# Profiling Fruit Volatiles in the Progeny of a 'Royal Gala' × 'Granny Smith' Apple (*Malus* × *domestica*) Cross

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Volatile flavor compounds from the fruit of the progeny of two apple (*Malus* × *domestica*) cultivars with distinctive flavor and volatile profiles, 'Royal Gala' and 'Granny Smith', were measured by headspace gas chromatography–mass spectrometry over two fruiting seasons. Principal component analysis separated the volatile profiles into two groups according to the amounts produced of butyl, 2-methybutyl, pentyl, and hexyl acetates and of ethyl butanoate, butanol, 2-methylbutanol, and hexanol. Fruit containing the four acetate esters clustered with the 'Royal Gala' parent and were scored more similar to 'Royal Gala' than to "Granny Smith" in flavor. Fruit clustering with the 'Granny Smith' parent contained higher levels of ethyl butanoate and alcohols. Levels of acetate esters correlated to levels of their alcohol precursors, and control of this trait segregated in Mendelian fashion. The locus was mapped to the top of 'Royal Gala' linkage group 2 close to the *Rvi4* (*Vh4*) locus for resistance to *Venturia inaequalis*, the causal agent of apple scab.

## INTRODUCTION

Flavor is an important quality attribute of fruit. However, despite extensive research concerning the volatile flavor compounds of apple (*Malus* × *domestica* Borkh.) fruit (*1*, *2*), the genetic factors controlling the production of these compounds are only now being described (*3*, *4*). As apple is a self-incompatible and highly heterozygous species, cross breeding results in a diverse progeny with few being superior to their parents. Apple breeding therefore usually involves backcrossing to concentrate traits of interest from high-quality parents into new cultivars. Historically, many apple cultivars arose as chance seedlings, for example, 'Granny Smith' (1860s), 'Delicious' (1870s), 'Golden Delicious' (1890s), and 'Braeburn' (1940s), but traditional breeding has provided cultivars such as 'Royal Gala' ('Kidd's Orange Red'×'Golden Delicious'), 'Jonagold' ('Delicious'×'Jonathan'), and 'Fuji' ('Ralls Janet'×'Delicious'), with newer varieties, such as 'Jazz', continuing to appear in the market.

Modern plant-breeding methods, based on screening seedlings from breeding progenies with DNA-based markers associated with traits of interest (marker-assisted selection, MAS), offer more efficient tools for cultivar improvement, especially for fruit trees with long juvenile periods. Accurate genotyping of seedlings would enable selection for important fruit characters, including

flavor, long before a tree reached maturity, offering significant savings of orchard space and time. A prerequisite to the use of MAS is the construction of genetic maps to enable the linkage of phenotypes with discrete genetic (or molecular) markers. The genetic mapping of apple initially focused on pest and disease resistance as apple has proved to be a rich source of simply inherited resistance genes (*5*), although attention is increasingly being paid to traits under more complex genetic control (*6*). A number of genetic maps of apple have been constructed on the basis of a range of genetic markers such as simple sequence repeats (SSRs), amplified fragments length polymorphisms (AFLPs), and single nucleotide polymorphisms (SNPs), enabling a good coverage of the 17 apple chromosomes (*7*−*10*). The availability of large numbers of apple-expressed sequence tags (*11*, *12*) and the forthcoming whole genome sequence for apple (*13*) will provide an extensive resource of genetic markers for fine mapping of breeding traits and MAS.

To date, some 350 volatiles including esters, alcohols, aldehydes, ketones, and sesquiterpenes have been reported from apple fruit and processed apple products; however, only a small number of these compounds are important for apple flavor and aroma (*1*, *2*). Character impact odorants reported from apple include (*E*)-*β*-damascenone, aldehydes such as (*Z*)-3-hexenal, and a number of esters including ethyl butanoate and 2-methylbutanoate, and butyl, 2-methylbutyl, and hexyl acetates; however, the relative contribution of these volatiles to aroma is also dependent

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**Table 1.** Semiquantitative Analysis of the Major Volatile Compounds in the Headspace of Fruit from a 'Royal Gala' × 'Granny Smith' Population Showing Retention Indices, Mass Spectral Ions Used for Quantification, and Interquartile Range, Mean, and Maximum Concentrations as Nanograms of Tetradecane Equivalents Released per Square Centimeter of Fruit Surface Area per Hour

