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RESEARCH

Profiling apple volatile organic compounds in a New Zealand collection of germplasm as a resource for breeding cultivars with desirable flavors

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Abstract Flavor is a major component of the apple eating experience, along with taste, texture and appearance. Apples produce a wide array of volatile organic compounds that impart particular flavors. Cultivars producing increased or novel flavors may have more desirability for consumers and help to differentiate the fruit. Efficiently breeding more flavorful apples requires understanding the volatiles present in apple germplasm and their potential sensory impact. As an initial step towards the development of more flavorful apple cultivars, a New Zealand collection of germplasm was surveyed, and seventy-three volatiles were identified as being present in at least half the accessions. Substantial differences in the presence and relative abundance of specific volatiles were uncovered across the accessions and could sometimes be linked to an apple's flavor profile. The large number of volatiles analyzed allowed relationships between and among molecule classes to be established. Esters were found to be the main drivers of volatile differentiation across accessions. Apples tended to produce either ethyl or acetate esters, suggesting there is different genetic control for these two ester types. Additionally, esters generally had larger broad-sense heritabilities, indicating they could be easier targets for modifying apple flavor. This volatile dataset is a valuable resource for apple breeding, and increases the understanding of an important consumer trait.

Keywords Apple · *Malus* · Volatile organic compound · Ester · Flavor · Aroma · Breeding · GC–MS

Introduction

The primary objective for an apple (*Malus*×*domestica* Borkh.) cultivar breeding program is to release high quality apples that please and excite consumers. Fruit quality attributes such as appearance (color, free of blemish), texture (crispness, juiciness), and taste (sweetness, acidity) contribute to a pleasurable eating experience (Musacchi and Serra 2018). Apple cultivars also have the potential to engage consumers through novel traits such as fruit size, shape, and flesh color (Brown and Maloney 2013). Flavor and aroma may also impact eating experience and novelty. Aroma components can enhance perception of

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sweetness and acidity (Ting et al. 2015), while novel aromas or flavor profiles may help differentiate a cultivar (Jaeger et al. 2011). There is growing evidence that many consumers perceive modern cultivars of some crops to be lacking in flavor (Bartoshuk and Klee 2013; Bowen and Grygorczyk 2021; Folta and Klee 2016). Weariness with commercially available offerings suggests a desire from consumers for more flavorful and satisfying products, and consequently an opportunity exists for breeding programs to deliver such cultivars (Galmarini et al. 2013; Harker et al. 2003).

Apple flavor is determined by the concentration and ratios of sugars (primarily sucrose and fructose) and acids (primarily malic acid), plus diversity of volatile organic compounds (VOC) (Kim et al. 2023; Zhang et al. 2010). While apples can synthesize hundreds of VOC, it is considered that only a few contribute substantially to aroma, and consequently flavor (Farneti et al. 2015; Roberts and Spadafora, 2020; Ulrich et al. 2009). Additionally, combinations of VOC may produce unique flavor/aroma profiles. Acetate esters (especially butyl acetate and 2-methylbutyl acetate) impart a fruity-sweet aroma, typical of 'Gala' apples, while ethyl esters (especially ethyl butanoate and ethyl 2-methylbutanoate) impart a fruity-fresh aroma, typical of 'McIntosh' apples (Panasiuk et al. 1980; Yahia 1991; Young et al. 1996). Hexyl and hexanoate esters (such as hexyl 2-methylbutanoate and butyl hexanoate) contribute "green-grassy" aromas characteristic of unripe bananas and 'Cripps Pink' apples (López et al. 2007; Villatoro et al. 2008). Additionally, C_6 alcohols and aldehydes such as hexanol and hexenal, respectively can impart these "green-grassy" aromas (Yan et al. 2020). Some apple VOC such as the phenolics anethole and estragole provide specific flavors tasting of anise/black licorice (Yauk et al. 2015). Beyond cultivated apple, other *Malus* species produce unique VOC that could be breeding targets for introgression (Kumar et al. 2015; Sugimoto et al. 2015).

Apple volatile diversity has been explored in several studies incorporating germplasm collections and segregating families (Costa et al. 2013; Farneti et al. 2015; Kumar et al. 2015; Rowan et al., 2009ab). These studies have helped reveal the inheritance and genetic control of apple VOC. QTL controlling esters, alcohols, terpenes and aldehydes have been discovered through mapping and genome-wide association studies (Costa et al. 2013; Larsen et al. 2019; Souleyre et al. 2019). Genes controlling VOC production have been functionally validated, including *ALCOHOL ACYL TRANSFERASE 1* (*AAT1*; *Malus*×*domestica* cv. 'Gala' Diploid Consensus Whole Genome v1.0 Assembly & Annotation gene ID Mdg_02A001440) involved in ester biosynthesis, and *O-METHYLTRANSFERASE 1* (*OMT1*; *Malus*×*domestica* GDDH13 v1.1 Whole Genome Assembly & Annotation gene ID MD01G1051900), responsible for estragole biosynthesis (Souleyre et al. 2014; Yauk et al. 2015). Heritability of specific volatiles and volatile classes have been assessed (Costa et al. 2013; Kumar et al. 2015; Rowan et al. 2009a), with some compounds showing high narrow-sense heritabilities, suggesting they could be targets for improvement through breeding. VOC production is also subject to factors that are not genetic or physiological, such as orchard management practices or postharvest conditions (Mpelasoka and Behboudian 2002; Peck et al. 2006). The production of volatiles is also tightly linked to ripening through the action of ethylene (Defilippi et al. 2005; Schaffer et al. 2007). Ethylene inhibition, either by chemical applications or transgenics, suppresses VOC synthesis (Johnston et al. 2009; Kondo et al. 2005).

Given the consumer benefits and market opportunities that might arise from a distinctly flavorful new apple, the apple breeding program at the New Zealand Institute for Plant and Food Research Limited (Plant & Food Research) initiated a project to develop cultivars with enhanced and unique aroma and flavor profiles. The initial step of the aroma and flavor breeding objective was to conduct a broad survey of volatile diversity across Plant & Food Research and New Zealand germplasm. This study differs from previous surveys of New Zealand apple volatiles, in that a broad range of cultivated apple germplasm has been assessed, rather than focusing on material from segregating families (Rowan et al. 2009b), or hybrids between cultivated apple and other *Malus* species (Kumar et al. 2015; Rowan et al. 2009a). Its purpose was to better understand the genetic parameters (such as heritability) of important volatiles in this germplasm, clarify associations between VOC and sensory flavors, and to identify potential parents for future breeding.

