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Evaluation of leaf metabolite-based phenomic selection to shorten the apple breeding cycle

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Abstract

Advances in high-throughput genotyping have accelerated the adoption of genomic selection (GS) in breeding programmes. However, genotyping thousands of plants each year remains costly and may limit the implementation of GS in many fruit breeding programmes. Phenomic selection (PS) is increasingly gaining attention as a cost-effective alternative to GS for predicting phenotypic traits in plants. A population of approximately 300 seedlings derived from crosses between domesticated and wild apple species was genotyped using low-coverage sequencing and phenotyped for several fruit quality traits. Mature leaf samples from the same seedlings were phenotyped for secondary metabolites, including chalcones, cinnamic acids, flavanols, flavonols, and triterpenoids, using LC-HRAM-MS. GS, PS, and combined GS + PS models were subsequently developed using these data to predict fruit traits. The across-trait average prediction accuracy of PS was similar to that of GS (0.62 vs. 0.64), with a correlation of 0.94 between PS and GS accuracies. The highly significant correlation (0.76) observed between the metabolite relationship matrix (MRM) and the genomic relationship matrix (GRM) provides a theoretical basis for the application of the PS model. The comparable accuracies of PS and GS suggested that a low-cost strategy based on leaf secondary metabolites could be developed to screen thousands of apple seedlings in both training and selection populations. This proof-of-concept study demonstrates the first application of leaf metabolite-based PS in a fruit crop, and further studies are currently underway to validate and strengthen these findings.

Keywords: Phenomic selection, Genomic selection, Apple breeding, Fruit traits, Leaf metabolite

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Introduction

Apple (*Malus domestica*) occupies a central role in the global fruit economy and in human nutrition. It is among the most widely produced and consumed fruits worldwide, reflecting its importance not only as a source of livelihood for growers and traders but also as a staple component of many diets[1,2]. Apples are recognized for their considerable health benefits, as they provide dietary fibre, vitamins, minerals, and a diverse array of phytochemicals, particularly phenolic acids and flavonoids[3]. Evidence from epidemiological, clinical, and experimental studies suggests that regular apple consumption may contribute to a reduced risk of several chronic diseases, including cardiovascular disease, type 2 diabetes, and certain cancers[4]. Apple consumption may also support metabolic health by improving lipid metabolism, vascular function, and inflammation, and may benefit gut health through the combined effects of dietary fiber and bioactive compounds that influence the gut microbiota[5].

In perennial crops, many commercially important traits, such as fruit phenotypes, are expressed only at later developmental stages because of a prolonged juvenile period. This, combined with the time-consuming and costly measurement of traits in mature trees, results in long evaluation cycles and reduced genetic gain per unit time. Advances in high-throughput genotyping have accelerated the adoption of genomic selection (GS) as a central tool in modern plant breeding[6]. However, despite its promise, the predictive performance of GS for primary breeding targets can be limited by factors such as low trait heritability, environmental variability, and constrained training population sizes. To address these challenges, there is growing interest in incorporating secondary traits (e.g., high-throughput phenotyping indicators) into predictive models, as these traits can be measured rapidly, non-destructively, and early in plant development. By leveraging these indicators of later performance, breeders can accelerate selection decisions, shorten breeding cycles, and improve the efficiency of breeding programmes in long-lived perennial species[7–9].

A widely used secondary phenotype is the near-infrared spectrum (NIRS) measured from plant tissues. A study in wheat and poplar showed that phenomic selection (PS) based on NIRS obtained from various tissues (grains, leaves, wood) achieved predictive accuracies comparable to those of DNA marker-based genomic selection (GS) for developmental, stress tolerance, and productivity traits[8]. PS also appears to be less sensitive than GS to the degree of relatedness between individuals in the training and prediction sets[10]. GS using SNP markers generally achieved higher predictive accuracy than NIRS-based PS in annual crops[11] and grapevine[8]. However, the accuracy of PS based on the leaf-derived NIRS data was considerably lower than that of GS for several apple fruit phenotypes[12]. Although GS generally outperformed PS, models combining both sources of information often achieved higher predictive ability than either approach alone[9,13].

NIRS is an indirect measure of the metabolite composition of analysed tissues, as it captures a combined signal rather than identifying or quantifying individual metabolic compounds. Metabolites have been reported to be well suited for predicting phenotypes because they contain rich and condensed biological information and can be measured using high-throughput analytical platforms[14]. Using relationship matrices computed from metabolite profiles

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collected at early developmental stages, predictive accuracies comparable to those achieved with molecular markers have been reported in maize[15], wheat[16], and rice[17]. In this study, we evaluate whether leveraging DNA markers and leaf-derived secondary metabolites can improve the prediction of apple fruit phenotypes. By clarifying the factors that determine the predictive value of leaf metabolites, we aim to support breeders in designing more efficient phenotyping strategies and optimizing prediction models for fruit traits to accelerate genetic gain.

Materials and methods

Plant material

Malus spp. accessions were used as pollen parents to hybridise with commercial cultivars, generating four families: 'PremA17' × *M. trilobata*, 'PremA17' × *M. ioensis*, 'Scilate' × *M. trilobata*, and 'Scifresh' × *M. sieboldii*. The numbers of seedlings in the four hybrid families were 74, 16, 51, and 163, respectively. The seed parents share ancestry with 'Royal Gala' and 'Braeburn', providing genetic connectedness among the families. Two-year-old seedlings were grafted onto 'M9' rootstock and planted in 2020 at Havelock North, New Zealand, for fruit evaluation. Trees were managed according to standard commercial practices. Of the 304 seedlings, only 191 fruited in the first year, whereas 281 fruited in the second year, with 180 seedlings fruiting in both years. Some seedlings that fruited in the first year but did not fruit in the second year may be due to their biennial bearing tendency.