| volatile | code | RI[a] | quantification[b] | interquartile range[c] | mean | maximum | identification[d] | origin[e] |
|---|---|---|---|---|---|---|---|---|
| ethyl butanoate | V04 | 1023 | 71 | 0.00[f]–0.22 | 1.4 | 84 | S, MS, RI | Aldrich |
| propyl propanoate | V06 | 1032 | 57 | 0.00–0.16 | 0.15 | 1.6 | S, MS, RI | synthesis |
| ethyl 2-methylbutanoate | V07 | 1038 | 57 | 0.00–0.15 | 1.3 | 88 | S, MS, RI | Acros |
| butyl acetate | V08 | 1059 | 43 | 0.11–37 | 23 | 155 | S, MS, RI | Aldrich |
| hexanal | V13 | 1067 | 44 | 0.00–0.04 | 0.05 | 1.1 | S, MS, RI | Lancaster |
| 2-methylpropanol | V10 | 1087 | 43 | 0.01–0.10 | 0.10 | 0.96 | S, MS, RI | BDH |
| 2-methylbutyl acetate | V12 | 1109 | 73 | 0.00–5.9 | 8.2 | 70 | S, MS, RI | synthesis |
| propyl butanoate | V14 | 1110 | 71 | 0.06–36 | 24 | 159 | S, MS, RI | synthesis |
| propyl 2-methylbutanoate | V16 | 1125 | 103 | 0.08–1.2 | 0.92 | 5.9 | MS, RI | |
| butyl propanoate | V17 | 1127 | 57 | 0.02–1.7 | 1.2 | 8.1 | S, MS, RI | synthesis |
| butanol | V18 | 1135 | 56 | 0.99–6.2 | 6.4 | 56 | S, MS, RI | BDH |
| 2-methylpropyl butanoate | V20 | 1146 | 71 | 0.01–0.05 | 0.05 | 0.61 | MS | |
| pentyl acetate | V21 | 1159 | 43 | 0.01–0.89 | 0.93 | 7.6 | S, MS, RI | synthesis |
| methyl hexanoate | V22 | 1172 | 74 | 0.00–0.02 | 0.02 | 0.19 | S, MS,RI | Aldrich |
| 2-methylbutyl propanoate | V24 | 1175 | 57 | 0.01–0.14 | 0.13 | 1.7 | MS, RI | |
| 3-methyl-3-butenyl acetate | V25 | 1180 | 43 | 0.01–0.04 | 0.03 | 0.30 | MS | |
| 2-methylbutanol | V26 | 1200 | 57 | 0.78–6.8 | 7.2 | 65 | S, MS, RI | Aldrich |
| butyl butanoate | V27 | 1206 | 71 | 0.12–2.6 | 2.3 | 20 | S, MS, RI | synthesis |
| butyl 2-methylbutanoate | V28 | 1219 | 57 | 0.06–1.4 | 0.92 | 5.9 | S, MS, RI | synthesis |
| ethyl hexanoate | V29 | 1221 | 88 | 0.00–0.003 | 0.07 | 4.8 | S, MS, RI | Aldrich |
| 2-methyl-2-butenyl acetate | V31 | 1232 | 43 | 0.00–0.02 | 0.03 | 0.55 | S, MS, RI | synthesis |
| pentanol | V32 | 1242 | 42 | 0.01–0.09 | 0.07 | 0.46 | S, MS, RI | BDH |
| 2-methylbutyl butanoate | V33 | 1252 | 71 | 0.01–0.07 | 0.09 | 1.0 | S, MS, RI | synthesis |
| hexyl acetate | V34 | 1259 | 43 | 0.19–19 | 13 | 128 | S, MS, RI | synthesis |
| 2-methylbutyl 2-methylbutanoate | V35 | 1267 | 57 | 0.01–0.05 | 0.06 | 0.65 | S, MS, RI | synthesis |
| pentyl butanoate | V38 | 1304 | 71 | 0.00–0.02 | 0.03 | 0.75 | S, MS, RI | synthesis |
| propyl hexanoate | V39 | 1306 | 117 | 0.01–0.12 | 0.12 | 1.8 | S, MS, RI | synthesis |
| pentyl 2-methylbutanoate | V40 | 1316 | 57 | 0.00–0.03 | 0.03 | 0.29 | MS, RI | |
| 2E-hexenyl acetate | V43 | 1320 | 43 | 0.00–0.02 | 0.03 | 0.67 | S, MS, RI | synthesis |
| hexyl propanoate | V46 | 1327 | 57 | 0.01–0.05 | 0.29 | 2.5 | S, MS, RI | synthesis |
| hexyl 2-methylpropanoate | V48 | 1331 | 43 | 0.00–0.02 | 0.04 | 0.72 | MS, RI | |
| 2-methylpropyl hexanoate | V49 | 1248 | 99 | 0.00–0.01 | 0.06 | 1.7 | MS, RI | |
| hexanol | V50 | 1348 | 56 | 0.50–4.4 | 3.9 | 28 | S, MS, RI | Sigma |
| heptyl acetate | V52 | 1362 | 43 | 0.00–0.01 | 0.03 | 0.80 | S, MS, RI | synthesis |
| 2-ethylhexyl acetate[g] | V56 | 1373 | 43 | 0.00–0.02 | 0.02 | 0.34 | MS | |
| tetradecane (IS) | | 1400 | 57 | | | | S, MS, RI | BDH |
| butyl hexanoate | V57 | 1403 | 117 | 0.01–3.2 | 2.6 | 28 | S, MS, RI | synthesis |
| hexyl butanoate | V59 | 1406 | 89 | 0.23–1.3 | 1.6 | 15 | S, MS, RI | synthesis |
| hexyl 2-methylbutanoate | V62 | 1418 | 103 | 0.19–5.5 | 4.2 | 27 | S, MS, RI | synthesis |
| 2-methylbutyl hexanoate | V67 | 1448 | 99 | 0.00–0.04 | 0.04 | 0.48 | MS, RI | synthesis |
| pentyl hexanoate | V70 | 1501 | 99 | 0.00–0.02 | 0.05 | 2.5 | S, MS, RI | synthesis |
| propyl octanoate | V72 | 1509 | 145 | 0.00–0.01 | 0.01 | 0.05 | S, MS, RI | synthesis |
| hexyl hexanoate | V78 | 1598 | 117 | 0.01–0.55 | 0.92 | 12 | S, MS, RI | synthesis |
| butyl octanoate | V79 | 1601 | 56 | 0.00–0.02 | 0.05 | 1.2 | S, MS, RI | synthesis |
| estragole | | 1649 | 148 | 0.00–0.02 | 0.02 | 0.53 | MS, RI | |
| 2-methylbutyl octanoate | V87 | 1677 | 70 | 0.00–0.02 | 0.01 | 0.07 | MS, RI | |
| (Z,E)-α-farnesene | V93 | 1715 | 93 | 0.00–0.02 | 0.01 | 0.08 | S, MS, RI | apple[h] |
| (E,E)-α-farnesene | V94 | 1738 | 93 | 0.02–1.7 | 4.3 | 79 | S, MS, RI | apple[h] |
| geranyl acetate | V97 | 1749 | 69 | 0.01–0.18 | 0.12 | 0.74 | S, MS, RI | Aldrich |

[a] Retention index. [b] Mass fragments used for quantitation. [c] First to third interquartile range. [d] Identification obtained by using standard compounds(s) and/or mass spectra (MS) and/or retention indices (RI). [e] Source of the standard compound. [f] Not detected. [g] Possible contaminant. [h] Purified from apple skin (44).

on the apple cultivar, its maturity, and the methods used for volatile extraction (14−17). Volatile esters are associated with "fruity" flavor attributes, and as apple is a climacteric fruit, their levels are modulated by ethylene (18) and increase during both ripening and storage. The formation of esters is largely confined to the fruit tissue and more specifically the skin (19−21). Apple esters can be broadly separated into straight-chain esters, synthesized from fatty acids (22), and branched-chain esters derived from isoleucine (23). The final step in ester biosynthesis, the combination of the acid donor, acyl-coenzyme A, with an alcohol acceptor is assumed to be the key step in this pathway and is catalyzed by alcohol acyl-CoA transferases (AATs). Plants

contain multiple AAT enzymes, which belong to the BAHD superfamily (24). Genes encoding AATs have been isolated from melon (25), strawberry, and banana (26), as well as rose flowers (27). A recombinant AAT from apple (28) was able to utilize a broad range of substrates, suggesting that substrate availability rather than AAT specificity may explain aroma differences between fruits.

'Royal Gala' and 'Granny Smith' are two apple cultivars with distinctive flavors and volatile profiles as well as very characteristic skin colors. 'Royal Gala' is an earlier maturing, red-striped cultivar; its volatile profile exhibits an abundance of butyl, 2-methylbutyl, pentyl, and hexyl acetate esters (15, 29, 30).

