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Pedigree-Based QTL Mapping Identifies Major Loci Associated to Fruit Sugar and Organic Acid Content in Apple

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Abstract

Fruit taste in apple (*Malus* × *domestica*) is one of the most important quality attributes and it is primarily determined by the balance between soluble sugars (mainly glucose, fructose, and sucrose) and organic acid. The combination of these metabolites influences the consumer preference by impacting sweetness and acidity, and therefore represent the central target of several apple breeding programs. To investigate the genetic control of taste-related traits, a pedigree-based QTL (Quantitative Trait Loci) analysis (PBA) across six full-sub apple families was performed to identify major QTL-associated traits in elite accessions. Bayesian statistics and Markov Chain Monte Carlo (MCMC) methods revealed the presence of a common QTL for glucose and fructose on linkage group (LG) 16 as well as sugar-specific intervals mapped on LG1 for fructose and LG12 for glucose. Furthermore, phenotypic sugars variation enabled also the identification of a QTL on LG4 for sucrose, while a QTL for malic acid content was detected on LG8. The LG16 was consistent between QTL-estimated genotypes, supporting its potential for marker-assisted selection. Our findings identified the genetic basis of apple taste traits, which can contribute to the development of improved apple cultivars with optimized sweetness and acidity balance and ultimately improved apple consumer appeal.

Keywords: apple; fruit quality; soluble sugars; malic acid; PBA; breeding

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1. Introduction

Apple (*Malus* × *domestica*) is the third economically most important temperate fruit crop worldwide [1] and largely appreciated for its quality features, mainly represented by size, shape, firmness, color and flavor (sugars, organic acids and aroma) together with nutritional components (vitamin C, minerals, phenolic compounds) [2]. Fruit quality also drives market competitiveness [3], since it is prioritized by producers [4] and represent an important indicators to the consumers [5]. However, fruit edibility and taste depends primarily on the quantitative and qualitative balance between different sugars and organic acids, resulting in different level of fruit sweetness and acidity [6,7]. Malic acid is the dominant organic acid in apple fruits and a key driver of acidity [8], while fructose, sucrose, glucose, and sorbitol are the main contributors to sweetness [7].

These primary metabolites are mainly synthesized via photosynthesis by the central carbon metabolism. During the Calvin cycle in the chloroplast, inorganic carbon is converted into glucose and fructose, with glucose 6-phosphate being the precursor for sorbitol biosynthesis [9]. Glucose and fructose are converted into sucrose, which together with sorbitol constitute the main carbohydrates translocated through the plant vascular system [10]. These sugars support the metabolism by providing energy in the form of adenosine triphosphate (ATP), while the sugar fraction not metabolized in the mitochondria is stored in the vacuole contributing to sweetness [11]. The tricarboxylic acid cycle generates also organic acids, with malic acid being the predominant compound in apple [12]. Because of the importance of these primary metabolites in the determination of fruit taste, sugars and acidity normally rank as top targets in breeding programs oriented to select for high flavored and consumers-appealing varieties in apple [13–15].

Historically, genetic studies of fruit acidity and sweetness in apple have relied on a diverse type of genetic materials, including crop wild relatives and elite cultivars with extreme and contrasting phenotypes [13,16,17]. Although these approaches gained knowledge about major genes involved in these metabolic processes, many of the resulting segregating material were unsuitable for commercial breeding programs because of the undesirable horticultural traits introgression [17]. In this context, molecular markers offered a valuable tool for DNA-informed selection and several studies have already identified major loci potentially associated to sugar and organic acid content [18–21]. These studies were carried out employing a diverse type of materials, such as bi-parental families [18–20,22] or cultivar collections [13,23,24]. Classical breeding in apple is still a resource-intensive and long process, due to seedling juvenility, high heterozygosity, and complex genetic control of targeted traits [25,26]. Therefore, the understanding of content variability of these metabolites in apple germplasm, that is directly relevant to breeding programs, is crucial for translating genetic knowledge into practical breeding applications for the improvement of the final fruit quality [8,13,17]. In this context, Pedigree-based QTL analysis (PBA) offers a powerful framework to analyze several inter-connected families sharing a common pedigree scheme, allowing detection of QTL segregating between different parents [27]. In apple breeding programs, many cultivars share similar parentage and founder relationships. Therefore, in pedigreed-structured genetic schemes, the PBA approach can be used to identify QTLs by capturing the diversity and recombination present in a multi-parental based breeding germplasm. Unlike traditional biparental QTL mapping, this approach increases the relevance of the detected alleles, improves QTL resolution, and allows the estimation of allele effects from diverse parents [28]. The capacity of PBA to detect genetic loci with stable effects across pedigrees makes it particularly suitable for the genetic differentiation of sweetness traits across different crops [29,30]. Prior studies using PBA have validated this methodology demonstrating its potential for marker-assisted breeding for apple fruit firmness [30–32], pear (*Pyrus communis*) fruit firmness [33] but also for individual fruit sugars and soluble solids in sweet cherry (*Prunus avium*) [34,35], and peach (*Prunus persica*) [36,37].

