0.59 (0.142)	0.43 (0.147)	−0.05 (0.120)	1.07 (0.143)	1.30 (0.191)	0.40 (0.191)	0.77 (0.045)
hexyl 2-methylbutanoate	0.60 (0.117)	0.57 (0.128)	0.31 (0.101)	0.86 (0.127)	0.69 (0.175)	0.57 (0.114)	0.55 (0.080)
ethyl octanoate	−0.51 (0.099)	−0.33 (0.115)	−0.67 (0.088)	−0.17 (0.113)	0.39 (0.130)	0.62 (0.103)	0.39 (0.088)
E,*E*-α-farnesene	1.66 (0.102)	1.80 (0.108)	1.56 (0.087)	1.90 (0.107)	0.57 (0.119)	0.34 (0.069)	0.63 (0.068)

[a] The narrow sense heritabilities h^2 are also shown. Standard errors are given in parentheses.

pentanoate and octanoate (**Table 3**). As a consequence, these metabolites had the lowest heritability (0.15−0.39). The highest heritability estimate was for hexyl acetate (0.85) but with two (butyl acetate, 0.76; 2-methylbutyl acetate, 0.82) of the other three acetates measured also in the top four heritabilities. Most heritability estimates were similar (within one standard error) when recomputed using the data set restricted to those genotypes with at least five relatives (data not shown) except for those volatiles with heritability estimates less than 0.5 (methyl, ethyl, and propyl 2-methylbutanoate, 1-methylethyl butanoate, and ethyl pentanoate and octanoate) where the recomputed heritabilities were more than one standard error lower.

Volatiles with low heritability are expected to show a poor response to selection in a breeding program; however, selection efficiency can be increased by minimizing sources of environmental variation such as differences in fruit maturity at harvest (*7*, *29*) and in volatile production between sites (*36*) or years. Comparison of the volatile profiles by hierarchical cluster analysis (data not shown) showed that major clusters contained profiles overwhelmingly from a single year, while all duplicate profiles collected from the same genotype in the same year were closely associated. These observations suggest a marked effect of year on the volatile profiles, which may be attributable to differences in fruit maturity at harvest. Alternatively, certain related genotypes may be consistently picked immature due to a genetic deviation from normal ripening cues, and if production of particular volatiles was sensitive to maturity, then this would inflate the estimated heritability. In general, however, the concentrations of apple volatiles increase, although not equally, with increasing fruit maturity (*2*, *36*, *37*), and because the majority of the volatiles show high heritability, environmental influences ought to be relatively unimportant. The influence of nonadditive factors such as dominance and epistasis could not be investigated given the experimental design.

Genetic and phenotypic correlations were similar whether computed using the entire data set (**Table 4**) or that restricted to genotypes with at least five relatives (data not shown). Almost all phenotypic correlations (93%) were positive (only two were less than −0.2) reflecting both a genotype effect where genotypes tend to range from being high or low overall producers of volatiles and also the general rise in volatile production, which occurs with increasing fruit maturity (*2*). High positive correlations were found between biosynthetically related esters (ethyl, propyl, and butyl esters and acetate, proprionate, and butanoate esters), as would be expected based on biosynthetic experiments (*31*, *38*), which show the conversion of alcohol and acid precursors into multiple ester products and extensive interconversion of esters derived from common precursors (**Figure 1**). For example, postharvest exposure of apples to ethanol vapor led to the production of ethyl butanoate, 2-methylbutanoate, hexanoate, and octanoate (*39*). Negative phenotypic correlations were found only between the most biosynthetically distant ester volatiles in particular between methyl 2-methylbutanoate and the four acetate esters and between butyl 2-methylbutanoate and ethyl esters such as ethyl hexanoate and ethyl octanoate. These negative correlations may reflect both differences in the activity of specific alcohol acyl-CoA transferases (AAT) (*9*, *15*) and the presence of different concentrations of acyl-CoA precursors in the different genotypes.