This study detected considerable differences in volatile profiles across the New Zealand apple

germplasm dataset. Differentiation among varieties is driven primarily by ester content and abundance, especially between acetate-type and ethyl-type esters. The large number of compounds profiled allowed for associations among VOC and volatile classes to be detected. A description of the flavor and aroma of each accession was also produced, providing a link between VOC content and specific aromas/flavors in certain apples.

Materials and methods

Plant materials

Ninety-eight apple accessions (Online Resource 1, Table S1) maintained at the Plant & Food Research Orchard, and New Zealand National Apple Repository (Havelock North, New Zealand; 39° 39′ S 176° 53′ E) were selected. Germplasm accessions comprised 24 modern cultivars (commercialized after 1980), 24 heritage cultivars, 24 Plant & Food Research advanced breeding selections, 25 Plant & Food Research Stage 1 seedlings (from un-replicated breeding families), and one crabapple of unknown parentage. Fifty-eight accessions had two replicates, and 41 were sampled over two seasons, allowing stability of volatile production to be determined across year and environment. Trees were managed following standard horticultural practices for pruning, thinning, irrigation, fertilization and pest/disease control. Scions were grafted onto 'M9' or 'MM106' rootstocks at 1.5 × 3.5 m spacing.

In 2020 and 2021, ten fruit per accession (five fruit from each of two replicate trees where possible) were harvested at commercial maturity (3–5 on the 0–7 point ENZA Starch Pattern Index scale). Fruit were cold-stored (1°C) for 10 weeks, then placed at ambient temperature (20 °C) for one week prior to fruit sampling for gas chromatography—mass spectrometry (GC–MS). For each fruit, a 1 cm plug was removed at the equator from the two sun-shade interfaces with a Number 4 (8.8 mm diameter) cork borer, and sealed in a 20 mL headspace vial (Thermo Scientific) with 2 g of NaCl (Fisher). Each headspace vial contained 1.2 cm^3 of cortex tissue and 1.2 cm^2 of skin from the two core samples. Samples were stored at −80 °C prior to volatile analysis. Either three or five fruit per replicate were sampled in 2020, while two fruit per replicate were sampled in 2021 (Online Resource 1, Table S2).

Fruit flavor evaluation

Following tissue collection, two fruit from each replicate (two to four fruit total) were used to generate a flavor profile. A longitudinal slice was taken from near the GC–MS sampling point and evaluated by smell and chewing. Flavor and aroma descriptors were noted and used to make a composite description of the accession. The same apple breeder tasted all fruit, and accessions were evaluated for a single year.

Gas chromatography—mass spectrometry analysis of apple volatiles

Samples were analyzed using a Shimadzu TQ8050 GC–MS equipped with a GL Sciences Optic-4 cryo-focusing injector unit using a 65 µm DVB-PDMS SPME fiber (Agilent Technologies). The GC column was a DB-Wax column (Agilent Technologies) 20 m × 0.18 mm ID × 0.18 µm film thickness, using helium at 0.9 mL min^{-1} column flow.

Vials were equilibrated at 50°C for 5 min, before sampling by SPME for 15 min at 50°C. The sample was injected with a 5:1 split ratio with an injection temperature of 240°C.

The cryo-trap was held at −120 °C for 165 s, then heated at 60 °C sec^{-1} to 220 °C. The GC temperature program was 35 °C for 2 min, then 8 °C min^{-1} to 80 °C, then 12 °C min^{-1} to 240 °C, with a hold at 240 °C for 2 min. Volatiles were identified from their retention indices and by comparison with commercial mass spectral databases and authentic compounds. The data were processed using Shimadzu GC–MS PostRun Analysis and GC–MS Browser software. Seventy-three volatiles were chosen for further analysis, with their numerical abundance representing the area under the spectral peak.

Statistical analysis

The distributions of VOC were skewed, so most of the plotting and data analysis was done using log-transformed peak area. Principal component analysis (PCA) was performed using the covariance matrix of the log-transformed peak area. This method focuses on groups of compounds with large fold-changes in

peak area. Clustering was done using a Euclidean distance on the log-transformed peak area, and Ward's method for forming clusters. Heritability calculations were made using both log and raw data, with mixed effects models with Accession + Accession:Year as random effects for the two years of data combined.

Several analyses involved whether two groups of samples had different volatile profiles. Two approaches were used for this; random forest models, where the variable importance plots were used to identify compounds that had a strong effect on the Gini coefficient; and sparse partial least squares-discriminant analysis (PLS-DA) models, where resampling was used to identify compounds which consistently differed between the two groups. All analysis was done using R, including the packages FactoMineR (PCA), Stats (clustering), Sommer (heritability calculations), random Forest, and mixOmics (sparse-PLS-DA).

Results

Germplasm and volatile overview

Ninety-eight apple accessions were screened for volatile organic compounds using GC–MS; 47 in 2020, of which 41 were sampled again in 2021 along with 51 additional accessions (Online Resource 1, Table S1 and S2). Table 1 presents the maximum, minimum and mean peak areas for each of the seventy-three VOC. Nine classes of organic molecules were detected, with the majority (forty-eight) being esters. Only 13 VOC were not detected in every accession, and these compounds tended to have small peak areas (Online Resource 2, Fig. S1). β-pinene was the least abundant VOC measured, while (E,E)-α-farnesene had the greatest average peak area.

Correlations among VOC derived from log10 peak area are presented in Online Resource 2, Fig. S2. Most within-class (Table 1) correlations were slightly (0.1–0.5) to highly (> 0.5) positive. Alcohols had a high positive within-class relationship. Alcohols also tended to have a negative relationship with aldehydes, but often had strong positive associations with esters. Esters, the largest class (forty-eight of seventy-three) of detected compounds, had strong positive self-correlations. Compounds that were present in low amounts and missing from many accessions (for example, (Z)-hex-2-enyl acetate, β-pinene, and ethyl 3-methylthiopropionate) were negatively correlated with most other compounds.