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'Granny Smith' is a late maturing, green-skinned cultivar; its flavor volatiles are characterized by the presence of ethyl and propyl esters and free alcohols such as 2-methylbutanol (*31*). Gas chromatography–olfactory analysis of 'Royal Gala' identified 2-methylbutyl acetate, butyl acetate, hexyl acetate, and butanol as important contributors to the aroma of the fruit, whereas analytical sensory panels indicated that 2-methylbutyl acetate, hexyl acetate, and butanol had the greatest effect on flavor attributes considered to be important to 'Royal Gala' flavor (*15*). The differences between the volatile profiles of these two cultivars seem to have a genetic basis as 'Granny Smith' apples are able to produce only trace quantities of 2-methylbutyl acetate from exogenous 2-methylbutanol (*23*), whereas hexanol is readily converted to hexyl acetate by both cultivars (*32*). The existence of multiple AAT enzymes that may define the profile of ester volatiles produced by apple has been proposed by a number of authors (*32, 33*).

The eventual fruiting of a 'Royal Gala' × 'Granny Smith' population enabled us to investigate the inheritance of flavor volatiles from these parents. We hypothesized that the contrasting volatile profiles of the parents would aid in the identification of genetic markers linked to flavor in their progeny. We report here the use of GC-MS volatile profiling to map a major QTL affecting the production of acetate esters that are significant flavor compounds (*15*) in this population. This QTL is in close proximity to the *Rvi4* (*Vh4*) locus for resistance to apple scab (*Venturia inaequalis*), a major fungal pest of apple (*34*), suggesting the possibility of simultaneous selection for both pest resistance and flavor during marker-assisted apple breeding.

## MATERIALS AND METHODS

**Plant Material.** Six hundred seedling trees, obtained from a controlled cross between the apple cultivars 'Royal Gala' and 'Granny Smith' (pollen parent), were planted on their own roots in the Plant and Food Research Orchard (Havelock North, New Zealand; 39° 39′ S 176° 53′ E) in 2002 in northwest facing rows at an average spacing of 0.6 m. Fruit maturity was tested in the field on the basis of background color and relative starch index (*35*). Generally, fruit were harvested at a starch index of 5, such fruit being considered as eating ripe. During 2006, duplicate samples of fruit collected from each of 39 individual trees were analyzed for headspace volatiles immediately upon receipt of fruit in the laboratory. In 2007, the volatile profiles of fruit from 155 trees, including 30 already sampled in 2006, were measured without replication after a maturation period of 15 days at 1 °C followed by 5 days at 20 °C. Soluble solids (Brix) were measured on the blush side of each fruit using a hand-held refractometer (Atago P1, Tokyo). In 2006, fruit surface area was calculated from the average diameter measured using a hand-held micrometer, assuming fruit were spherical. In 2007, surface area was calculated from the weight increase resulting from submerging individual fruit in water. Fruit samples were photographed and assigned to one of nine color classes (1 (white) 2 (yellow), and 3 (green) 'Granny Smith' (GS) types; 4 (light), 5 (normal), and 6 (heavily striped) 'Royal Gala' (RG) types; and 7 (blush), 8 (block), and 9 (100% red-skinned fruit)). After headspace analysis, a fruit slice was tasted by an informal panel familiar with apple flavors and scored as to similarity to either 'Royal Gala' or 'Granny Smith' flavor on a nine-point continuous ascending scale. For comparison, fruit of the parent cultivars 'Granny Smith' and 'Royal Gala' were obtained from a local supermarket in 2006 and a nearby orchard in 2007.

**Volatile Analysis.** Typically, three whole fruits were placed in 1.5 L glass jars (160×105 mm i.d.), and charcoal-filtered air was drawn into the bottom of each jar for 3 h at 40 mL min$^{-1}$. Volatile compounds were absorbed onto Tenax-TA (ca. 350 mg) contained in glass traps attached to the top of each jar (*23*). After volatile collection, the adsorbent traps were eluted with diethyl ether (2×1 mL) containing tetradecane at 10 nL/mL. Samples were stored at −20 °C prior to analysis.

GC-MS separations were carried out using an Agilent 6890N gas chromatograph coupled to a GCT time of flight mass spectrometer (Waters, Manchester, U.K.) using a 20 m × 0.18 mm i.d. × 0.18 μm film
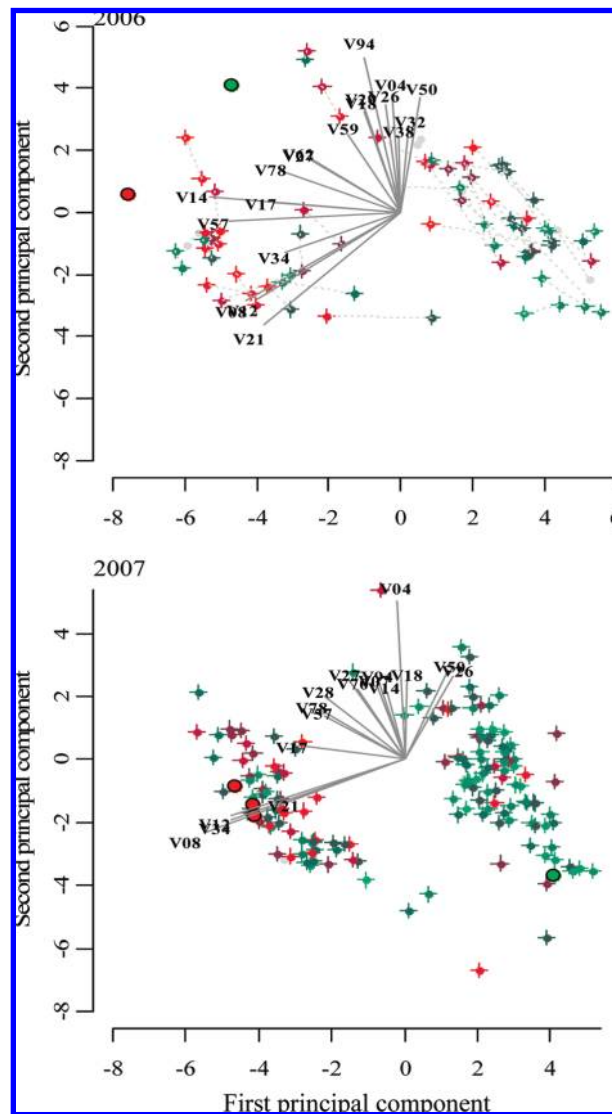


**Figure 1.** Biplots of the first and second principal components from the principal component analysis of the 2006 and 2007 apple headspace volatile profiles of a 'Royal Gala'×'Granny Smith' progeny. Points show the PCA scores of individual samples and are color coded according to the degree of 'Royal Gala' to 'Granny Smith' flavor (red to green, respectively) determined by sensory analysis. Duplicate samples, measured in 2006, are linked by dotted lines. Parental values are shown as larger circles with black surrounds: red, 'Royal Gala'; green, 'Granny Smith'. Lines indicate the loadings with an absolute value >0.18 on at least one of the axes. Volatiles: V04, ethyl butanoate; V08, butyl acetate; V12, 2-methylbutyl acetate; V14, propyl butanoate; V17, butyl propanoate; V18, butanol; V21, pentyl acetate; V26, 2-methylbutanol; V32, pentanol; V34, hexyl acetate; V38, pentyl butanoate; V50, hexanol; V57, butyl hexanoate; V59, hexyl butanoate; V78, hexyl hexanoate; V94, (*E,E*)-α-farnesene. Gray points did not have a flavor score.