Cluster analysis was used to more clearly show the relationships between the phenotypic correlations (**Figure 2a**). The phenotypic correlations were grouped into five clusters broadly consisting of the alcohols and α-farnesene, the ethyl esters, the butyl and propyl esters, the proprionate and 2-methylbutanoate esters, and a clearly defined cluster of four acetate esters. Alcohol precursors of ester volatiles (*38*, *39*) (**Figure 1**) were not clustered with their biosynthetically associated esters, indicating the occurrence of genotypes, which accumulated either esters or alcohols.

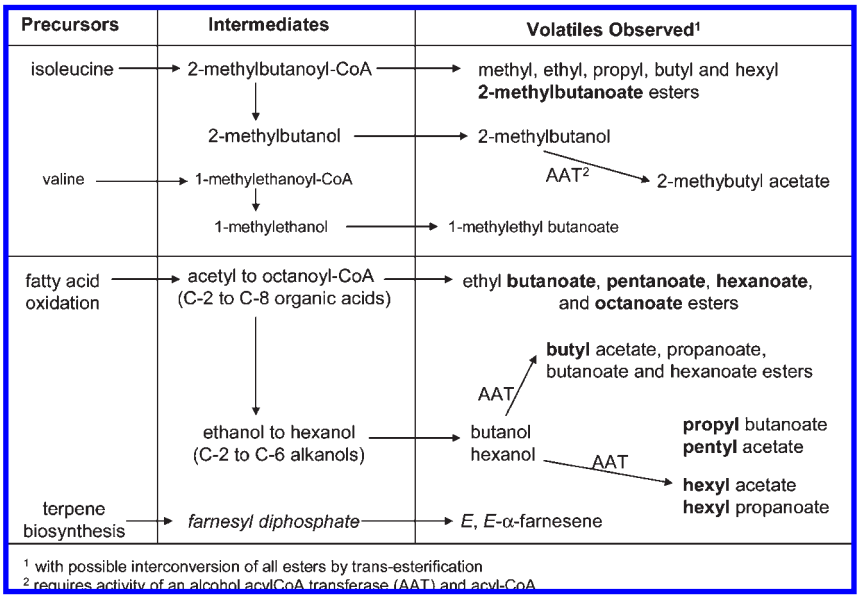

Figure 1. Overview of biochemical pathways for the biosynthesis of apple flavor volatiles.