Leaf sampling for secondary metabolite phenotyping

Mature leaves were collected from all available seedlings in 2022. Three grams of crushed tissue were placed in a 50 mL screw-capped tube, and 30 mL of extraction solvent (ethanol:water:formic acid, 80:20:2) containing naringenin (1.05 mg/L) as an internal standard was added immediately. Samples were vortexed and extracted overnight at 1 °C. Extracts were then shaken, centrifuged, and an aliquot collected, diluted 50-fold with methanol, and analysed by LC–HRAM–MS for secondary metabolites, including chalcones, cinnamic acids, flavanols, flavonols, and triterpenoids. This LC–HRAM–MS system comprised a Vanquish Horizon UHPLC (Thermo Fisher Scientific, Germering, Germany) coupled to a timsTOF high-resolution mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization source. The LC column was a Luna Omega C18 column (100 mm × 2.1 mm, 1.6 μm; Phenomenex, California, USA) maintained at 40 °C. The flow rate was 400 μL/min. The mobile phases were A, 0.2% formic acid, and B, 100% acetonitrile. The gradient program was as follows: 90% A and 10% B from 0 to 0.5 min; a linear gradient to 60% A and 40% B from 0.5 to 7 min; a linear gradient to 5% A and 95% B from 7 to 12 min; held at 5% A and 95% B from 12 to 15 min; returned linearly to 90% A and 10% B from 15 to 15.2 min; and maintained at the initial conditions until the next injection at 18 min. The injection volume for both samples and standards was 1 μL. The timsTOF parameters were as follows: source temperature, 250 °C; drying N_2 flow, 8 L/min; nebuliser N_2 pressure, 1.8 bar; endplate offset, 500 V; mass range, 100–1,500 Da; and acquisition rate, 5 scans/s. Negative electrospray ionization was performed using a capillary voltage of 3,500 V. Post-acquisition internal mass calibration was performed using sodium formate clusters, with sodium formate delivered by a syringe pump at the start of each chromatographic run. Data were processed using the targeted quantitation workflow TASQ (Bruker Daltonics, Bremen, Germany). AA-2βG was analysed using UHPLC and quantified as AA-2αG equivalents based on authentic standards[18].

Fruit sampling and phenotyping

Fruit sampling was conducted over 2 years (2023–2024), depending on availability. Six fruit were harvested from 191 of the 304 seedlings in 2023 and from 281 seedlings in 2024, with 180 seedlings assessed in both seasons. Harvesting and storage followed the protocol described by Kumar et al.[18]: fruit was harvested at maturity, stored at 0.5 °C for 10 weeks, and then held at 20 °C for 1 week prior to evaluation. Thin cortical wedges from two fruit per seedling were used for sensory assessment. Sweetness, sourness, astringency, and overall eating quality (EQUAL) were scored from 0 (lowest) to 9 (highest). The EQUAL score was calculated as the average of the fruit firmness, crispness, juiciness, and sweetness scores. Fruit size was visually rated on a scale of 1 (< 30 g) to 9 (> 240 g). Following Kumar et al.[18], fruit were also assessed for the secondary metabolites AA-2βG and trilobatin, as these compounds are known to have antioxidant properties and are being considered as potential target traits for future cultivar breeding.

Phenotypic data analysis

As fruit data were collected over two consecutive years, each trait was analysed using a mixed-effects model that accounted for permanent environmental effects[19]:

$$y = Xb + Za + Zp + e$$

where, y is the vector of observations, b represents the fixed effects (overall mean, year); a, p, and e denote the random additive genetic effects, permanent environment effects, and residual effects, respectively; and X and Z are incidence matrices. The variances associated with the random effects, a, p, and e were σ^2_a, σ^2_p, and σ^2_e, respectively. For each trait, variance components and best linear unbiased predictions (BLUPs) of additive genetic effects for individual seedlings were estimated using the ASReml-R software[20]. Narrow-sense heritability (h^2) was calculated as the ratio of additive genetic variance (σ^2_a) to total phenotypic variance (= $\sigma^2_a + \sigma^2_p + \sigma^2_e$). Genetic correlations between traits were estimated as product–moment correlations between their respective BLUPs. For the analysis of leaf traits, the permanent environmental effect was omitted as the seedlings were assessed only once.

DNA sequencing and variant calling

DNA was extracted from young leaves using a CTAB-based method, followed by clean-up with AMPure XP beads. DNA was eluted in TE buffer (pH 7.5). Approximately 100–200 ng of DNA per sample was fragmented for 9 min using the NEBNext® Ultra™ II FS DNA Library Prep (half-volume reactions), yielding an average fragment size of ~300 bp. Libraries were indexed using NEBNext multiplex oligos for Illumina (96 Unique Dual Index Primer Pairs, cat. # E6441A, E6443A, E6445A, E6447A), purified with AMPure XP beads, and showed an average size of ~ 450 bp. Equal amounts of each library were pooled based on the amount of DNA quantified by fluorescence (Qubit HS dsDNA kit, ThermoFisher), one pool per plate. To refine the fragment distribution, a second AMPure XP magnetic bead size selection was performed on each pool, at a 0.6/0.2X ratio. Sequencing was performed on the NovaSeq platform (paired-end 150 bp) at Novogene. Low-coverage whole-genome resequencing generated ~ 3.6 Gb of data per seedling.