thickness Agilent DB-Wax capillary column after a 20 s splitless injection. The helium flow rate was 1 mL min$^{-1}$; the injection temperature, 220 °C; and the oven temperature, 35 °C (1 min), raised at 2.9 °C min$^{-1}$ to 100 °C and at 8 °C min$^{-1}$ to 200 °C (5 min). Electron ionization was used at 70 eV. A standard containing ethyl 2-methylbutanoate, 2-methylbutanol, tetradecane, hexyl butanoate, and hexanol was injected after every 10 samples. Volatiles, identified by comparison with NIST02 and Wiley 7 mass spectral databases and against authentic standards (**Table 1**), are reported as nanograms of tetradecane equivalents released per square centimeter of fruit surface area per hour. Authentic ester standards were obtained from commercial suppliers or by standard chemical methods from the

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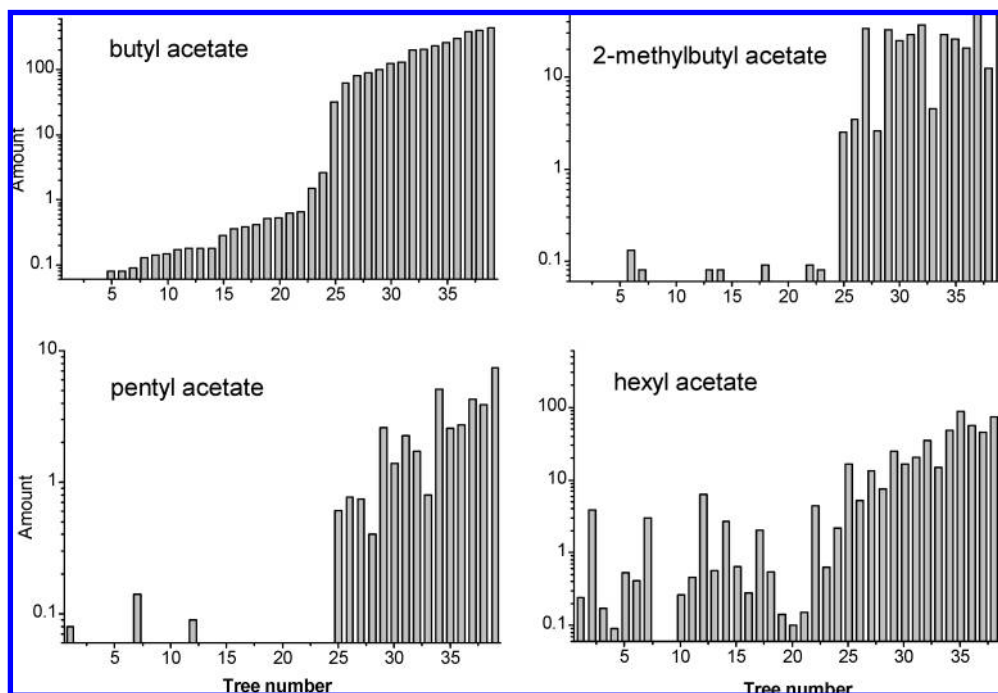


**Figure 2.** Amounts (ng equiv cm$^{-2}$ fruit skin h$^{-1}$) of butyl acetate, 2-methylbutyl acetate, pentyl acetate, and hexyl acetate produced by apples harvested from trees of 41 'Royal Gala' × 'Granny Smith' progeny in 2006.

corresponding alcohols and acids (*23*) as indicated in **Table 1**. Only 2-methylpropyl hexanoate does not appear to have been previously reported in apple fruit. Peak areas were obtained from the GC-MS data files using Waters QuanLynx software setting the minimum peak area to zero such that some minimum (noise) peak area was generally measured for all volatiles. Peak areas were reported for 47 volatiles in 2006 and for 89 in 2007 with detection limits estimated as 0.01−0.17 ng of tetradecane (*m*/*z* 57) equiv released cm$^{-2}$ of fruit surface area h$^{-1}$. Major volatiles and quantitation ions are listed in **Table 1**. The variability (RSD) between individual Tenax traps was measured, using octyl acetate, as 19.7% (*n* = 24) (*36*). The mean percent difference in volatile concentrations between duplicate samples collected from the same tree was 40.9 ± 6.9% (SD) for 48 major volatiles where peaks for both duplicates were detected by the GC-MS software.

**Statistical Analysis.** Volatile data were log$_{10}$ transformed after the addition of half the minimum value for each volatile. Hierarchial cluster analysis demonstrated that the best pairing of duplicate samples was obtained using unscaled data. Principal component analysis (PCA) was therefore undertaken using this method after exclusion of those volatiles present in only a few samples. All statistical analyses were undertaken in R 2.9.0 (*37*).

**Molecular Marker Screening and Genetic Mapping.** Leaf tissue samples from the population were collected in the spring and stored at −80 °C prior to DNA extraction (*38*). Extracted genomic DNA was quantified, diluted to 1 ng/μL, and stored at −20 °C prior to use. A three-step strategy was used to locate the locus controlling the production of 2-methylbutyl acetate (2MBA). First, a cosegregation test was carried out using DNA from both parents and from seedlings showing extreme phenotypes (three seedlings with high fruit 2-methylbutyl acetate and three without), in order to identify coincidence between segregating marker alleles and the phenotypes. This was done by screening a large set of 137 evenly spaced SSR markers covering the entire apple genome (*7*, *8*, *10*). The second step consisted of validating the initial cosegregation using a larger number of fruiting seedlings. Once a cose-gregation had been established, further published markers from these regions of the genome were screened over the complete set of trees to construct a genetic map around the phenotypic locus. The PCR conditions for SSRs were as described (*7*). PCR products were separated indepen-dently (no multiplexing) by nonfluorescent capillary electrophoresis using a Cepro9600 (Advance Analytical, Ames, IA) and analyzed using DNA Size software (Advance Analytical). Genetic maps were constructed using

Joinmap v3.0 (Kyazma, The Netherlands) (*39*) using the Kosambi function with a minimum LOD score of 3.0 for grouping.

## RESULTS AND DISCUSSION

The apple cultivars 'Royal Gala' and 'Granny Smith' have distinctive flavors and volatile profiles. A controlled cross made between these cultivars resulted in a population of 600 trees, which began fruiting in 2006; 47 headspace volatiles from 39 fruit samples were measured in duplicate by GC-MS (**Table 1**). In 2007, fruits from 155 trees were measured, including 30 trees already sampled in 2006, and concentrations of 89 volatiles were measured.