Volatile organic compound heritabilities

Using both 2020 and 2021 data, broad-sense heritability (H^2) was calculated from the log10 peak area for each volatile (Table 1), which ranged from 0.935 (butyl acetate) to 0 (β-pinene), and varied considerably based on class. The 15 highest heritabilities were for ester compounds, while esters accounted for only six of the lowest 15 VOC H^2 (Table 1). Heritabilities also varied based on VOC abundance. Volatiles with low mean peak area tended to have both lower H^2 and peak area means that varied substantially between years (Fig. 1).

Principal component analysis

Principal component analysis (PCA) was performed on the combined 2020 and 2021 dataset using all accessions and volatiles. The first three principal components (PCs) explained 53.6% of the variability within the dataset (Fig. 2). PC1 (Fig. 2A) explained 25.7% of the variability and separated samples based on differences in VOC abundance between years. Only the 20 most influential compounds are plotted, of which most are minor VOC, indicating that the majority of between-year variability derives from compounds that had a low peak area and were rare across the accessions (with correspondingly low H^2). Figure 2B demonstrates how samples within an accession separate across the PCA graph according to year. All accessions plotted onto the PC1 and PC2 graph are presented in Online Resource 2, Fig. S3 A.

PC2 explained 14.7% of variability, while PC3 explained 13.1% (Fig. 2C). PC2 separated accessions based on whether they produced esters or not. Accessions such as the crabapple ABGS0836, which do not produce many esters clustered negatively along PC2, while those that produce abundant esters such as 'PremA17' and 'Cortland' clustered neutrally or positively (Fig. 2D). PC3 separated accessions based on whether they are predominantly acetate ester-producers or ethyl ester-producers. Accessions like 'Cortland' that produce ethyl esters clustered negatively along PC3 while acetate ester producers like 'PremA17' clustered positively (Fig. 2D). Unlike

Table 1 The seventy-three volatile organic compounds surveyed in this study from selected apple (*Malus* spp.) germplasm

Volatile	Class	Min	Max	Mean	Number of accessions volatile was undetected	H^2	Std Error
		(Peak area)	(Peak area)	(Peak area)			
(3E)-3-hexenoic acid	Acid	79	115,358	8642		0.866	0.031
(E)-2-hexenal	Aldehyde	224	51,601	9297		0.168	0.124
(E)-2-hexenol	Alcohol	4771	778,873	99,934		0.379	0.108
(E,E)-α-farnesene	Terpene	181,743	24,484,119	7,347,269		0.684	0.068
(Z)-2-hexenal	Aldehyde	27,597	2,030,635	462,556		0.199	0.130
(Z)-hex-2-enyl acetate	Ester	0	153,265	18,966	14	0.076	0.113
(Z,E)-α-farnesene	Terpene	843	254,384	81,635		0.545	0.085
1-butanol	Alcohol	0	6,834,983	1,042,516		0.641	0.062
1-hexanol	Alcohol	127,752	9,512,993	2,194,265		0.764	0.052
1-pentanol	Alcohol	2779	274,034	44,838		0.830	0.037
1-propanol	Alcohol	327	116,084	15,866		0.730	0.062
2-methyl butanol	Alcohol	6789	3,878,806	556,009		0.851	0.035
2-methyl propanol	Alcohol	0	203,575	45,959		0.490	0.084
2-methyl-2-butenyl acetate	Ester	0	57,079	2880	1	0.535	0.082
2-methylbutyl 2-methylbutanoate	Ester	80	794,054	38,177		0.793	0.049
2-methylbutyl 2-methylpropanoate	Ester	0	389,610	34,285		0.608	0.072
2-methylbutyl acetate	Ester	2091	4,482,971	890,368		0.928	0.018
2-methylbutyl octanoate	Ester	63	152,886	19,825		0.751	0.057
2-methylbutyric acid	Acid	0	3,711,141	421,938		0.674	0.069
2-methylpropyl 2-methylbutanoate	Ester	0	802,828	74,503	5	0.437	0.111
2-methylpropyl acetate	Ester	12	117,510	25,042		0.833	0.040
2-pentylfuran	Furan	0	18,723	2085	2	0.095	0.088
3-methylthiopropyl acetate	Ester	0	7699	769		0.605	0.068
5-hexenyl acetate	Ester	34	122,305	14,001		0.917	0.020
6-methyl-5-hepten-2-one	Ketone	3068	610,862	73,259		0.581	0.076
6-methyl-hept-5-en-2-ol	Alcohol	447	1,242,848	77,536		0.677	0.065
Anethole	Ether	0	7166	413		0.119	0.067
Benzaldehyde	Aldehyde	44	605,826	3721		0.190	0.079
Benzyl acetate	Ester	0	181,919	5827	1	0.277	0.104
β-pinene	Terpene	0	3609	224	47	0.000	0.079
Butyl 2-methylbutanoate	Ester	3723	1,222,448	211,087		0.862	0.033
Butyl 9-decenoate	Ester	49	93,417	7148		0.840	0.036
Butyl acetate	Ester	2209	5,793,830	1,159,371		0.935	0.017
Butyl butanoate	Ester	4367	987,808	183,642		0.856	0.033
Butyl decanoate	Ester	0	141,721	13,693	1	0.557	0.096
Butyl heptanoate	Ester	357	145,329	26,457		0.714	0.064
Butyl hexanoate	Ester	26,718	4,032,042	930,109		0.747	0.056
Butyl octanoate	Ester	2897	1,186,884	211,820		0.823	0.041
Butyl propanoate	Ester	426	1,636,972	168,376		0.854	0.035
E-ethyl tiglate	Ester	0	40,987	1131	49	0.361	0.124
Estragole	Ether	4392	3,700,160	265,841		0.745	0.060
Ethanol	Alcohol	319	2,329,570	56,441		0.791	0.049
Ethyl (4E)-4-octenoate	Ester	0	44,006	6113		0.559	0.087
Ethyl 2-methylbutanoate	Ester	0	1,566,269	29,659		0.815	0.040