In the present study, we investigated the genetic control of fruit taste in apples using a PBA on a multi-parental cross design scheme sharing a common pedigree composed by six high-quality cultivars. The aim of this work is to analyze the genetic architecture underlying fruit sweetness and acidity, providing a genetic roadmap to guide marker assisted selection in pedigree programs oriented to improve apple quality taste profiles.

2. Materials and Methods

2.1. Plant Material

The plant material was represented by a pedigree-based scheme consisting of six bi-parental families generated by crossing six common parental cultivars [33]. The bi-parental families were planted in the experimental orchards of Laimburg Research Centre and Fondazione Edmund Mach, both located in the South Tyrol region of Italy. The families were defined as: FjDe = 'Deleary' × 'Fuji' (n = 69); GDFj = 'Golden Delicious' × 'Fuji' (n = 84); GaPL = 'Royal Gala' × 'Cripps Pink' (n = 32); GaPi = 'Royal Gala' × 'Pinova' (n = 43); FjPi = 'Fuji' × 'Pinova' (n = 93); FjPL = 'Cripps Pink' × 'Fuji' (n = 82). Each replicate was bearing fruit at the time of sampling. Plants were maintained according to standard agronomic practices commonly adopted in South Tyrol apple orchards, including fruit thinning, pest and disease control, and water supply management.

2.2. Fruit Quality Traits Phenotyping and Statistical Analysis

Around 10 to 12 apple fruits for each genotype were harvested for two years (2012 and 2014) at the time of horticultural maturity, assessed using standard maturity indices (penetrometer firmness, iodine-starch staining, and skin color change) [38,39]. After harvest, the apple fruits were placed in cold storage (2 °C, 95% relative humidity) for two months. Before phenotyping, fruit were equilibrated at room temperature (20 °C) overnight.

Relative trait variability was quantified using the squared coefficient of variation, calculated as the ratio of the phenotypic variance to the square of the trait mean ($CV^2 = \sigma^2/\mu^2$) to compare the variability across traits independently of scale units. Apple fruit taste traits (glucose, fructose, sucrose, and malic acid) were also evaluated using linear mixed models (LMM) implemented with the function 'lmer' from the lme4 R package (v.1.1-35.3) [40]. In the model, the genotype and family were treated as random factors, whereas year was included as a fixed effect. In this context, the family effect accounted for variation among the different crossing families, whereas the genotype effect accounted for variation among individual seedlings and repeated observations of the same genotypes across years. Variance components for genotype and genotype by family were extracted from the model output using 'VarCorr' from the lme4 R package to estimate their contribution to the total phenotypic variance. The variance associated with the fixed year effect was calculated from predicted values obtained after removing all random components from the model. Best linear unbiased predictors (BLUPs) for genotypic effects were obtained using the function 'ranef' from the lme4 R package to the LMM fit. Principal component analysis (PCA) was performed with the R package FactoMineR (v.2.12) [41]. Broad-sense heritability (H^2) was estimated for each metabolite (glucose, fructose, sucrose, and malic acid) as $H^2 = \frac{V_G}{V_G + V_F + V_R}$, where ($V_G$) is the variance among genotypes, (V_F) is the variance attributable to the family, and (V_R) is the residual variance. For consistency with the variance-decomposition analysis, H^2 was calculated using the same LMM and the same variance-component estimation as described before.