Raw reads were assessed for quality using FastQC (v0.11.7) and cleaned with BBDuk (BBMap v38.33 package). Filtered reads were aligned to the GDDH13 v1.0 reference genome[21] using HISAT2 (v2.2.1)[22] with the --sensitive, --no-spliced-alignment, and -I 100 options. Alignments were sorted and indexed with SAMtools (v1.12). Variants (SNPs and INDELs) were called using bcftools 'mpileup' and 'call' (v1.12)[23], and the resulting VCF files were indexed with tabix (v0.2.6). SNPs and INDELs were further separated and filtered in VCFtools (v0.1.14) based on minor allele frequency (MAF) (--maf 0.05 --max-maf 0.9). Multiallelic SNPs were removed using PLINK2 (v2.00a2lm)[24]. SNPs with > 2% missing data were excluded, and remaining missing genotypes were imputed using a k-nearest neighbour approach.

Genomic prediction analysis

A double kernel model, combining genomic data and leaf-derived secondary metabolites, each represented by its own kernel (similarity matrix), was fitted to predict the primary fruit traits:

$$y = 1\mu + Z_1 g_1 + Z_2 g_2 + \varepsilon$$

where, y is an $n \times 1$ vector containing the adjusted means (best linear unbiased estimates; BLUEs) of n seedlings for each trait; $g_1 \sim \mathcal{N}\left(0, K_1\sigma_g^2\right)$ represents the genomic effects, $g_2 \sim \mathcal{N}(0, K_2\sigma_s^2)$ represents the effects associated with leaf-derived secondary metabolites, $\varepsilon \sim \mathcal{N}(0, I\sigma_e^2)$ is the residual error, K_1 = genomic relationship matrix (GRM) built from genome-wide SNP markers, K_2 = metabolomic relationship matrix (MRM) built from leaf-derived secondary metabolites. The variance components σ_g^2 and σ_s^2 represent the proportions of variance explained by the genomic and metabolomic kernels, respectively. The residual variance (σ_e^2) was calculated by subtracting σ_g^2 and σ_s^2 from the total phenotypic variance. Z_1 and Z_2 are design matrices for the genomic and metabolomic kernels, respectively. The GRM was calculated following the method of VanRaden[25], and the metabolite relationship matrix (MRM) was obtained using the following steps: Leaf metabolite data were first standardised to zero mean and unit variance. The MRM was then constructed as the cross-product of the scaled metabolite matrix and subsequently normalised by the average diagonal value to obtain a covariance matrix describing pairwise phenomic similarity among individuals[9]. This two-kernel best linear unbiased predictor (GBLUP) model, in which GRM and MRM were fitted as independent random effects, was implemented using the 'BGLR' package[26]. Single-kernel models fitting either the GRM or MRM as a random effect were also evaluated for comparisons. The performance of single-kernel and multi-kernel models was assessed using five-fold cross-validation where 80% of the data were used to train the models and the remaining 20% for testing. Prediction accuracy was obtained as the Pearson correlation coefficient between the adjusted phenotypes and the predicted breeding values.

Results

Phenotypic variation of leaf metabolites and fruit traits

Twenty-four targeted compounds (21 phenolic compounds and 3 triterpenoids) commonly found in apple leaves were quantified. Additionally, AA-2βG was analysed in both leaf and fruit samples. The range in the concentration of leaf metabolites is presented in Table 1. Among the chalcone group of compounds, the average concentration of phloridzin was the highest (28,161.7 μg/g), followed by trilobatin (6,333.7 μg/g). Chlorogenic acid was the most dominant cinnamic acid with an average concentration of 1,625.7 μg/g, and epicatechin was present at the highest average concentration (586.3 μg/g) in the group of flavanol compounds (Table 1). The average concentration of AA-2βG in leaves was 64.8 μg/g, with the highest value being 256.5 μg/g. The trilobatin content in fruit was much lower (4.2 μg/g) than in the leaf, whereas the AA-2βG content in fruit

Table 1. Distribution of the content (μg/g) of leaf metabolites. Q1_25% and Q3_75% represent the 25th and 75th percentile, respectively.

Metabolite	Group	Code	Q1_25%	Mean	Q3_75%	Maximum
3-hydroxyphloridzin	Chalcone	3-OHPhlz	1,709.2	3,359.5	5,036.9	14,622.6
Phloretin	Chalcone	Phlt	957.4	4,707.8	6,090.0	47,272.8
Phloridzin	Chalcone	Phlz	17,787.3	28,161.7	38,830.3	61,781.4
Sieboldin	Chalcone	Sb	0.0	3,174.5	1,295.1	40,078.7
Trilobatin	Chalcone	Tb	0.0	6,333.7	12,339.0	42,781.9
Phloretin-2'-O-xyloglucoside	Chalcone	xyloPhlz	136.5	493.4	615.0	3,708.7
Chlorogenic acid	Cinnamic acid	CGA	498.9	1,625.7	2,131.6	9,954.7
Neochlorogenic acid	Cinnamic acid	neoCGA	0.0	21.2	24.2	341.5
Trans-4-p-coumaroyl quinic acid	Cinnamic acid	t4pCouQA	23.5	268.7	430.0	1,793.8
Trans-5-p-coumaroyl quinic acid	Cinnamic acid	t5pCouQA	159.8	382.1	466.5	2,503.6
Epicatechin	Flavanol	epiCat	64.1	586.3	727.3	11,840.9
Procyanidin B1	Flavanol	PCB1	0.0	7.5	11.4	107.6
Procyanidin B2	Flavanol	PCB2	25.8	244.3	283.1	5,902.3
Procyanidin B5	Flavanol	PCB5	8.9	73.0	87.9	1,778.6
Procyanidin B7	Flavanol	PCB7	0.0	13.9	19.1	291.1
Quercetin 3-arabinopyranoside	Flavonol	Qarapy	1,145.5	1,817.6	2,286.6	5,133.7
Quercetin 3-galactoside	Flavonol	Qgal	2,322.7	3,919.9	5,350.0	11,555.3
Quercetin 3-glucoside	Flavonol	Qglu	1,842.3	3,558.6	4,822.7	11,907.1
Quercetin 3-rhamnoside	Flavonol	Qrha	3,246.0	5,595.0	7,497.8	24,610.3
Quercetin 3-rutinoside	Flavonol	Qrut	215.1	567.6	564.6	4,223.6
Quercetin 3-xyloside	Flavonol	Qxyl	512.8	871.6	1,148.7	2,678.3
Ascorbyl 2-beta-glucoside	Glycoside	AA-2βG	40.8	64.8	79.8	256.5
Annurcoic acid	Triterpenoid	AnnA	363.0	1,015.5	1,273.4	12,873.8
Betulinic acid	Triterpenoid	BA	16.3	33.6	47.7	105.6
Ursolic acid	Triterpenoid	UA	274.1	518.4	739.6	1,102.2