**Principal Component Analysis.** PCA was used to analyze the differences between the volatile profiles of the various genotypes (**Figure 1**). Data for each year were analyzed separately, as in 2006 fruit volatiles were measured immediately after picking, whereas in 2007 volatiles were analyzed after a short period of cold storage, which was used to advance fruit maturity. In both years the samples fell into two distinct clusters defined by the first two principal components (PC) (**Figure 1**). The first principal compo-nent (PC1) accounted for 36.0 and 33.6% and the second principal component (PC2) for 9.2 and 12.3% of the variance of the 2006 and 2007 data, respectively. All other PCs accounted for 6.7% or less of the variance. In 2006, the year in which duplicate fruit samples were taken from each tree, all of the duplicates fell within the same cluster. The parental samples were each associated with a separate cluster, particularly in 2007 when parental fruits were collected from a nearby orchard rather than purchased from a supermarket.

In both years, the loadings for the first two PCs for butyl, 2-methylbutyl, pentyl, and hexyl acetates (V08, V12, V21, and V34, respectively) were very similar to one other and of approxi-mately the same direction and magnitude (**Figure 1**). These acetates occurred at higher levels in 'Royal Gala', and their vectors point toward the Royal Gala cluster associated with this parent in the biplot (**Figure 1**). Other volatiles characteristic of 'Royal Gala' apples, namely, butyl propanoate, butyl hexanoate,

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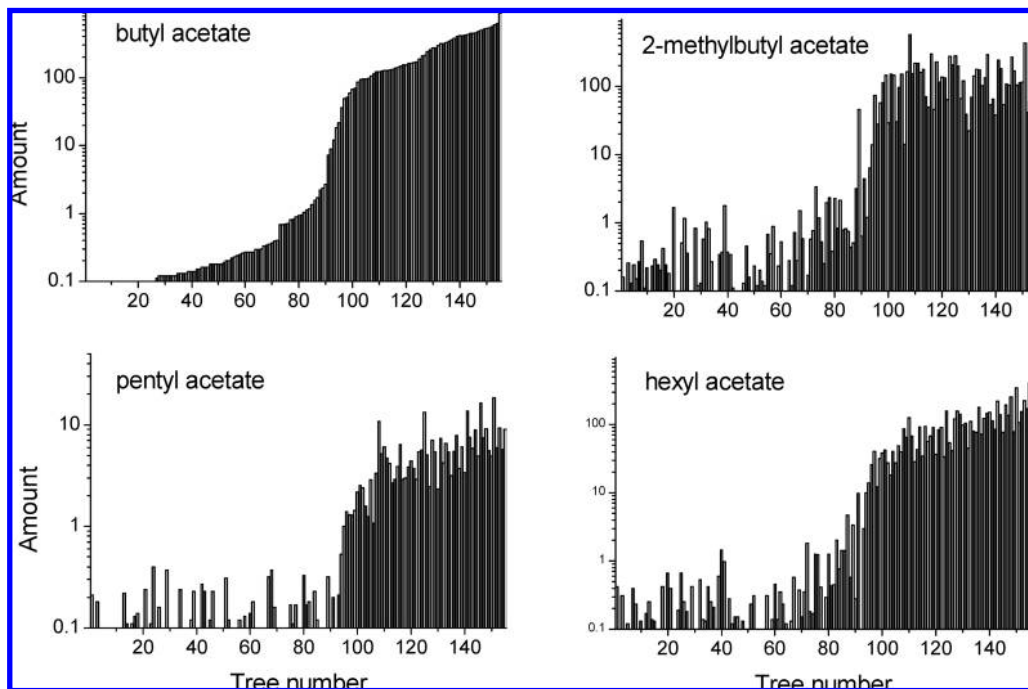


**Figure 3.** Amounts (ng equiv cm$^{-2}$ fruit skin h$^{-1}$) of butyl acetate, 2-methylbutyl acetate, pentyl acetate, and hexyl acetate produced by apples harvested from trees of 155 'Royal Gala' × 'Granny Smith' progeny in 2007.

butyl butanoate, butyl 2-methylbutanoate, and hexyl hexanoate (V17, V57, V27, V28, and V78, respectively), were not so closely associated with respect to the first two PC loadings, but still tended toward the Royal Gala cluster. PC1 thus divided the volatile profiles into two groups, essentially on the basis of the concentration of total, or major, volatiles such as butyl and 2-methylbutyl acetate. This interpretation was supported by a strong correlation between the PC1 loading and the mean concentration of each volatile (2006; $r = -0.63$, arbitrary sign). The exceptions were the alcohols (butanol, 2-methylbutanol, and hexanol) and ethyl hexanoate. Of the volatiles particularly associated with the Granny Smith cluster, ethyl butanoate, butanol, 2-methylbutanol, and hexanol (V04, V14, V26, and V50, respectively), all had negligible (low) loadings on PC1, but relatively high (positive) loadings on PC2 (**Figure 1**). PC2 was a contrast between volatiles, in particular, between the four acetate esters (2-methylbutyl, butyl, pentyl, and hexyl acetates) characteristic of 'Royal Gala' and volatiles including ethyl butanoate, butanol, 2-methylbutanol, and hexanol, which are characteristic of the 'Granny Smith' parent.

Analysis of the distribution of flavor scores between the two PCA clusters (**Figure 1**) showed that in both years, the average flavor score for the Royal Gala cluster was lower on the Granny Smith-type flavor scale than that for the Granny Smith cluster (unpaired *t* test: 2006, Royal Gala cluster score = 4.0, Granny Smith cluster = 5.3, $P = 0.018$; 2007, Royal Gala cluster score = 5.1, Granny Smith cluster = 6.7, $P < 0.001$). Fruit in the Royal Gala cluster contained higher concentrations of the acetate esters, consistent with the published association of butyl, 2-methylbutyl, and hexyl acetate, and butanol, with varietal 'Royal Gala' flavor (*15*), but also contained higher concentrations of other major volatiles. Flavor scores were not related to soluble solids (Brix) and only weakly to scores for skin color (data not shown), suggesting these factors were only weakly associated with the panelists' perception of 'Royal Gala' or 'Granny Smith' flavor.