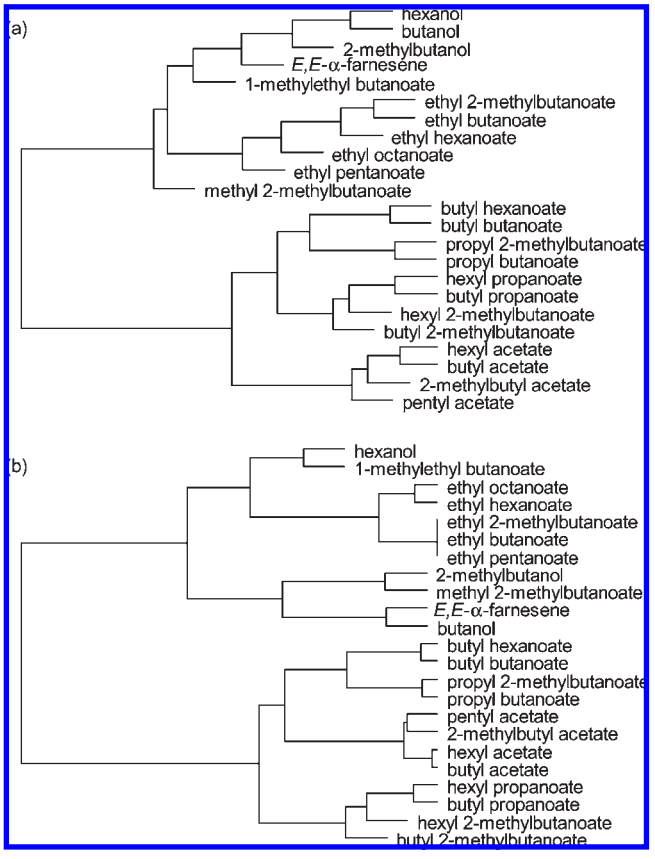

Figure 2. Cluster analysis of the phenotypic (**a**) and genetic (**b**) correlation matrices of the 23 volatiles (log_{10} transformed), arranged so that more closely correlated volatiles are on the same branch.

Ethyl esters were also clearly distinguished from other esters, suggesting different responses of ethanol and ethyl esters to those of other volatiles (*36*, *37*).

In general, the genetic correlations (**Table 4**) were of larger magnitude than the phenotypic correlations as found by Waitt and Levin (*40*) for 85% of the plant studies covering mostly agronomic and morphological traits that they reviewed. Most genetic correlations were positive as expected from the volatile analysis where genotypes tended to range from being low to high overall producers of volatiles and from biosynthetic studies, which show considerable interconversion of metabolites due to both trans-esterification and the partial reversibility of many biosynthetic reactions (*15*, *38*) (**Figure 1**). Esters derived from common alcohol or acid precursors were generally highly correlated, and high positive genetic correlations were found between ethyl, propyl, butyl, and hexyl esters and between acetate, proprionate, and butanoate esters. Pleiotropy, where multiple traits are controlled by the same genes, is the likely explanation of many of these correlations as the last enzymes of ester biosynthesis show broad substrate specificity (*2*, *15*, *41*). Linkage disequilibrium, with nonrandom association of alleles from different loci, is unlikely since there were low levels of relatedness and no inbreeding (*27*).

Cluster analysis of the genotypic correlations (**Figure 2b**) showed a tighter clustering of volatiles according to what is known of their biosynthetic pathways, suggesting that the method used for their estimation was broadly appropriate. All five ethyl esters formed a well-defined cluster distinct from the other esters and inferring the operation of a common gene for ethyl ester synthesis or for high ethanol production. Similarly, the accumulation of acetate esters appeared as a highly associated trait. Evidence for a major QTL for this trait has recently been found in a 'Royal Gala' × 'Granny Smith' apple population (*30*). The remaining ester clusters appeared to associate genes for the synthesis of the chemically similar butyl and propyl esters and for the synthesis of propanoate and 2-methylbutanoate esters. Genetic correlations for the production of the remaining 2-methylbutanaote esters were distributed across the four main cluster branches. Methyl 2-methylbutanoate was positively correlated with ethyl 2-methylbutanoate and 2-methylbutanol (**Table 4**) but negatively correlated (r_G, −0.40) with 2-methylbutyl acetate, consistent with the branching in this pathway between production of 2-methylbutyl or 2-methylbutanoate esters from isoleucine (*31*). Hexyl 2-methylbutanoate was genetically correlated with propyl and butyl 2-methylbutanoates but not with the corresponding methyl or ethyl esters consistent with evidence, based on precursor feeding studies (*15*), for the involvement of multiple enzymes in the biosynthesis of the 2-methylbutanoate esters.

Table 4. Genetic (Upper Triangle) and Phenotypic (Lower Triangle) Correlations Between Pairs of the 23 Volatiles Measured in 240 Apple Genotypes[a]