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(122.7 μg/g) was almost double that in the leaf (Table 2). Compared to other sensory traits, the average astringency (ASTR) score was the lowest, while average sourness was the highest (3.8) (Table 2).

Estimated heritability and genetic correlations

Estimated heritability (h^2) of leaf metabolites suggested that most of these traits have moderate-to-high heritability (Fig. 1). Phloretin-2'-O-xyloglucoside (xyloPhlz) and trans-5-p-coumaryl quinic acid (t5pCouQA) were the least heritable ($h^2 < 0.25$) traits, while the flavonol group of compounds (particularly the quercetins), along with phloridzin (Phlz) and ursolic acid (UA), were among the highly heritable ($h^2 > 0.75$) traits. Heritability of leaf and fruit AA-2βG was very similar (≈ 0.45), but trilobatin (Tb) was found to be more heritable in leaves (0.54) than in fruit (0.13) (Fig. 2). Fruit size and overall eating quality (EQUAL) were highly heritable (≈ 0.80), and estimated h^2 for SWEET, ASTR, and SOUR varied between 0.40 and 0.60 (Fig. 2). Fruit quality traits showed high correlations (> 0.70) between years, and relatively consistent heritability estimates were also obtained for most traits, indicating good phenotypic stability across seasons (Supplementary Table S1).

Estimated genetic correlations between leaf metabolites and fruit traits are shown in Fig. 3. In general, fruit traits such as SIZE, SWEET, and EQUAL were negatively correlated with leaf metabolites, whereas SOUR, ASTR, Tb, and AA-2βG showed positive relationships with leaf metabolites. However, converse relationships with these fruit traits were found for two cinnamic acid compounds (t4pCouQA, t5pCouQA) (Fig. 3). Genetic correlations between leaf and fruit samples for AA-2βG and Tb were 0.70 and 0.81, respectively. The flavanol group of leaf metabolites generally displayed stronger correlations with fruit traits than flavonols. Among fruit sensory traits, SWEET and ASTR showed the highest (|−0.63|) and lowest (0.54) average correlation magnitudes with leaf metabolites, respectively. Overall, all leaf metabolites except quercetin 3-rutinoside were highly significantly correlated with fruit traits, providing biological support for leaf metabolite-based PS.

Population structure

Following quality control measures, approximately 186,000 SNPs were retained, and the imputation accuracy reached approximately 90%. Principal component analysis (PCA) of the SNP data matrix was conducted to evaluate clustering patterns among all seedlings (Fig. 4a). A plot of the first two principal components (PC1 and PC2) grouped seedlings largely according to their familial relationships. Some seedlings did not cluster within their pedigree-assigned full-sib family groupings, which could suggest pollen contamination and/or mislabelling (Fig. 4a). Seedlings from different families sharing a common parent clustered more closely than those from unrelated families (e.g. 'Scifresh' × MS). Clustering patterns based on leaf metabolites (Fig. 4b) displayed similar trends to those observed in the SNP-based clustering (Fig. 4a). A group of seedlings from the 'Scifresh' × MT family formed a separate cluster away from their respective full-sibs (Fig. 4a, b).

Comparison between phenomic selection and genomic selection

The proportion of phenotypic variance in fruit traits explained by the GRM kernel varied between 25% (SIZE) and 45% (AA-2βG) (Fig. 5). The MRM kernel explained nearly twice as much variance as the GRM kernel for SIZE and Tb content. For SOUR and AA-2βG, the GRM kernel explained slightly more variance than the MRM. A highly significant correlation ($r = 0.76$, $p < 0.001$) was observed between the GRM and MRM. The variance explained by both kernels together varied between 0.62 (ASTR) and 0.83 (Tb) (Fig. 5).

The prediction accuracy of the GRM-based single-kernel model varied between 0.41 (ASTR) and 0.89 (SIZE) (Fig. 6). Prediction

Table 2. Distribution of the content of fruit-derived traits. Q1_25% and Q3_75% represent the 25th and 75th percentile, respectively.