**Consistency of Phenotype and Effect of Fruit Maturity.** In 2006, 25 of the 39 of the genotypes examined showed an absence, or

greatly reduced levels, of butyl, 2-methybutyl, and pentyl acetates (**Figure 2**). Some fruit also showed an absence of α-farnesene, a sesquiterpene normally found in stored apples. To check if the absence of these volatiles resulted from insufficient maturity of the fruit at harvest, the headspace volatiles of 23 of these fruit samples were remeasured after an additional storage period ranging from 6 to 14 weeks at 1 °C (followed by 5 days at 20 °C). After cold storage, α-farnesene was present in all fruit samples (average increase = 7.6 fold) with increased concentrations observed in 22 of 23 of the fruit samples. All fruits, however, were still negative for, or produced only traces of, the four acetate esters. This result suggested that whereas the presence or absence of acetate esters was insensitive to fruit maturity, our assessment of fruit maturity using starch index (*35*) and fruit color was inadequate when applied to this range of genotypes. For this reason a postharvest treatment (15 days of cold storage treatment followed by 5 days at 20 °C) was applied to all fruit harvested in 2007. After use of this additional period of cold storage, α-farnesene was no longer a major contributor to the discrimination between fruit genotypes by PCA (**Figure 1**).

The four acetate esters (butyl, 2-methylbutyl, pentyl, and hexyl acetate) had high loadings in both PC1 and PC2 and were present in significant amounts only in fruit from seedlings that clustered with the 'Royal Gala' parent (**Figure 1**). Significant amounts of butyl, 2-methylbutyl, and pentyl (but not hexyl) acetates occurred together in 15 of the 39 fruits in 2006 (**Figure 2**), suggesting the operation of a major gene controlling ester biosynthesis. In 2007, fruit producing these esters again clustered with the 'Royal Gala' parent, whereas the ratio of high to low acetate ester producers was ca. 65:90, depending slightly on the individual ester and concentration threshold used (**Figure 3**), but again suggestive of control by a single major gene. Comparison of the volatile profiles of genotypes measured in both 2006 and 2007 revealed that, although the general relationship between the amounts of volatiles produced between the two years was poor, there were good linear correlations between the concentrations of butyl, 2-methylbutyl, pentyl, and hexyl acetates measured in 2006 and 2007
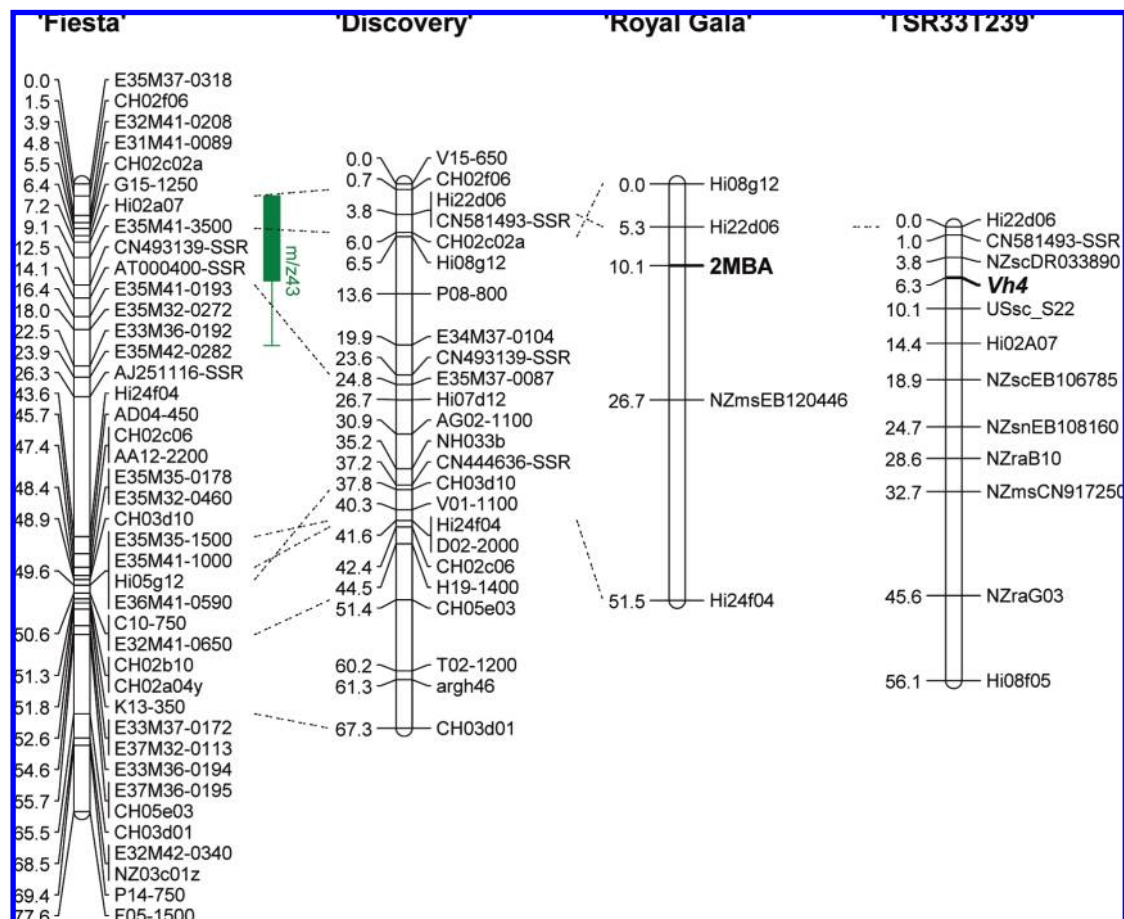
**Figure 4.** Genetic mapping on apple 'Royal Gala' linkage group 2 of a locus (2MBA) controlling concentrations of 2-methylbutyl acetate in fruit. The position of 2MBA was compared to a quantitative trait locus (QTL) corresponding to *m/z* 43 detected in the population 'Fiesta' × 'Discovery' (*3*). The reference maps for 'Fiesta' and 'Discovery' (*3*) are shown. The genetic map around the 2MBA locus is also compared to the genetic map developed for TSR33T239 around the Rvi4 (Vh4) locus for scab resistance (*5, 34*).

($r^2 = 0.73$, 0.80, 0.50, and 0.65, respectively), suggesting that production of these esters was highly genotype dependent. The relative amounts of butyl, 2-methylbutyl, and pentyl acetates and, to a lesser extent, hexyl acetate, also showed a similar pattern of presence or absence from fruit of the same genotype whether collected in either 2006 or 2007 (see the Supporting Information). As the differences between acetate ester producing and nonproducing fruits were most distinct for the production of 2-methylbutyl acetate, this trait was subsequently referred to as "2MBA".