2.3. Extraction and Determination of Sugars and Organic Acids by Ion Chromatography (IC)

Primary metabolites (glucose, fructose, sucrose and malic acid) were quantified in apple fruit pulp by ion chromatography (IC). At each sampling date, six apple fruits were collected and used as replicates for analysis. To prepare the samples, an equatorial disc (1.5 cm thick) was taken from each of five peeled apples and immediately frozen in liquid nitrogen. Frozen discs were powdered and stored in glass vials at −80 °C until further analysis.

For extraction, 200 mg of frozen powder was suspended in 5 mL of aqueous ethanol (80% *v*/*v*) in a microcentrifuge tube. The mixtures were shaken in a Thermomixer for 15 min at 20 °C. Centrifugation followed at 10,000× g for 10 min. The supernatant was separated and on the solid part a second extraction with water-methanol was performed. Both organic phases were put together and the samples acids were diluted with MiliQ water to 50 mL (organic acids) and to 1:50 *v*/*v* (soluble sugars). Prior to injection samples were filtered with a 0.2 μm syringe filter.

The analysis of soluble sugars and organic acids content levels was carried out using high performance liquid chromatography (HPLC) performed with the instrument DionexTM ICS-5000 DP (Thermo Fisher Scientific, Waltham, MA, USA). Chromatography of soluble sugars (glucose, fructose and sucrose) was performed using a DionexCarboPac® PA1 capillary column (4 × 250 mm) (Thermo Fisher Scientific, Waltham, MA, USA), using isocratic conditions with a solution of NaOH 10 nM; while chromatography of malic acid was performed according to application note 273 of Thermo Scientic with some modifications.

2.4. SNPs Genotyping

Genotypic data used in this study were the same as used by [32]. Genomic DNA was extracted from young leaf tissue using the DNeasy Plant Kit (Qiagen, Venlo, The Netherlands). DNA concentration and purity were assessed using a Nanodrop ND-8000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Genotyping was performed with the Illumina 20 K Infinium SNP array [42]. Raw SNP calls were initially processed in GenomeStudio® 2.0 Data Analysis Software [43] and subsequently curated using ASSIsT [44].

2.5. Linkage Mapping and SNPs Marker Consensus Order

SNPs segregating in the six families were used as previously described by [32] to generate individual bi-parental maps with JoinMap 4 [45]. Markers were grouped at a minimum LOD of 3.0 and ordered using a recombination threshold of 0.45 with the Haldane function. The six maps were then combined into a consensus map using the ConsMap module of BioMarcator v4.2 [46].

2.6. QTL Mapping by Pedigree-Based Analysis (PBA)

A PBA detection was performed by using FlexQTL™ (https://github.com/senresearch/FlxQTL.jl), which implements a Bayesian modeling strategy to evaluate alternative QTL configurations across a pedigree structure [27,31]. Through MCMC sampling, the software explored the posterior distribution of QTL number, position, and effects to estimate the parameters derived analytically due to the uncertainty in founder allele phases and segregating QTL genotypes. For each analysis, MCMC chains were set to 500,000 cycles with 500 thinning using additive genetic models with normal prior distributions and convergence was verified through inspection of trace diagnostics. The effective chain size (ECS) was used to evaluate the sensitivity of posterior estimates with ECS values exceeding 100, considered to be indicative of robust inference for the phenotypic mean (μ), additive QTL variance ($\sigma 2_a$), residual variance ($\sigma 2_e$), and the prior expectation of QTL number (NQTL). FlexQTL™ indicates the level of evidence provided for the presence of a QTL as two times the natural logarithm of the Bayes factors (2lnBF) for an incremental number of QTLs per LG, through a pair-wise comparison. A 2lnBF value above 2 (2lnBF(1/0) ≥ 2) is considered to indicate positive evidence of a model and used to discriminate between alternative models containing different numbers of QTLs. The percentage of phenotypic variance explained (PVE) by each single identified QTL was calculated as the ratio between the additive variance of the trait and the total phenotypic variance,

which was calculated directly by the software FlexQTL™. Furthermore, this software also assumed a QTL bi-allelic additive architecture with three possible genotypic conformations: 'QQ' with value of 1, 'Qq' with value of 0, and 'qq' with value of –1, inferring QTL genotypes by tracking allele transmission from founders to descendants throughout the pedigree.