Trait (unit)	Code	Q1_25%	Mean	Q3_75%	Maximum
Ascorbyl 2-beta-glucoside (μg/g)	AA-2βG	0.7	122.7	187.2	1,317.5
Trilobatin (μg/g)	Tb	0.0	4.2	2.3	107.2
Size (1–9 scale)	SIZE	1.0	4.0	6.0	9.0
Sweetness (0–9 scale)	SWEET	1.0	2.7	4.0	6.0
Sourness (0–9 scale)	SOUR	2.6	3.8	5.0	7.0
Astringency (0–9 scale)	ASTR	0.0	1.0	1.5	7.0
Eating quality (0–9 scale)	EQUAL	2.0	2.7	3.7	5.3

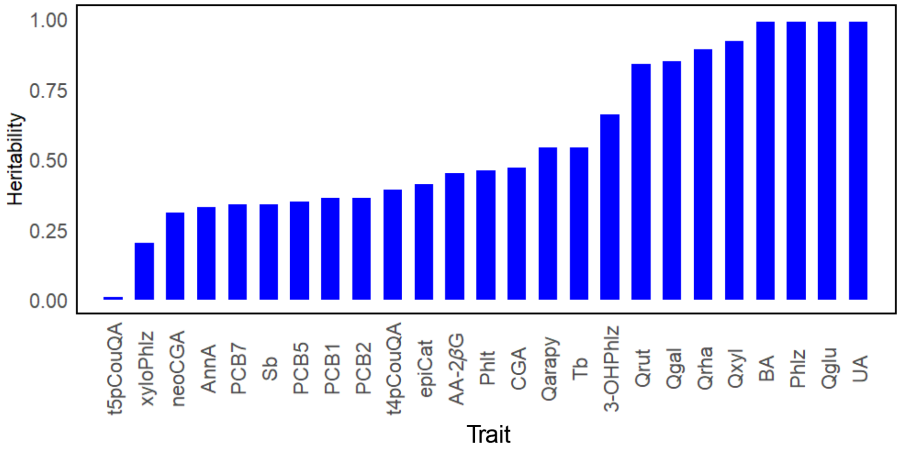

Fig. 1 Estimated heritability of leaf metabolites in apple seedlings. Refer to Table 1 for metabolite abbreviations.

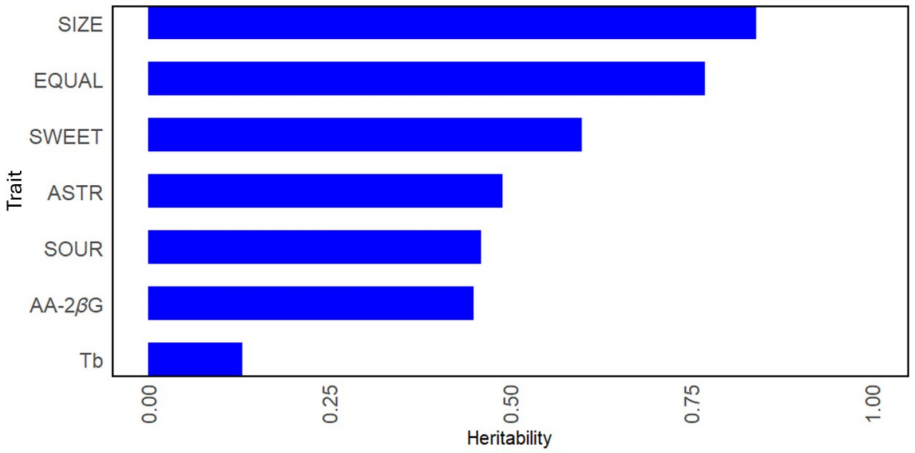

Fig. 2 Heritability of the apple fruit traits (SIZE: fruit size, EQUAL: overall eating quality, SWEET: sweetness, ASTR: astringency, SOUR: sourness, AA-2βG: ascorbyl 2-beta-glucoside, Tb: trilobatin).

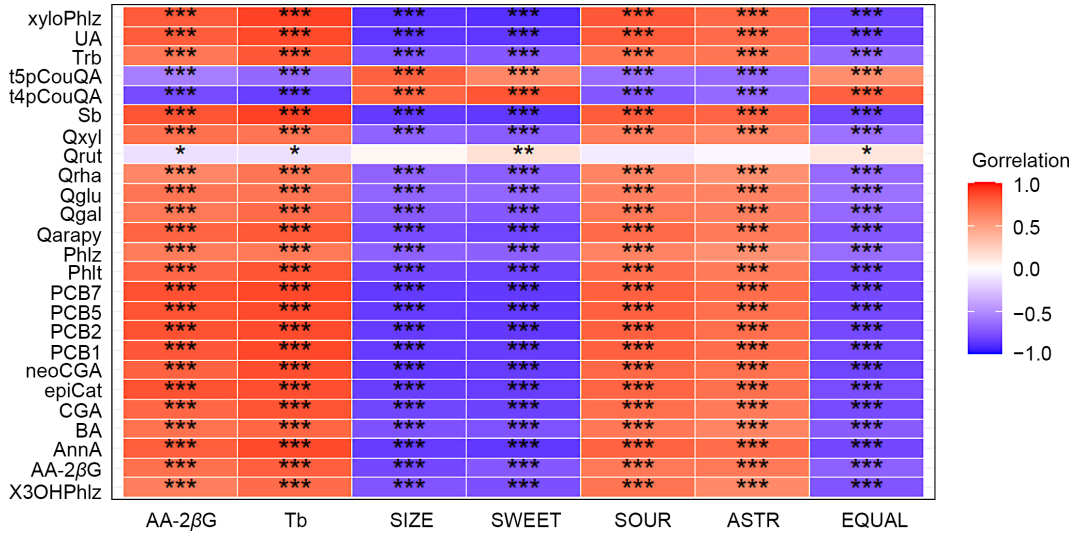

Fig. 3 Genetic correlation between apple leaf metabolites (*y*-axis) and apple fruit traits (*x*-axis). Significance levels of correlation coefficients: *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$. Refer to Table 1 for metabolite abbreviations.