Table 1 (continued)

Volatile	Class	Min	Max	Mean	Number of accessions volatile was undetected	H^2	Std Error
Ethyl 3-methylthiopropionate	Ester	0	37,672	329	44	0.027	0.128
Ethyl acetate	Ester	23	525,079	7385		0.702	0.062
Ethyl butanoate	Ester	0	5,272,656	109,448		0.885	0.028
Ethyl E/E 2,4-decadienoate	Ester	0	19,421	1459	5	0.268	0.114
Ethyl heptanoate	Ester	0	7409	1057	1	0.068	0.127
Ethyl hexanoate	Ester	313	5,593,604	82,454		0.870	0.031
Ethyl octanoate	Ester	90	2,806,476	36,691		0.770	0.052
Ethyl propanoate	Ester	0	105,662	2174	3	0.650	0.080
γ-decalactone	Lactone	0	26,327	975	29	0.186	0.109
Hexanal	Aldehyde	2850	455,764	103,030		0.447	0.112
Hexyl 2-methylbutanoate	Ester	120,015	11,349,247	2,575,583		0.793	0.046
Hexyl 2-methylpropanoate	Ester	571	369,343	45,271		0.806	0.042
Hexyl acetate	Ester	17,938	11,084,630	1,923,814		0.930	0.018
Hexyl butanoate	Ester	22,955	4,107,514	583,686		0.785	0.047
Hexyl hexanoate	Ester	25,798	7,043,972	1,536,292		0.760	0.055
Hexyl octanoate	Ester	247	521,397	57,093		0.834	0.038
Hexyl propanoate	Ester	3064	1,689,626	217,382		0.848	0.036
Hexyl tiglate	Ester	2137	725,601	72,702		0.561	0.084
Methional	Aldehyde	121	68,181	7561		0.567	0.080
Methyl 2-hydroxy-3-methylpentanoate	Ester	35	36,823	2852		0.752	0.053
Methyl 2-methylbutanoate	Ester	0	176,949	12,132		0.742	0.058
Pentyl 2-methylbutanoate	Ester	129	353,932	37,798		0.816	0.042
Pentyl acetate	Ester	14	498,717	89,029		0.902	0.025
Pentyl hexanoate	Ester	50	68,039	7923		0.674	0.068
Propyl acetate	Ester	0	1,115,734	120,739		0.750	0.058
Propyl hexanoate	Ester	658	504,266	64,796		0.756	0.055
Propyl octanoate	Ester	0	115,870	13,142		0.679	0.068
Propyl propanoate	Ester	14	377,261	28,833		0.771	0.048
Unknown sesquiterpene	Terpene	0	27,867	9204		0.040	0.098

Values and H^2 are based on combined 2020 and 2021 data. Values across year and accession can be found in Online Resource 1, Table S2

PC1, there was consistency within each accession across sampling years for PC2 and PC3 (Fig. 2D). All accessions plotted onto the PC2 and PC3 graph are presented in Online Resource 2, Fig. S3 B.

Relationships among volatiles

The relationship between alcohols and their corresponding esters (for example, 1-butanol and butyl acetate) was investigated and the results are presented in Online Resource 1, Table S3. Correlations ranged from −0.28 (ethanol–ethyl heptanoate) to 0.75 (ethanol–ethyl hexanoate), with most relationships being positive. Several trends were revealed when correlations were graphed (Fig. 3). Some relationships such as ethanol–ethyl hexanoate (corr = 0.75; Fig. 3A) were clearly positively linear, suggesting that ethyl hexanoate production is very dependent upon ethanol abundance. Often, no relationship existed between the alcohol and ester, for example, 1-hexanol–hexyl tiglate (corr = 0.08; Fig. 3B), suggesting the availability of 1-hexanol has little impact on hexyl tiglate production. This lack of correlation was consistent for all hexyl- and pentyl-derived esters. A

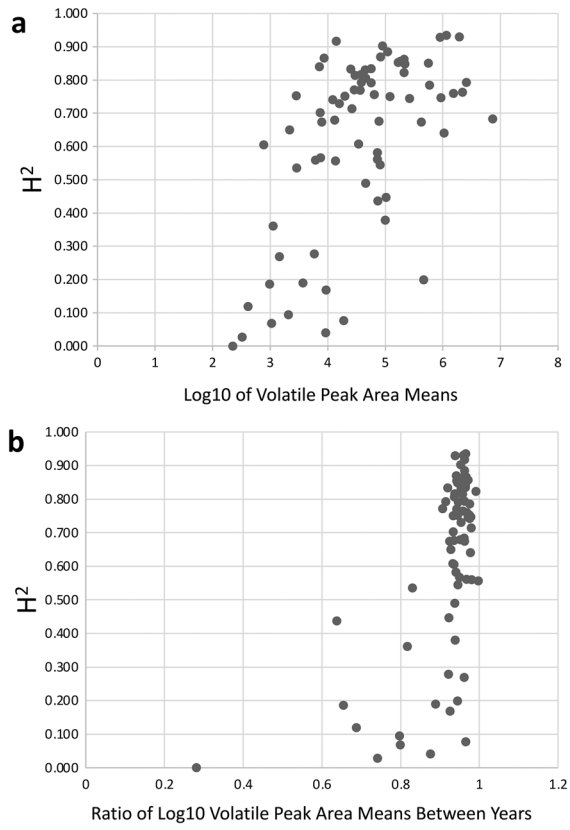

Fig. 1 **a** Plot of H^2 and volatile peak area means from apple (*Malus* spp.). Volatiles with smaller mean peak area tended to have smaller H^2. **b** Plot of H^2 and the ratio of volatile means between 2020 and 2021 assessments. Volatiles with consistent means between years (ratios close to one) tended to have higher H^2. Mean peak area data of each sample are presented on a log10 scale

few relationships such as ethanol–ethyl heptanoate (corr = −0.28; Fig. 3C), had a slight negative correlation. Many accessions did not produce ethyl esters, even though ethanol was represented by a large peak area, as shown in Fig. 3C. Finally, among acetate esters, there were two distinct clusters of accessions, as in 1-butanol–butyl acetate (corr = 0.23; Fig. 3D). The clusters appear largely independent of alcohol concentration, but rather more determined by ester content. All alcohol-ester correlations are presented in Online Resource 2, Fig. S4.