**Genetic Mapping.** Whereas the concentrations of most volatiles showed a continuous distribution of values, suggesting a polygenic control (data not shown), for butyl, 2-methylbutyl, pentyl, and hexyl acetates a disjunct distribution of volatile concentrations was observed (**Figures 2** and **3**). The segregation ratio for 2-methylbutyl acetate was 65:90 (presence/absence), which differed slightly from a 1:1 ratio ($\chi^2 = 4.03$, $P = 0.04$), but suggestive of control by a single major gene or a strong effect of a quantitative trait locus (QTL). We therefore focused on the genetic mapping of 2-methylbutyl acetate (2MBA) as a Mendelian trait. As coding 2MBA as "present" or "absent" seemed to be appropriate, seedlings bearing fruit producing $< 5$ ng equiv cm$^{-2}$ of fruit skin h$^{-1}$ were considered to be "negative" phenotypes. The mapping strategy used was the double pseudo testcross (*40*), which consists of building genetic maps for each parent of a cross using informative markers for these parents (i.e., heterozygous and segregating as a backcross type). Because levels of 2MBA were high in 'Royal Gala' and almost absent in 'Granny Smith', we hypothesized that the locus controlling the presence of 2MBA was heterozygous in 'Royal

Gala', with a dominant 2MBA allele, and homozygous in 'Granny Smith'. As a consequence, only genetic markers that were heterozygous in 'Royal Gala' could be used for the cosegregation test.

Some 137 SSR markers, covering the whole genome, were tested on a subset of the population consisting of both parents and three individuals that produced 2-methylbutyl acetate and three that did not. As apple has 17 genetic linkage groups (LG), screening using three individuals per phenotype was an efficient strategy using the minimum number of individuals to quickly find a genomic region linked to the production of 2-methylbutyl acetate. In this first stage of the mapping strategy, some cosegregation was found with markers located on several linkage groups: Hi22d06 and Hi08g12 on LG2, NH55b and NH201a on LG8, CH05h05 on LG13, and Hi22f06 on LG16. When these markers were screened over a larger population of 159 fruiting individuals (41 individuals measured in 2006 and 118 in 2007), only the cosegregation with Hi22d06 and Hi08g12 was confirmed, which indicated that the locus controlling 2MBA was located on LG2. In the third stage, 10 published SSR markers from LG2 (CH02c02a, CN581493, Hi22d06, Hi08g12, Hi05g12, Hi24f04, 186610b, CN493139, 90483, and NB124b) were tested over the 159 genotypes available. One individual that gave no genetic marker data and seven genotype−phenotype incongruent (GPI) (*41*) individuals, 4.4% of the population, were discarded. These GPI plants may have arisen from the presence of rogue plants in the population resulting from stray pollen, through experimental errors in sample collection or measurement, or through the action of genetic modifiers changing the phenotype. Two markers (CH02c02a and CN581493) were not considered for
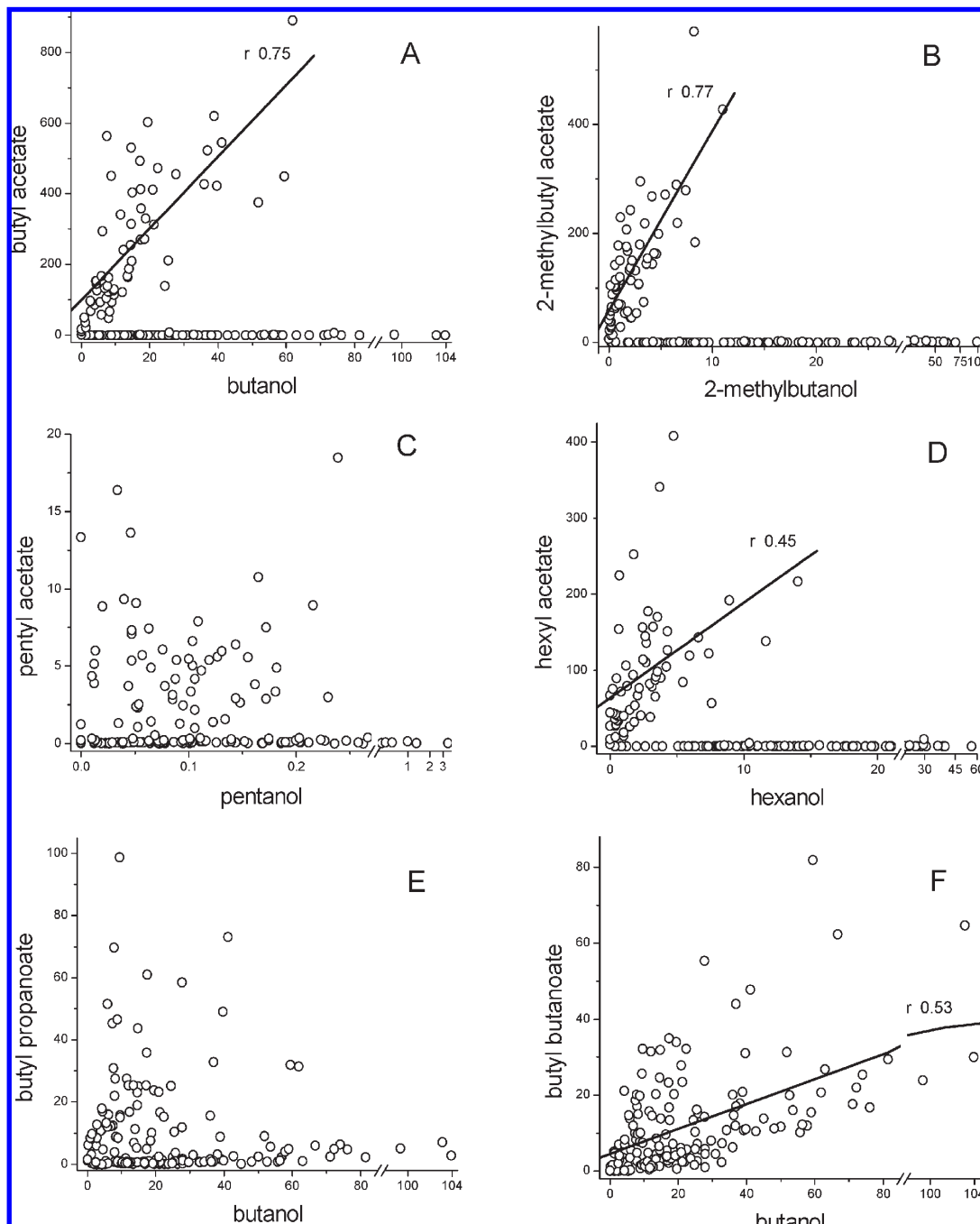
**Figure 5.** Amounts (ng equiv cm$^{-2}$ fruit skin h$^{-1}$) of butyl acetate (**A**), 2-methylbutyl acetate (**B**), pentyl acetate (**C**), hexyl acetate (**D**), butyl propanoate (**E**), and butyl butanoate (**F**) and of their corresponding alcohol precursors produced by apples harvested from 155 trees of 'Royal Gala' × 'Granny Smith' progeny in 2007.

mapping as they were not scorable in this population. Four markers (NB124b, NZmsEB149808b, CN493139, and Hi05g12) remained unlinked at a LOD score of 3.0. A group of five markers, including 2MBA, Hi22d06, Hi08g12, NZmsEB120446, and Hi24f04, were grouped with a minimum LOD score of 8.0. A genetic map located 2MBA 5 centimorgans (cM) below the marker Hi22d06 and 17 cM above the marker NZmsEB120446 (**Figure 4**). This map covered 52 cM, and the genetic distances between SSRs and their order were consistent with published apple maps, such as 'Fiesta'×'Discovery' (*7*, *8*). When butyl, pentyl, and hexyl acetates were similarly coded as dominant loci, they mapped adjacent to 2MBA at the top of LG2 (data not shown) as expected on phenotypic and biosynthetic grounds.