accuracies of the GRM (0.67) and GRM + MRM (0.66) models were significantly ($p < 0.001$) higher than that of the MRM (0.53) model for AA-2βG. For Tb, the GRM + MRM model provided significantly higher accuracy (0.63) than the GRM model (0.49). For all other fruit traits, the double-kernel model resulted in insignificant improvement in prediction accuracy compared with the single-kernel models. Overall, the across-trait average prediction accuracies of the GRM + MRM, GRM, and MRM models were 0.65, 0.64, and 0.62, respectively (Fig. 6). The genetic correlation (Fig. 3) between leaf and fruit content of Tb was higher than that for AA-2βG (0.81 vs. 0.72), which may explain the slightly higher prediction accuracy of the MRM model for Tb. A high correlation (0.94) was observed between prediction accuracies of the GRM and MRM kernel models for different traits.

Discussion

Genomic prediction in *M. domestica* has been actively applied to predict breeding values for quantitative traits by fitting genome-wide SNP marker data to statistical models such as genomic best linear unbiased prediction (G-BLUP), enabling breeders to select promising genotypes early in the breeding cycle[19]. Recent studies using high-density SNP arrays and diverse training populations have demonstrated moderate to high predictive ability for many traits, including harvest date, fruit quality, and growth characteristics, with prediction accuracy strongly influenced by training population size, relatedness to selection candidates, marker density, and trait heritability[12].

Due to the advent of DNA sequencing technology, genotyping costs have decreased drastically during the past decades, but they can still be prohibitive when thousands of seedlings must be genotyped. Rincent et al.[8] proposed replacing genomic markers with NIRS measured on plant tissues, as the relationship matrix based on NIRS is expected to share similarities with the GRM because a reflectance spectrum is determined by the metabolite composition of the analysed tissues[9]. Based on a significant positive correlation between the predictive ability of genomic and phenomic predictions, it was hypothesised that PS is driven by similar factors to GS[9]. Practical considerations such as the age of measurement, the biological relevance of the secondary trait to the primary target, cost of phenotyping, and the risk of model overfitting have been identified as key factors determining whether a secondary trait is worth integrating into predictive-breeding pipelines[27].

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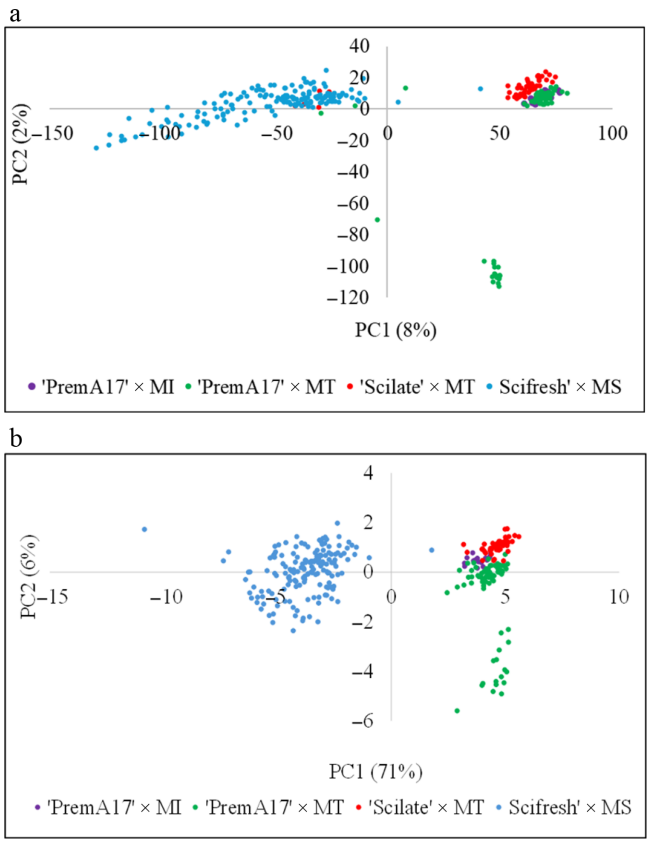

Fig. 4 Clustering pattern of apple seedlings from different families based on (a) SNP markers, and (b) leaf-derived secondary metabolites.

Using multispectral imaging of strawberry canopies, it was reported that PS outperformed GS for yield-related traits but was less effective for fruit quality traits[28]. Similarly, NIRS-based PS in apple showed a 0.35 decrease in average predictive ability across several fruit traits compared with SNP-based GS, suggesting that the tested PS approach was ineffective[12]. The premise of indirect selection assumes that secondary traits (e.g., NIRS, metabolites) are genetically correlated with the target traits. Except for dry matter content and SSC, NIRS measured on the fruit itself has been shown to be a less reliable predictor of several fruit traits[29]. Therefore, it is not surprising that the PS based on the leaf-derived NIRS was overall a poor predictor of apple fruit traits in an earlier study[12].