metabolite	methyl 2-methyl butanoate	ethyl butanoate	1-methyl ethyl butanoate	ethyl 2-methyl butanoate	butyl acetate	2-methylbutyl acetate	propyl butanoate	ethyl pentanoate	propyl 2-methyl butanoate	butyl propanoate	butanol	pentyl acetate	2-methyl butanol	butyl butanoate	butyl 2-methyl butanoate	ethyl hexanoate	hexyl acetate	hexyl propanoate	hexanol	butyl hexanoate	hexyl 2-methyl butanoate	ethyl octanoate	*E,E*-α-farnesene
methyl 2-methyl butanoate		0.13	0.37	0.55	−0.47	−0.40	0.15	0.61	0.06	−0.05	0.46	−0.63	0.79	0.08	0.13	0.20	−0.43	0.07	0.51	−0.12	0.16	−0.03	0.50
ethyl butanoate	0.13		0.48	**1.06**	0.17	−0.06	0.61	**1.19**	0.41	0.02	0.63	0.08	0.22	0.29	−0.41	**0.89**	0.13	−0.18	0.43	0.34	−0.05	0.76	0.54
1-methylethyl butanoate	0.11	0.21		0.52	0.10	−0.08	0.34	0.70	0.30	0.06	0.47	−0.22	0.43	0.20	−0.08	0.31	0.04	−0.14	0.45	0.20	0.01	0.34	0.22
ethyl 2-methyl butanoate	0.16	0.79	0.17		0.34	0.12	0.70	**1.29**	0.60	0.16	0.63	0.19	0.33	0.40	−0.31	**0.96**	0.17	−0.16	0.24	0.50	0.09	**0.88**	0.64
butyl acetate	−0.14	0.22	0.09	0.21		**0.92**	0.58	−0.04	0.60	**0.92**	0.32	**0.95**	−0.12	0.47	0.43	−0.02	**0.98**	0.59	−0.10	0.61	0.27	−0.09	0.18
2-methylbutyl acetate	−0.12	0.15	0.06	0.22	**0.83**		0.37	−0.32	0.53	0.76	0.10	**0.88**	−0.14	0.40	0.53	−0.26	**0.86**	0.57	−0.29	0.51	0.34	−0.08	0.18
propyl butanoate	0.08	0.55	0.36	0.56	0.44	0.49		0.36	**0.94**	0.61	0.73	0.42	0.43	**0.81**	0.30	0.32	0.53	0.46	0.30	0.76	0.51	0.25	0.58
ethyl pentanoate	0.31	0.44	0.20	0.39	0.04	0.02	0.25		−0.08	−0.21	**1.10**	−0.19	0.60	0.06	−0.58	**1.27**	−0.15	−0.48	0.42	0.25	−0.18	**1.12**	**1.12**
propyl 2-methyl butanoate	0.13	0.45	0.28	0.53	0.40	0.55	**0.86**	0.22		**0.85**	0.47	0.57	0.51	0.65	0.53	−0.03	0.54	0.57	0.05	0.63	0.77	0.08	0.35
butyl propanoate	−0.07	0.25	0.17	0.29	0.65	0.70	0.75	0.07	0.69		0.42	0.69	0.13	0.74	0.62	−0.27	**0.80**	**0.90**	−0.03	**0.82**	0.71	−0.01	0.37
butanol	0.08	0.48	0.29	0.47	0.34	0.21	0.61	0.24	0.44	0.50		0.13	0.51	0.63	−0.01	0.61	0.36	0.25	0.75	0.53	0.21	0.44	0.79
pentyl acetate	−0.14	0.17	0.05	0.21	0.77	0.72	0.40	0.02	0.38	0.56	0.26		−0.32	0.39	0.41	−0.16	**0.87**	0.45	−0.26	0.48	0.31	−0.02	0.04
2-methyl butanol	0.21	0.31	0.30	0.37	0.03	0.09	0.50	0.25	0.55	0.32	0.56	0.01		0.11	−0.03	0.26	−0.20	0.02	0.35	0.01	0.10	0.19	0.36
butyl butanoate	0.00	0.34	0.31	0.32	0.55	0.51	0.76	0.18	0.58	0.77	0.62	0.43	0.29		0.46	0.05	0.54	0.64	0.26	**0.93**	0.49	0.09	0.52
butyl 2-methyl butanoate	0.03	−0.07	0.05	0.04	0.41	0.56	0.50	−0.06	0.60	0.66	0.21	0.38	0.27	0.56		−0.71	0.40	0.65	−0.22	0.43	0.67	−0.59	0.06
ethyl hexanoate	0.19	0.75	0.13	0.69	0.06	−0.04	0.29	0.46	0.21	−0.01	0.35	0.05	0.23	0.13	−0.35		0.02	−0.49	0.55	0.20	−0.35	**0.91**	0.26
hexyl acetate	−0.14	0.18	0.08	0.19	**0.88**	0.78	0.41	0.03	0.35	0.58	0.33	0.75	0.00	0.52	0.37	0.06		0.56	0.01	0.62	0.32	−0.03	0.32
hexyl propanoate	0.00	0.12	0.16	0.17	0.47	0.58	0.65	0.03	0.65	**0.86**	0.39	0.44	0.33	0.66	0.67	−0.11	0.46		−0.01	0.61	**0.80**	−0.36	0.22
hexanol	0.15	0.36	0.26	0.31	0.07	−0.06	0.37	0.24	0.21	0.23	0.72	0.01	0.53	0.38	0.04	0.33	0.12	0.23		0.10	0.02	0.23	0.44
butyl hexanoate	−0.01	0.38	0.25	0.41	0.61	0.60	0.71	0.23	0.59	0.75	0.55	0.48	0.23	**0.84**	0.48	0.19	0.54	0.62	0.27		0.48	0.24	0.66
hexyl 2-methyl butanoate	0.06	0.18	0.15	0.28	0.35	0.47	0.59	0.14	0.63	0.71	0.41	0.35	0.36	0.61	0.69	−0.03	0.33	0.75	0.32	0.60		−0.24	0.13
ethyl octanoate	0.18	0.50	0.13	0.49	0.07	0.04	0.19	0.37	0.18	0.01	0.18	0.04	0.13	0.12	−0.21	0.54	0.04	−0.08	0.15	0.21	−0.01		0.11
E,E-α-farnesene	0.08	0.40	0.21	0.41	0.31	0.28	0.55	0.25	0.44	0.45	0.55	0.25	0.37	0.56	0.24	0.30	0.32	0.38	0.40	0.60	0.43	0.25	