2.7. Candidate Gene Functional Annotation

For most probable QTL for each trait, the confidence interval was identified by the first and last focal points flanking the QTL peak, and therefore delimiting the genetic boundaries of the QTL region. To anchor these genetic intervals to the assembled-genome, the probe nucleotide sequences of the SNP markers corresponding to the first and last focal points were retrieved and aligned against the *Malus* × *domestica* 'Golden Delicious' doubled haploid genome assembly (GDT2T assembly; [47] available at the Genome Database for Rosaceae, https://www.rosaceae.org/). Sequence alignment was performed using BLAST to determine precise physical coordinates on the reference genome. Physical positions for all markers within each QTL interval were subsequently retrieved for each haplome based on their alignment coordinates. On the same reference genome was also carried out the QTL anchoring and the related candidate genes in-silico search. Similarly, candidate genes identified within each QTL region were functionally annotated by assigning them to Gene Ontology (GO) terms. GO annotations were retrieved using the 'GO.db' R package (v. 3.20.0) [48]. The GO terms associated with each candidate gene were extracted from the annotation table obtained by the GDR [49]. After extracting GO identifiers, candidate genes within each QTL were mapped to their corresponding ontology terms and descriptions, and their distribution within each QTL and trait was summarized.

3. Results

3.1. Fruit Sugars and Malic Acid Variation

Raw variance and CV^2 were used to assess trait variability in absolute and relative terms, respectively (Figure 1). Glucose exhibited a raw variance of 0.35 and a CV^2 of 0.17, representing 16.6% of the mean squared and indicating moderate variability across different fruit samples. Fructose showed the highest absolute variability, with a raw variance of 2.08, but a low CV^2 of 0.07 (6.9% of the mean squared). Sucrose showed both relatively high absolute and relative variability (raw variance = 1.61; CV^2 = 0.15, corresponding to 15.4% of the mean squared) and a greater flexibility in sucrose levels among samples. In contrast, malic acid was characterized by the lowest absolute variability (raw variance = 0.05) and the highest relative variability (CV^2 = 0.37, or 37% of the mean squared), indicating that a low mean concentration for which even small absolute changes represent a large proportion of the average level.

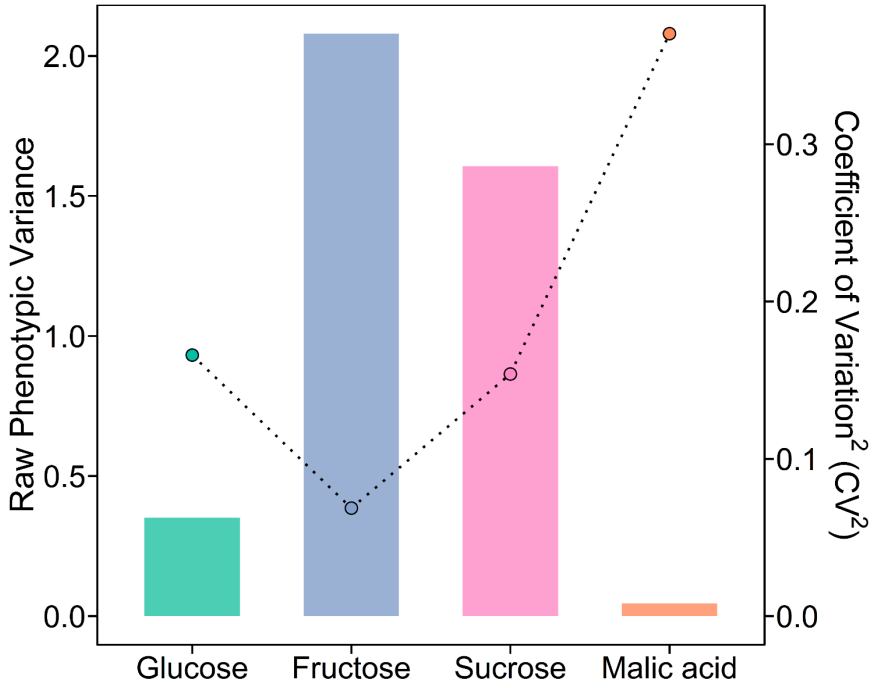

Figure 1. Apple fruit sugar composition and malic acid variance variation. Raw variance, shown by the columns, was used to account for the variability in the original measurement units, whereas squared coefficient of variation (CV^2), shown by the points, quantified variance relative to the trait mean for each trait: glucose (green colored), fructose (blue colored), sucrose (pink colored) and malic acid (orange colored).