Multi-kernel models can potentially outperform single-kernel models, but the magnitude of improvement depends on the degree of independence between kernels. The superiority of multi-kernel models has been reported to vary across traits. A study in maize[30] reported that these models achieved high prediction accuracy for kernel weight, but not for grain yield. Similarly, a study in strawberry[28] showed that multi-kernel models performed better for yield-related traits. In our study, the average prediction accuracy (0.65) of the multi-kernel model (GRM + MRM) was similar to that of the GRM (0.64) and MRM (0.62) models (Fig. 6). This minimal improvement may be due to the lack of independence between the two kernels, as we observed a highly significant correlation ($r = 0.76$) between the GRM and MRM.

PCA plots based on leaf metabolites and SNP markers showed similar clustering patterns (Fig. 4), suggesting that leaf metabolite profiles of seedlings reflect both within- and between-family genetic structure. These observations were supported by the highly significant correlation between the MRM and GRM. *M. trilobata* leaves are distinctively three-lobed and maple-like, and a group of seedlings that inherited this leaf shape from the paternal parent in the 'Scifresh' × MT family clustered distinctly in both the SNP-based and metabolite-based PCA graphs, providing further evidence that a relationship matrix derived from leaf secondary metabolites may serve as a reliable proxy for the SNP-derived GRM.

Source–sink relationships play a fundamental role in fruit crops, as fruit development relies heavily on the translocation of metabolites and mineral elements, primarily from photosynthetic leaves[31]. The source–sink relationship of secondary metabolites (e.g., tannins, organic acids, phenolic acids, and flavonoids) between apple leaves and fruit may be governed by shared genetic pathways[32–34]. However, the accumulation of specific phenolic compounds differs between tissues according to their physiological functions. Leaves activate these pathways to produce defence-related compounds (e.g. phloridzin), whereas fruit regulate pathway activities in response to developmental cues and environmental factors such as light exposure, contributing to quality traits including skin pigmentation[35]. Among the various groups of polyphenols, procyanidins predominate in apple fruits, whereas dihydrochalcones (DHCs) are the major phenolic compounds in leaves[36]. Several genes, including phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS), play key roles in the phenylpropanoid and flavonoid biosynthetic pathways, leading to the production of numerous secondary metabolites in both apple leaves and fruit[35]. In addition, the *MdPGT1* gene has

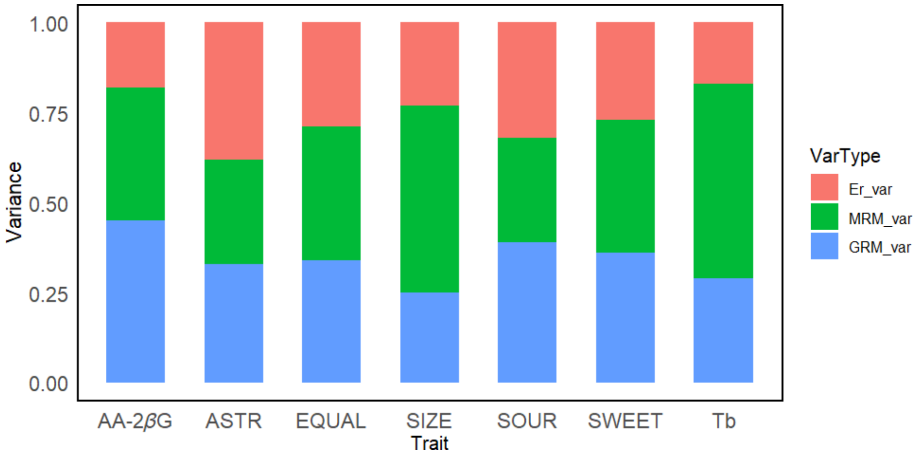

Fig. 5 Proportion of phenotypic variance in apple fruit traits captured by different kernels in the dual-kernel model analyses. (GRM: genomic relationship matrix, MRM: leaf metabolites relationship matrix, Er: error variance).

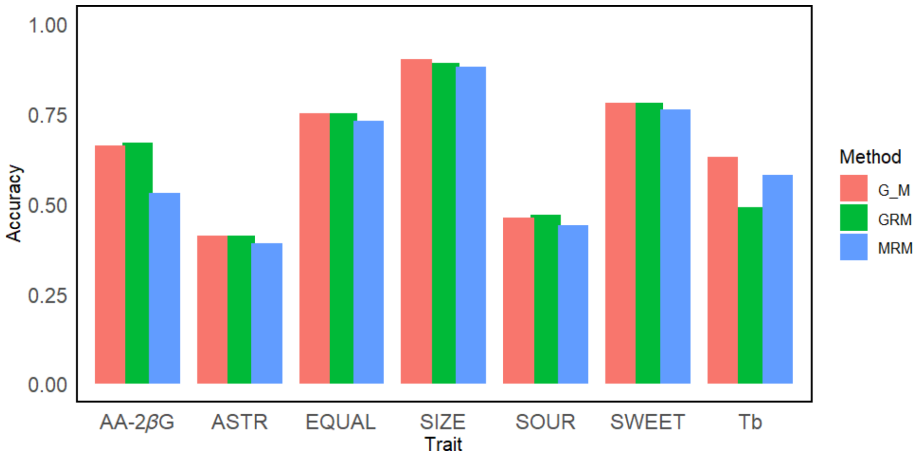

Fig. 6 Accuracy of genomic predictions in apple using models incorporating genomic relationship matrix (GRM), leaf metabolites relationship matrix (MRM), and GRM + MRM (G_M).

been shown to play a major role in DHC accumulation in both apple leaves and fruit[37].