[a] The average standard error for the genetic correlations was 0.214, and for the phenotypic correlations, it was 0.054. Correlations greater than 0.80 are presented in bold font and are significantly greater than zero after applying the Bonferroni correction (α = 0.05) to the standard error of the genetic correlations.

The accumulation of butanol, 2-methylbutanol, and hexanol was genetically correlated with the accumulation of ethyl and of 2-methylbutanoate esters but not with the accumulation of most other esters (**Figure 2b**). Levels of butanol were correlated to ethyl, propyl, and butyl esters and with the presence of 2-methylbutanol and hexanol (**Table 4**). The presence of 2-methylbutanol correlated with methyl and propyl 2-methylbutanoates but not with 2-methylbutyl acetate. Similarly, accumulation of hexanol correlated with levels of ethyl hexanoate but not hexyl acetate, hexyl propanoate, or hexyl 2-methylbutanoate, the immediate products of ester biosynthesis. In apple cultivars such as 'Red Delicious' and 'Granny Smith', exogenous alcohols such as hexanol are primarily or exclusively esterified to the corresponding esters with minimal oxidation (*38*). In this genetically diverse population, this did not generally occur. The control of ester biosynthesis by alcohol acyl-CoA transferase (AAT), the last enzyme in the ester biosynthetic pathway, has been proposed (*9*,*42*), and suppression or loss of the activity of this enzyme may lead to the accumulation of alcohol precursors, which could undergo oxidation and esterification to alkanoate esters. The activity and expression of AAT is regulated by ethylene (*9*) providing an additional point of metabolic and genetic control. AAT enzymes generally have broad substrate specificities (*41*,*43*) so the level of one precursor may affect the production of multiple ester volatiles. Accumulation of butanol and 2-methylbutanol was also associated with accumulation of methyl 2-methylbutanoate and of the sesquiterpene α-farnesene. Interestingly, a number of old apple cultivars such as 'Merton Russet' and 'Wilmont Russet' demonstrate this pattern of volatile production.