Esters comprise both an alkyl (alcohol-derived) and alkanoate (acid-derived) group, which may be synthesized from the same pathway (for example, 1-butanol and butanoic acid). For a particular group of esters, alkyl- and alkanoate-derived values were plotted, to determine if there was a preference for using either the alcohol or acid substrate in ester production (Fig. 4; Online Resource 1, Table S4). Seven relationships were identified, of which four (propyl–propanoate, butyl–butanoate, hexyl–hexanoate, 2-methylbutyl–2-methylbutanoate) had a significant positive correlation, and three had no correlation.

Both straight-chain and branched-chain esters were detected in apple (Online Resource 1, Table S5). Plotting the log10 peak area of straight- and branched-chain esters yielded a correlation of 0.61 (Online Resource 2, Fig. S5), with ratios averaging ~ 1.0. Based on VOC peak areas, this suggests that across the germplasm set, apples tend to have consistent ester ratios of "straight-chain" and "branched-chain" types.

Linking sensory perception to volatile organic compound relative abundance

A description of the fruit flavor following ten weeks of cold storage plus seven days at ambient temperature (20 °C) was developed for every accession (Online Resource 1, Table S6). For most accessions, the "fruity, acetate ester" aroma reminiscent of 'Gala' apples predominated. These apples produced large amounts of acetate esters (especially 2-methylbutyl acetate, butyl acetate, and pentyl acetate) which are known to impart a fruity-sweet aroma (Chitarrini et al. 2021). Some older heritage varieties, such as 'Cortland,' 'Empire,' and 'Fleuritard Rouge,' were noted as having a strong "fruity-fresh" aroma, characteristic of 'McIntosh' apples. This aroma is typical of several ethyl esters (especially ethyl butanoate, ethyl 2-methylbutanoate, and ethyl propanoate), and these "fruity-fresh" accessions produced large amounts of those esters (Aaby et al. 2002; Online Resource 1, Table S2).

Apples are known to produce VOC such as C_6 aldehydes and alcohols, and hexyl esters that impart "green-grassy" aromas (Aparicio et al., 1998; Hongsoongnern and Chambers 2008). Based on sensory profiling, 'Cripps Pink' and 'Granny Smith' were regarded as having a distinct "green-grassy" aroma, and both apples produced large amounts of "green-grassy" compounds. 'Granny Smith' contained large amounts of C_6 aldehydes and alcohols, including (E)-2-hexenol, (E)-2-hexenal, and (Z)-2-hexenal. Relative to ethyl, butyl and pentyl esters, 'Cripps Pink'

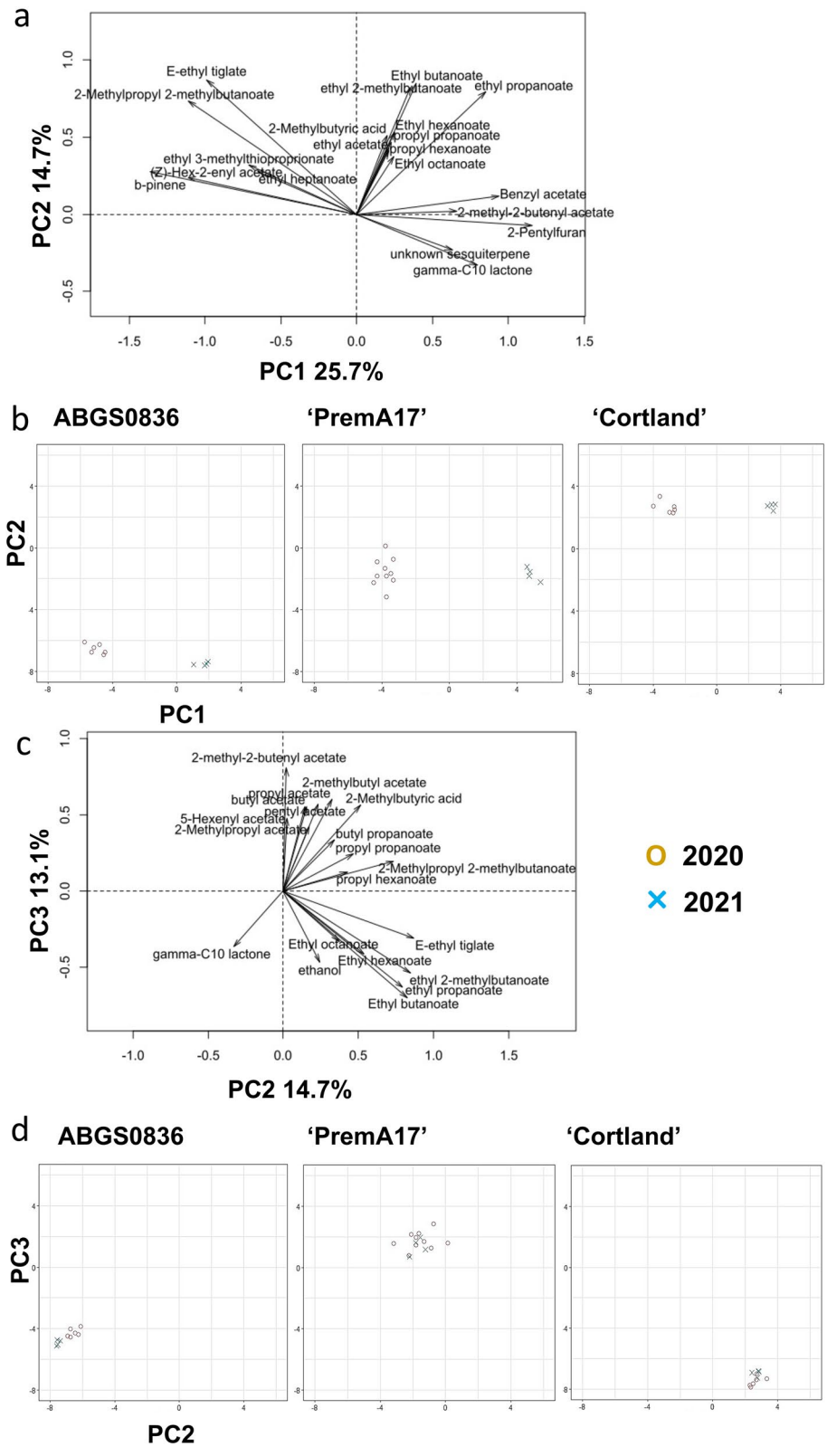

Fig. 2 **a, c** Plots of the top twenty volatile organic compounds from apple (*Malus* spp.) that distinguish Principal Components (PC) one and two and Principal Components two and three. **b, d** Overlay of ABGS0836, 'PremA17,' and 'Cortland' onto the Principal Component plots in 2020 and 2021