3.2. Different Effects on Fruit Sugars, Malic Acid, and Phenotypic Variance Decomposition

PCA based on BLUPs was performed to investigate the multivariate relationships among the sugars and malic acid to explore potential clustering patterns among families (Figure 2A). The PCA represented by the first two PCs and explaining 70.28% of the total phenotypic variation (PC1: 43.1% and PC2: 27.18%) did not reveal any distinct separation by family (Figure 2A). The projection of variables evidenced that fructose and glucose showed a positive and significant ($r_p = 0.58$, $p < 0.001$) correlation, as also evidenced by the acute angles between their vectors' projection in the PCA. Similarly, fructose and sucrose exhibited a positive and significant correlation ($r_p = 0.37$, $p < 0.001$). In contrast, the correlation between sucrose and malic acid was not statistically significant ($r_p = 0.14$, $p > 0.05$).

To further explore the phenotypic variability of these traits, phenotypic variance was modelled using LMMs with the year as a fixed effect and genotype, family, and their interactions as random effects (Figure 2B). The proportion of variance attributed to genotype was highest for glucose (29.2%), followed closely by sucrose (28.3%). Glucose also exhibited the largest contribution of the family variance (41.2%), with substantial family effects also observed for fructose (25.0%) and sucrose (21.2%). In contrast, malic acid variance was dominated by year effects, which accounted for 50.1% of the total phenotypic variance. H^2 varied among the four metabolites, indicating differences in the proportion of phenotypic variation explained by genetic effects. Estimated H^2 values were 0.30 for glucose, 0.12 for fructose, 0.29 for sucrose, and 0.37 for malic acid (Figure 2B).