In contrast to the phenotypic correlations, 21% of the genetic correlations were negative (**Table 4**). However, large negative genetic correlations ($r_G < -0.4$) were found for only 10 pairs of volatiles and were notable for only four of these (methyl 2-methylbutanoate with pentyl acetate and butyl 2-methylbutanoate with ethyl pentanoate, hexanoate, and octanoate). Methyl esters constitute a small proportion of the total headspace of apple and may arise from reaction of the corresponding acid with S-adenosyl-methionine (*44*). While the biosynthetic origin of methyl esters in apple is unclear, this analysis suggests that it is unrelated to that of the other esters as methyl 2-methylbutanoate does not cluster with the other 2-methylbutanoate esters (**Figure 2b**). Butyl 2-methylbutanoate arises in part from isoleucine (*38*) and from butanoyl-CoA from the oxidation of lipids (*38*). The distance between these biosynthetic pathways may contribute to the above negative genetic correlation; however, ethyl esters as a group were also poorly correlated with all other esters.

Large genetic correlations between traits indicate that selection for one trait will have a correlated response of selection for the other trait, enabling enhanced progress to be made in breeding for traits of low heritability or rapid gain in multitrait selection (*12*). Two volatiles that may respond to such indirect selection were methyl 2-methylbutanoate (h^2, 0.15), which showed strong genetic correlations to 2-methylbutanol (h^2, 0.42) and to pentyl acetate (h^2, 0.64) (r_G, 0.79 and −0.63, respectively) (**Table 4**) and the important flavor volatile ethyl 2-methybutanoate (*3*,*7*) (h^2, 0.39), which showed a strong genetic correlation (r_G, 1.09) to ethyl butanoate (h^2, 0.66).

The genetic parameters estimated in this study are based on sampling populations with low effective replication (low levels of relatedness) and should be regarded as preliminary. Indeed, seven of the genetic correlations were above unity, and all had large standard errors. Furthermore, we were unable to determine a year × genotype variance component as the computational process failed to converge, probably as a result of the low levels of replication across years. For this reason, we repeated the analyses using only those genotypes with at least five relatives and were encouraged that similar results were obtained. Likewise, that most of the genetic correlations correspond with known biosynthetic pathways lends credence to these results. To obtain further insight as to how volatile production was linked to genotype, we calculated empirical breeding values for each genotype for each of the 23 volatiles for which heritability values were obtained. The breeding value identifies the value of individual genotypes as genetic parents for each trait and is that part of an individual's genotypic value that is due to additive, and therefore transmittable, gene effects. Cluster analysis (**Figure 3**) was used to examine the relationship between the empirical breeding values of those genotypes with at least five relatives for each of the 23 volatiles. As expected on biosynthetic grounds (**Figure 1**), the breeding value of genotypes for α-farnesene (a sesquiterpene) was least related to that for any of the fatty acid or isoleucine-derived esters or alcohols. Encouragingly, the remaining volatiles preserved some of the pattern shown by their genetic correlations with high breeding values for butyl, 2-methylbutyl, and hexyl acetates being associated with one major cluster of genotypes. Further examination of the genotype clustering (**Figure 3**) also showed that although genotypes from any particular kinship group were typically represented on both main branches, related genotypes were clustered together more in one branch than would be expected by chance ($P < 0.001$, Monte Carlo simulation).