Our mapping of 2MBA as a major gene locus is consistent with previous QTL mapping studies on apple flavor. Zini et al. (*3*) mapped several QTL in a 'Fiesta'×'Discovery' population, using proton transfer reaction−mass spectrometry. One QTL, corresponding to *m/z* 43 in the mass spectrometer and explaining approximately 20% of the variation, mapped to the top of LG2 and, as *m/z* 43 is a major ion in the mass spectrum of 2MBA, may be the same gene. More recently, Dunemann et al. (*4*) used solid-phase microextraction to identify QTL for 27 apple volatiles in a 'Discovery' × 'Prima' apple population. QTL for volatiles were distributed over 12 of the 17 apple chromosomes but were mainly clustered on LG2, LG3, and LG9 with evidence presented for colocalization of QTL on the basis of their biochemical

relationships. A number of QTL from this study colocate with the 2MBA locus at the top of LG2, including 'Prima'-derived QTL for the related esters, butyl and pentyl acetate. Interestingly, a QTL for 2MBA, explaining 13% of the phenotypic variation, also mapped at the top of LG9 ('Discovery'), indicating that independent factors may control the level of 2MBA. Nevertheless, the colocation of the QTL for 2MBA derived from 'Royal Gala' with QTL for 2MBA derived from 'Fiesta' (*3*), and for related compounds in 'Prima' (*4*), suggests that this locus is stable across varieties and environment.

Our map of LG2 was constructed with a LOD score of 8, which is relatively high, but not unexpected, given that we removed GPI individuals. As the flanking SSR markers Hi22d06 and NZmsEB120446 are both reliable, easy to score, and lie within a 22 cM interval, they should have a high success rate when used in MAS, selecting for individuals producing 2MBA. Several disease-resistance genes have also been identified on apple LG2 (*5*). In particular, the *Rvi4* (*Vh4*) scab resistance gene has been mapped at the top of LG2 of the resistant parent TSR33T239 using a 'Royal Gala' × TSR33T239 segregating population (*5*, *34*). The *Rvi4* scab resistance locus has since been mapped in relation to additional markers (Gardiner, unpublished results), indicating that the Hi22d06 marker mapping above the 2MBA locus at 4.8 cM is only a little more distant from the *Rvi4* locus (6.3 cM) and suggesting that the loci are quite close. One could envisage a series of markers (**Figure 4**) being used for selection of a recombinant seedling carrying both loci and this then being used as a parent in breeding for a new scab-resistant cultivar with high levels of acetate esters in the fruit.

**Nature of the 2MBA Gene.** The products of the 2MBA gene are all short-chain acetate esters requiring alcohol acylCoA transferase (AAT) activity for the final step of their biosynthesis, even though the alcohol moieties involved are produced by different biosynthetic pathways. 2-Methylbutyl acetate is a branched-chain ester derived from isoleucine (*23*), whereas the butyl, pentyl, hexyl, and acetate moieties may be produced by oxidation of straight-chain fatty acids (*22*). AAT activity has been demonstrated to be a key step in ester biosynthesis (*20*, *42*), and AAT enzymes from fruit typically show low specificity for both their alcohol and CoA substrates (*28*, *43*). To determine whether the 2MBA gene is involved in the last step of ester biosynthesis or elsewhere in the pathway, we examined the relationship between the concentrations of acetate esters and their alcohol precursors in the 154 apple genotypes measured in 2007 (**Figure 5**). For those genotypes that accumulated butyl, 2-methylbutyl, and hexyl acetates the concentrations of these acetates were correlated ($P < 0.001$) with the concentration of their corresponding alcohol precursors (**Figures 5A,B,D**), although those fruit that accumulated esters tended to have lower levels of alcohols than fruit which did not. This trend suggested that for these genotypes, alcohol rather than enzyme availability was limiting for esterification, which is consistent with previous publications (*18*, *33*). For fruit accumulating pentyl acetate, no correlation ($r = 0.01$, $P = 0.94$) was apparent between the levels of this ester and its alcohol precursor, although the absolute concentrations in this case were much smaller. That the effect of the 2MBA gene is specific to the acetate esters is exemplified by the absence of correlation between levels of butyl propanoate and butanol ($r = -0.06$, $P = 0.29$, **Figure 5E**) and the overall correlation between levels of butyl butanoate and butanol ($r = 0.33$, $P < 0.001$, **Figure 5F**) in all fruit. Overall, these results suggest that the 2MBA gene product is likely to be the AAT enzyme involved in the last step of ester biosynthesis.

Preliminary candidate gene mapping suggests that the *MpAAT1* gene of apple (*28*) is located on LG2 (Chagné et al.,

unpublished results). Recombinant MpAAT1, partially purified from *Escherichia coli* or expressed in *Nicotiana benthamiana* (*28*), showed a broad substrate specificity with a preference for the production of hexyl esters from mid-chain-length CoAs at both high and low alcohol substrate concentrations. At low alcohol substrate concentrations, MpAAT1 esterifies 2-methylbutanol at a greater rate than butanol and hexanol and at low alcohol and acetyl-CoA concentrations, as might occur in ripening fruit, it was suggested there might be enhanced relative production of 2-methylbutyl acetate. Work to further define the identification and mapping of the 2MBA gene in relation to *MpAAT1* is ongoing.

In summary, multivariate statistical analysis grouped the fruit volatile profiles of the progeny of a 'Royal Gala'×'Granny Smith' population into two clusters differentiated by their production of butyl, 2-methylbutyl, pentyl, and hexyl acetates. Production of these four acetate esters showed a Mendelian segregation, and this trait was mapped to the top of LG2 between two flanking SSR markers and close to the *Rvi4* scab-resistance gene. Being able to use genetic markers to select for both resistance and a quality trait such as flavor may greatly enhance the development of high-quality, disease-resistant, apple cultivars.

**Supporting Information Available:** Figure S1 showing year to year variation in levels of butyl, 2-methylbutyl, pentyl, and hexyl acetates in apples collected from the progeny and parental trees. This material is available free of charge via the Internet at http://pubs.acs.org.

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