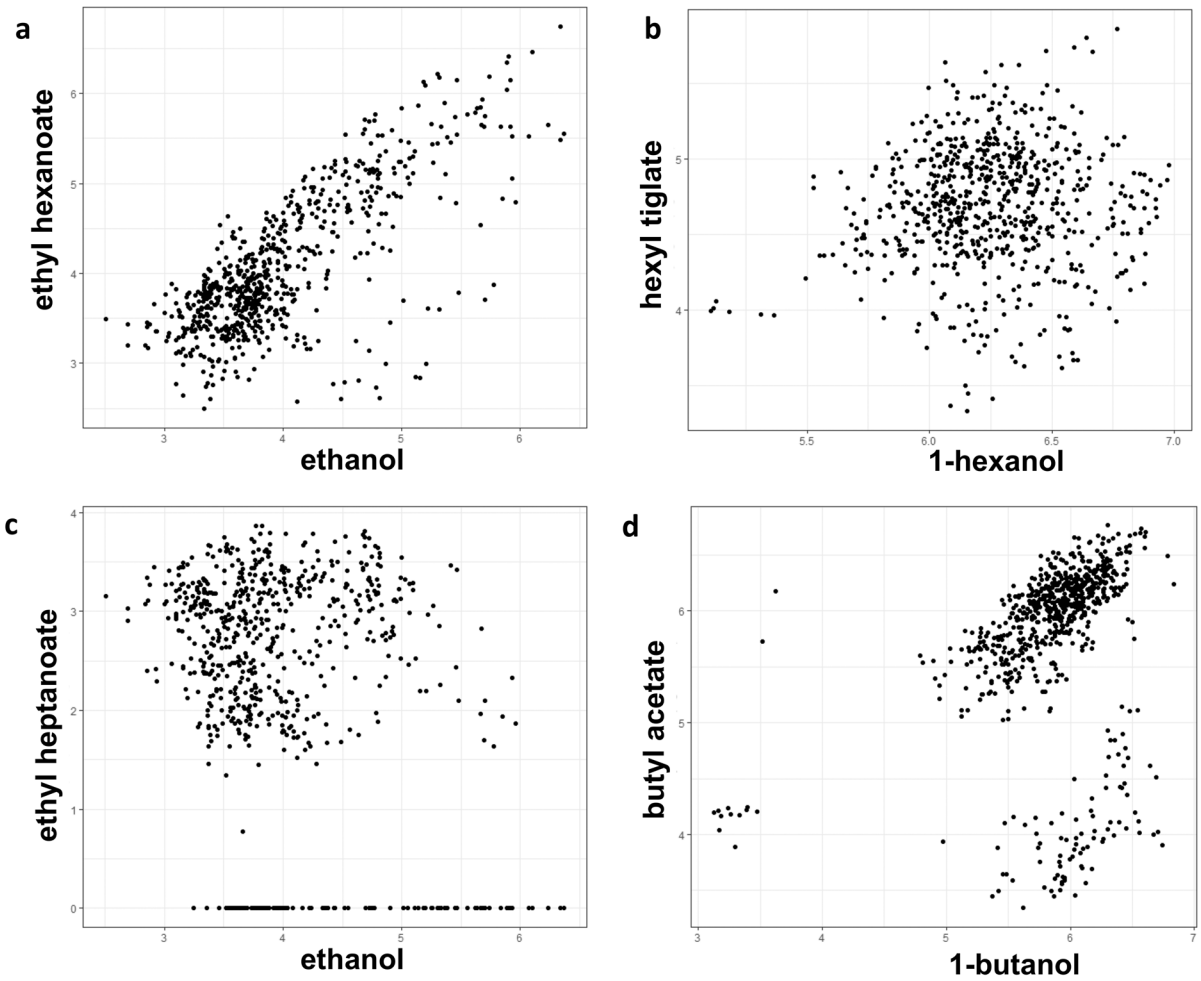

Fig. 3 Four plots of an ester and its alcohol precursor sampled from apple (*Malus* spp.). **a** Ethanol and ethyl hexanoate, corr = 0.75. **b** 1-hexanol and hexyl tiglate, corr = 0.08. **c** Ethanol and ethyl heptanoate, corr = −0.28. **d** 1-butanol and butyl acetate, corr = 0.23. Peak area data of each sample are presented on a log10 scale

produced large amounts of all eight hexyl esters, which may explain its "green-grassy" aroma. Finally, the "green-grassy" VOC (E,E)-α-farnesene was abundant in both 'Granny Smith' and 'Cripps Pink' (Yan et al. 2020).

Several aromas/flavors could be ascribed to specific compounds in some accessions. Anise/black licorice flavor was noted in the accessions 'Priscilla,' AA0787-0390, and A185R09T179, and these apples were among those with the largest peak areas for estragole (Yauk et al. 2015). AA0643-0070 had the largest peak area of 6-methyl-hept-5-en-2-ol, which may be why this accession was noted for its caraway-like flavor (Furdikova et al. 2017). A596S29R02T090, noted as having a distinct peach flavor had the largest peak area of γ-decalactone, the main constituent of peach aroma (Peng et al. 2020). However, other accessions noted for their peach aroma did not contain especially large amounts of γ-decalactone, suggesting that additional VOC may be responsible for this taste profile in apple (Online Resource 1, Table S6).