The presence of shared regulatory mechanisms in leaves and fruit may partly explain why leaf metabolite profiles can serve as predictors of fruit quality traits, even when the metabolites themselves are not directly translocated from leaves to fruit. Some of the metabolites investigated in this study were found to be associated with fruit quality parameters[18] and domestication selection signals[38]. This may have influenced our results, where the across-trait average accuracy of PS was similar to that of GS (0.62 vs. 0.64), with a correlation of 0.94 between PS and GS accuracies. Studies in grain crops using leaf secondary metabolites have also shown that PS accuracy could reach levels similar to those of GS[15,17]. We measured only a small number (25) of targeted secondary leaf metabolites and postulate that measuring a larger number of metabolites in apple leaves may further improve the prediction accuracy of PS. Non-targeted analytical approaches could be used to increase the number of potential metabolites that provide good discrimination between seedlings and are amenable to high-throughput analysis[39].

The comparable prediction accuracies achieved using leaf metabolite-based PS underscore its value as a potential alternative to GS. Strong genetic correlations between leaf metabolites and fruit traits (Fig. 3) are one of the reasons behind the success of the proposed PS method. Given its lower cost and rapid data acquisition, PS offers a practical approach for accelerating plant breeding programmes[8]. For the development of the PS model in this study, leaf metabolite data were obtained 1 year prior to the acquisition

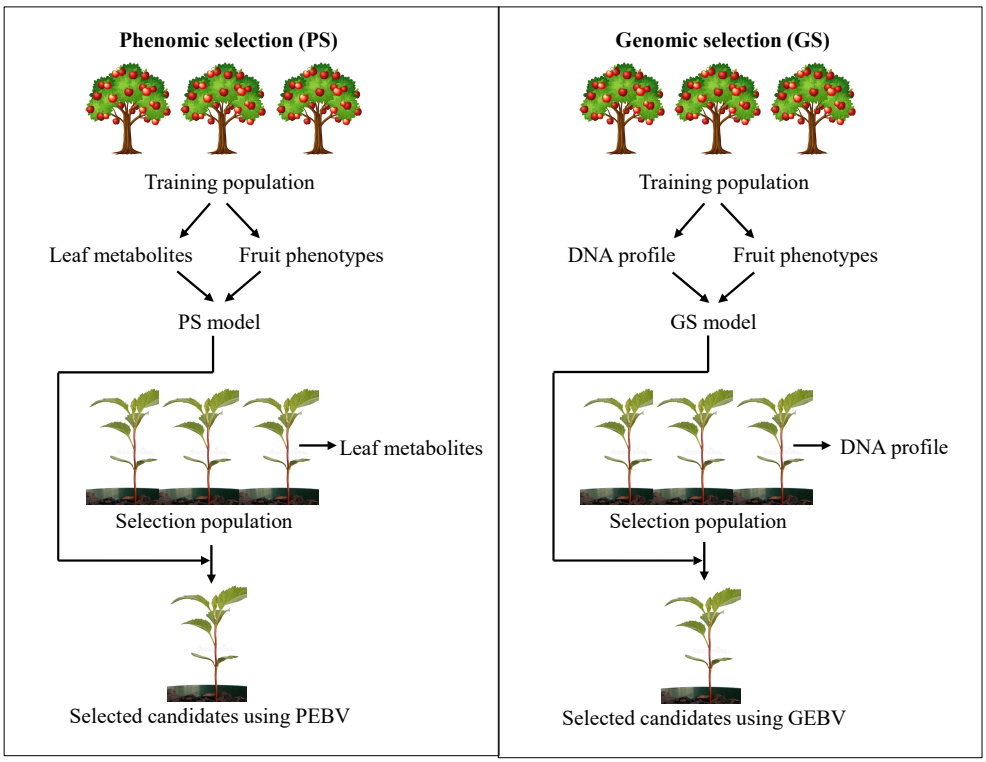

Fig. 7 Schematic presentation of the phenomic and genomic selection scheme for shortening the apple Stage 1 seedling evaluation cycle. GEBV: genomic estimated breeding value; PEBV: phenomic estimated breeding value.

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of fruit phenotypes, which was convenient for model development using existing training populations. For implementation of the models, the leaf metabolite data from selection populations would be obtained when seedlings are in the glasshouse or nursery. Conceptually, the proposed PS approach mirrors the GS approach (Fig. 7) and may help shorten the Stage 1 evaluation cycle in cultivar development programmes.

For the proposed PS model, the age at which leaf metabolites are measured will differ between the selection and training populations. Therefore, it is of interest to determine whether strong age–age genetic correlations exist for leaf metabolites. No significant seasonal variation in the content and distribution of metabolic compounds in apple leaves has been reported over 2 consecutive years[40], suggesting a strong age–age correlation. However, further studies are required to understand age-dependent metabolic profiles in the leaves of perennial fruit crops.

One possible limitation of the present study is the relatively limited population size and the use of targeted metabolites, which may not fully capture the diversity of leaf metabolites potentially associated with fruit quality traits. Future studies should therefore evaluate larger training populations, incorporate untargeted metabolomics approaches to expand metabolite coverage, and conduct multi-year and multi-location trials to assess the robustness and transferability of leaf metabolite-based PS models. Having successfully demonstrated the novel concept of leaf metabolite-based PS in fruit crops for the first time, we are now conducting further studies to strengthen these findings.

Author contributions

The authors confirm their contributions to the paper as follows: designed the project: Kumar S; coordinated field sampling and collected the lab data: Molloy C, Stoklosinski H, Hunt M; coordinated DNA extraction: Hilario E; conducted bioinformatics: Deng CH; analysed the data and prepared the first draft: Kumar S. All authors reviewed the results and approved the final version of the manuscript.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

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Conflict of interest

The authors declare that they have no conflict of interest.

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