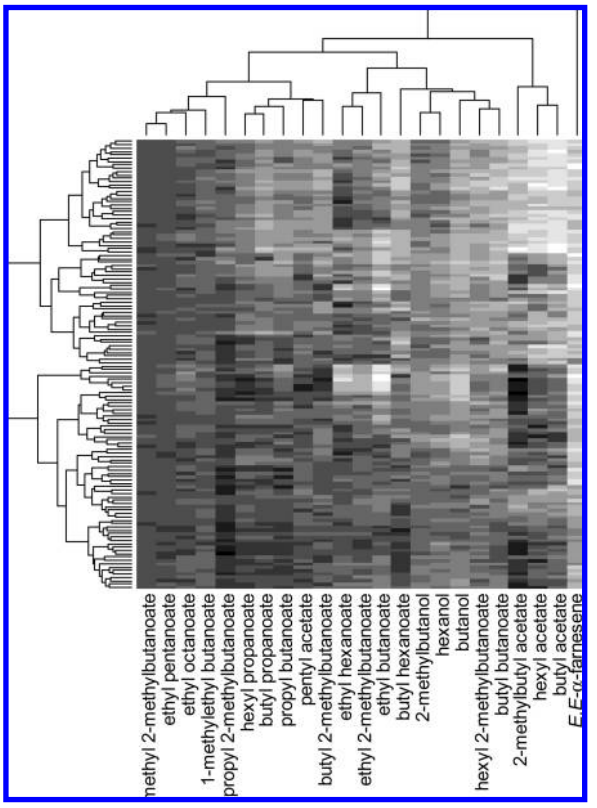

Figure 3. Heatmap of empirical breeding values for those genotypes with at least five relatives clustered according to Euclidean distance for 23 volatiles (log_{10} transformed). The intensity scale ranges from black (nondetected) to white (>3; i.e., >1000 on original scale).

Future directions for the volatile profiling and genetic analysis of apple volatiles may be prefigured by comparable research on tomato (*L. esculentum*) volatiles, which includes surveys of volatile concentrations in fruit of diverse genotypes (*22*, *23*) and of metabolites in related species (*45*). Volatile profiling of

94 tomato genotypes led to the identification of 322 compounds, which were ordered by cluster analysis into biosynthetically related groups (*23*). GC-MS profiling of interspecific introgression lines has also enabled the identification of QTL with potential for tomato improvement (*46*) and which influence the chemical composition and flavor (*24*). QTL for apple fruit volatiles have recently been identified using PTR-MS (*25*). PTR-MS is potentially a high-throughput technique at the cost of some loss of information as individual volatiles are not separated by chromatography and ions of the same mass but, arising from different volatiles, cannot be distinguished. Using 86 progeny from a 'Fiesta' × 'Discovery' population, putative QTL were detected for seven mass spectral ions, most of which were related to specific volatiles or volatile classes (*23*). Thus, the ions at m/z 43 and 61 were inferred to arise largely from acetate esters although m/z 43 can also arise from other alcohols and esters present in the apple headspace. More recently, Dunemann et al. (*26*) have used solid-phase microextraction GC to identify QTL for 27 apple volatiles in a 'Discovery' × 'Prima' apple population. QTL for flavor volatiles were distributed over 12 of the 17 apple chromosomes but mainly on linkage groups 2, 3, and 9. The use of high-throughput volatile profiling for the identification of QTL affecting flavor (*30*) may enable the establishment of marker assisted selection as a technology for improving flavor in apple breeding.

This study has shed new light on the modes of inheritance of apple flavor volatiles in a genetically diverse apple population. Narrow sense heritability estimates were mostly above 0.5 for 23 apple volatiles representative of all major flavor biosynthetic pathways and including compounds considered important in determining the aroma of apple fruit. Analysis of the genetic correlations between volatiles showed that volatiles were generally inherited in ways consistent with their biosynthesis, supporting our experimental and statistical methodology. The ability to extract this information by sampling from such a population of poorly related genotypes demonstrates the potential of GC-MS volatile profiling for the genetic analysis of fruit aroma.

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