Distribution of rare volatile organic compounds

Some VOC that are known to be important for characteristic aromas in other fruit species were detected in only a few apple accessions. However, these rare

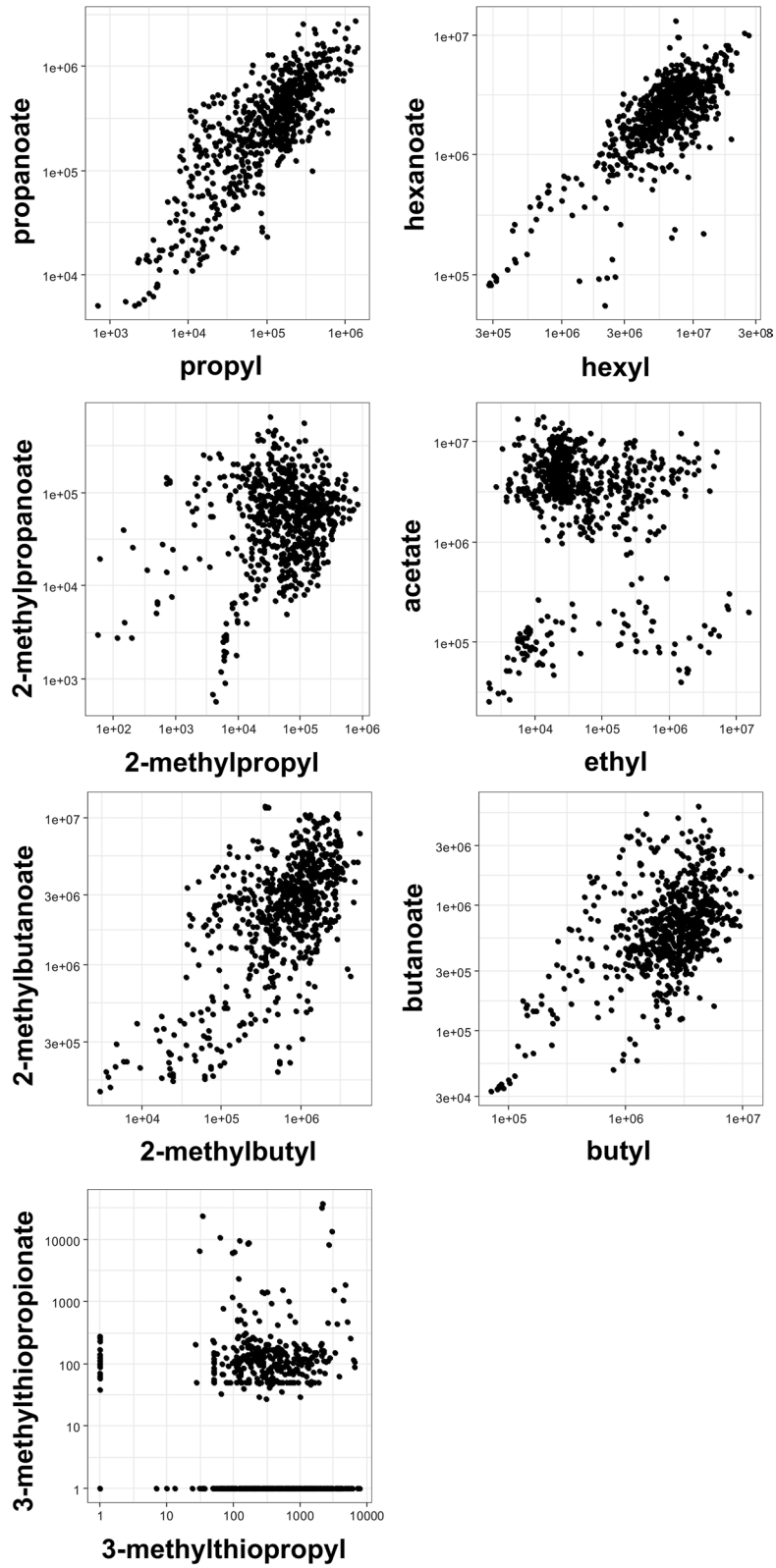

Fig. 4 Seven plots of alkyl and alkanoate groups derived from the same precursors in apple (*Malus* spp.). The correlations and list of esters included in each alkyl and alkanoate group can be found in Online Resource 1, Table S4

VOC did not impart the expected fruit aromas in this germplasm set, either because they were present in insufficient quantities or were masked by other VOC. 'Fleuritard Rouge' fruit produced a peak area of E-ethyl tiglate (ethyl (E)-2-methylbut-2-enoate) that was three times greater than the next largest accession, 'Redgold' (Online Resource 1, Table S2). E-ethyl tiglate is a component of quince flavor (Schreyen et al. 1979), but no quince flavor/aroma was noted in 'Fleuritard Rouge' (Online Resource 1, Table S6). Similarly, relative to other acetate esters, 'Pinova' and 'Sciros' produced large amounts of 3-methylthiopropyl acetate, a component of melon flavor (Sakamoto et al. 2002), but no melon aroma was noted in these apples.

Discussion

Volatile organic compound profiling

Gas chromatography—mass spectrometry provided valuable insight into the diversity and relationships among volatile organic compounds in the New Zealand-based apple germplasm collection. This dataset serves as an important resource for breeding apples with increased or unique flavor profiles. In agreement with previous studies, esters were the most prevalent class of VOC detected, and among these, hexyl 2-methylbutanoate, hexyl acetate, hexyl hexanoate, and butyl acetate had the highest mean peak areas (Holland et al. 2005; Sugimoto et al. 2015; Ulrich et al. 2009; Yang et al., 2021ab). Even among germplasm that was unselected or unimproved by modern breeding programs, esters were the largest and most prevalent VOC class (Kumar et al. 2015; Rowan et al. 2009a). However, ethyl esters were more likely to make up a significant portion of VOC in older material, supporting the observation that there has been a shift from ethyl ester to acetate ester production in modern cultivars. In New Zealand germplasm, (E,E)-α-farnesene had the greatest average peak area, which agrees with findings of previous reports (Ban et al. 2010; Kumar et al. 2015).

Volatile genetic and biochemical control

Analysis of VOC relationships provided useful clues to the possible genetic and biochemical control of volatile biosynthesis, especially esters. Many alcohol-ester relationships were positive (Online Resource 2, Fig. S4; Online Resource 1, Table S3), indicating that more alcohols were produced than could be consumed by ester biosynthesis, and that alcohol concentration did not limit ester formation. If alcohols were limiting during ester production, a negative correlation would be expected because much of the available alcohol would be consumed to produce esters. Situations were also observed where there was a low correlation (for example, hexyl 2-methylbutanoate; corr = 0.23) or when many accessions do not produce an ester (for example, ethyl heptanoate), despite abundant alcohol being present, suggesting the alcohol precursor is not always a determinant of ester content. In these cases, there may be insufficient alkanoate-CoA precursor or the alkanoate is being utilized preferentially for other esters. There may also be no ester-forming enzyme (nonfunctional allele) or a regulatory block in these accessions. In most of the acetate ester correlations, there were two distinct clusters of accessions, which may represent accessions with different alleles (*A_* or *aa*) of an ester-producing enzyme with different catalytic rates. Allelic variants of *AAT1* with differing activities have been detected in apple and explain much of the difference in ester production among cultivars studied (Souleyre et al. 2014). Finally, abundant alcohols might be sequestered in flesh tissue and not available to the skin, which is the primary site of VOC production (Rudell et al. 2002).

The four positive alkyl–alkanoate correlations (propyl–propanoate, butyl–butanoate, hexyl–hexanoate, 2-methylbutyl–2-methylbutanoate) suggest that despite the alkyl and alkanoate groups being formed from the same pathway, there is sufficient precursor and little competition or preference. For example, where α-ketobutyrate (the precursor of propanol and propionyl-CoA) was in excess, there was no limit on the production of apple propyl- or propanoate-esters, leading to a high correlation between the two (Sugimoto et al. 2021). When there is no correlation, such as the ethyl–acetate relationship, the alkyl and alkanoate precursors may be derived from different sources. There may also be a genetic component, because in this germplasm collection, accessions tended to be "acetate-ester" producers or "ethyl-ester" producers (Online Resource 1, Table S2; Online Resource 2, Fig. S3 B).

Potential breeding targets

Esters are both the largest class of VOC detected in apple, and the primary components of apple flavor (Fellman et al. 2000), making them attractive targets for breeding increased or unique aromas. Ester profiles were also the most distinguishing features of accessions based on PCA. In the New Zealand germplasm, apples tended to be primarily acetate- or ethyl-ester producers. However, some accessions such as 'Honeycrisp,' 'Fuji,' 'Delicious,' and AA0742-0030 had large levels of both types, indicating no genetic dominance (Online Resource 1, Table S2). 'Honeycrisp' and AA0742-0030 in particular were noted as having a rich flavor, and the presence of both acetate and ethyl esters may contribute to a more complex and desirable flavor (Online Resource 1, Table S6). Most of the modern cultivars measured were dominated by acetate esters, while ethyl esters were found mostly in heritage varieties. It is unclear whether this reflects a change in consumer preference toward acetate ester flavor, or modern breeding programs relying on acetate producing germplasm as founders. However, with the extensive use of 'Honeycrisp' as a parent, many breeding programs may release cultivars producing significant ethyl esters (Brown and Maloney 2018).

Often, a relationship between VOC and flavor/aroma was detected, suggesting that targeting these VOC could affect apple flavor. However, breeding for such VOC may still be a difficult proposition: presuming additivity, only compounds with large H^2 or h^2 could be easily modified through selection. Heritability estimates from different studies may differ greatly depending upon the germplasm set used (relatedness, prior selection, populations or segregating families) and experimental design (horticultural management, replication and sampling). This is evident when comparing heritabilities of major apple esters across studies. The H^2 estimates presented herein were calculated from a collection of mostly unrelated accessions, while the VOC H^2 values of Costa et al. (2013) are derived from a full-sib family. Despite the differences in germplasm structure, five major apple esters (2-methylbutyl acetate, butyl acetate, ethyl 2-methylbutanoate, ethyl butanoate, and hexyl acetate) all had $H^2 > 0.6$ in both studies. However, for these same esters, Kumar et al. (2015) calculated much lower h^2 (0.09–0.39) compared with values (0.39–0.85) recorded by Rowan et al. (2009a), despite the studies utilizing much overlapping plant material. The h^2 differences may be attributed to the size and genetic background of the populations, and different tree management and experimental designs.

Some of the rarer VOC (for example, γ-decalactone responsible for peach aroma) detected have low H^2, and so may be challenging breeding targets. Another potential barrier to targeting specific VOC for breeding is the positive correlation among many of them (Online Resource 2, Fig. S2). Many VOC are produced from the same pathway (under the same genetic control), meaning it would be difficult to target just one without affecting others (Schaffer et al. 2007; Souleyre et al. 2014).

Breeding progress may be slowed by the quality of germplasm as well as heritability of the VOC. For example, only 'Fleuritard Rouge' produced a large peak area of E-ethyl tiglate. However, this variety is of poor eating quality, so it may take several generations to introgress appreciable E-ethyl tiglate content into a high-quality selection. Additionally, E-ethyl tiglate was not detected in 49 accessions and only has a H^2 of 0.361, indicating it will be difficult to breed for increased amounts.

Future perspectives

Although this dataset is a critical starting point for breeding apples with increased and unique flavors and aromas, it cannot recommend how new cultivars should taste. Some targets seem obvious, like increasing esters to develop highly flavorful cultivars akin to 'Scilate' and 'Honeycrisp.' Direction for other flavors/aromas and their corresponding VOC is less clear. Plant & Food Research plans to integrate consumer studies into the breeding pipeline to assist with decision-making around VOC targets. Consumer preferences can be inferred from tasting panels sampling fruit, or a recombinate system, whereby a VOC-of-interest is added to a solution that mimics a fruit juice base (Zeng et al. 2020).

Once breeding targets are established, genetic tools can be developed to make flavor/aroma breeding quicker and more efficient. Segregating families and fruit RNA-seq will provide further insight into the genetic control of VOC production. QTL and candidate genes can be used to develop markers for

MAS, targeting seedlings with putative desired taste profiles.

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