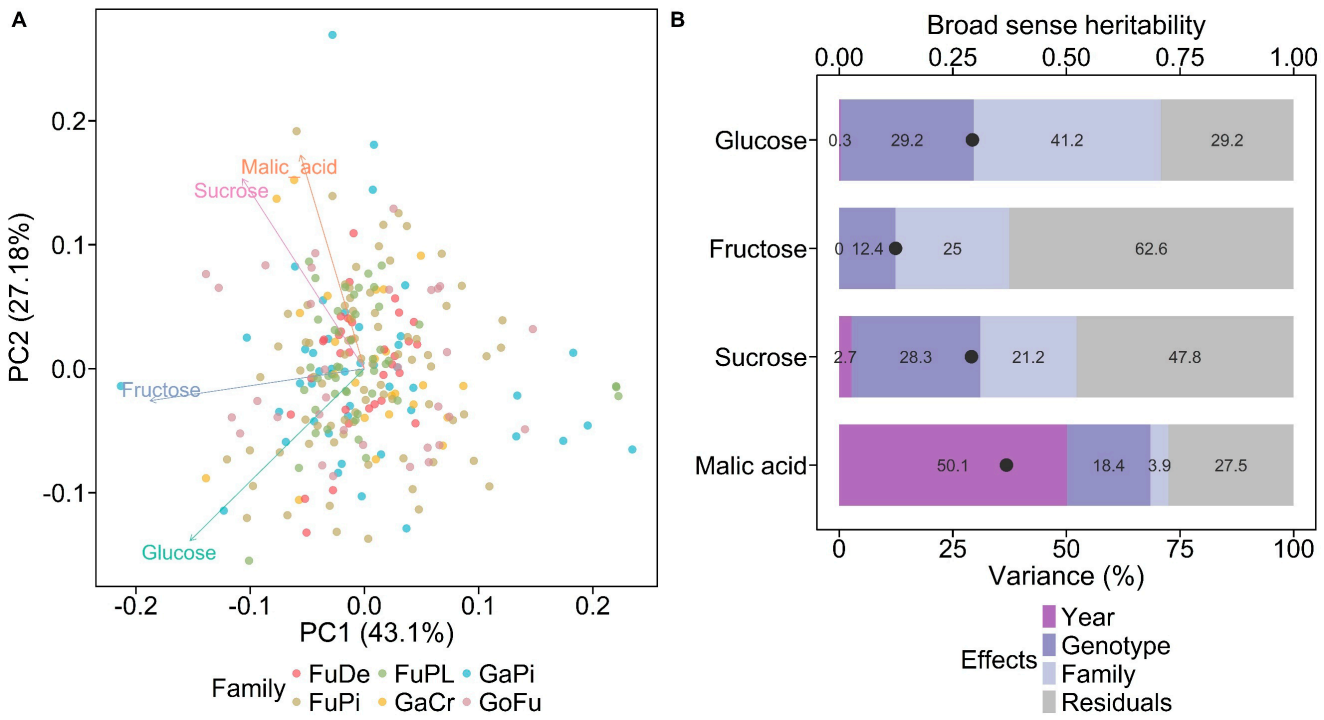

Figure 2. Different factors effects on fruit sugars, malic acid, and phenotypic variance decomposition for each trait. (**A**) Principal component analysis (PCA) biplot of the six families based on Best Linear Unbiased Predictors (BLUPs) for each trait: glucose (green colored), fructose (blue colored), sucrose (pink colored) and malic acid (orange colored). Plot of the first two principal components with their respective proportion of the total variance shown within brackets. The families defined as: FjDe = 'Deleary' × 'Fuji'; GDFj = 'Golden Delicious' × 'Fuji'; GaPL = 'Royal Gala' × 'Cripps Pink'; GaPi = 'Royal Gala' × 'Pinova'; FjPi = 'Fuji' × 'Pinova'; FjPL = 'Cripps Pink' × 'Fuji'. (**B**) Stacked bar plots showing the percentage of variance explained by the fixed effect of year and by the random effects (genotype, genotype by family interaction, and residuals), as calculated from the linear mixed model (LMM) fit. Black points indicate the estimated broad-sense heritability (H^2) for each trait.

3.3. Fruit Sugars and Malic Acid QTL Identification via PBA

The robustness of the model implemented in FlexQTL™ and the validity of the methodology applied was supported by the ECS, which exceeded the threshold value of 100 for all four traits analyzed (glucose, fructose, sucrose, and malic acid) indicating reliable posterior estimates and therefore minimizing possible errors (Table 1). In total, six QTLs were identified across five different LGs (1, 4, 8, 12, and 16; Table 1). Only loci showing evidence of association with $2\ln BF_{(1/0)}$ close to or greater than 2 were retained for further consideration (Table 1).

Table 1. QTL parameters for each fruit quality trait (glucose, fructose, sucrose and malic acid). For each fruit quality phenotypic trait glucose, fructose, sucrose and malic acid are reported: the linkage group on which the QTL was detected (LG), the length of the peak (Length), the interval mode peak (Mode), the posterior QTL probability (Prob), the variance expressed in percentage (PVE), and Bayes factor ($BF_{(1/0)}$) for the presence of one QTL.

Trait	LG	Length	Mode	Prob	PVE	$BF_{(1/0)}$
Glucose	12	26	6	0.497	7.35	2.7
Glucose	16	38	24	0.619	7.35	2.9
Fructose	1	48	28	0.341	4.29	1.1
Fructose	16	49	27	0.47	5.71	1.9
Sucrose	4	32	56	0.36	7.23	1.3

Malic acid	8	46	26	1.096	0	8.4

Across the genomic regions identified, a major QTL was detected for each trait, although their effect sizes and statistics varied among them (Table 1; Figure 3). For glucose, two QTLs were mapped on LG12 and LG16. The locus on LG12 showed a QTL intensity of 0.50, explaining 7.35% of the phenotypic variance, with a $2lnBF_{(1/0)}$ of 2.7. The QTL on LG16 showed a higher intensity (0.62), a similar proportion of explained variance (7.35%), and a slightly stronger Bayesian evidence ($2lnBF_{(1/0)}$ = 2.9). In contrast, QTLs associated with fructose and sucrose exhibited lower levels of Bayesian evidence. For fructose, two loci were detected: one on LG1, with a QTL intensity of 0.34, accounting for 4.29% of the phenotypic variance ($2lnBF_{(1/0)}$ = 1.1), and another on LG16, with an intensity of 0.47, explaining 5.71% of the variance and close to the 2lnBF threshold ($2lnBF_{(1/0)}$ = 1.9). For sucrose, a single QTL was identified on LG4, characterized by a QTL intensity of 0.36, a phenotypic variance explained of 7.23%, and a $2lnBF_{(1/0)}$ of 1.3. A stronger signal was observed for malic acid, where a major QTL mapped on LG8 showed high values for QTL intensity (Probability=1.1) and Bayes factor ($2lnBF_{(1/0)}$ = 8.4) (Table 1; Figure 3).

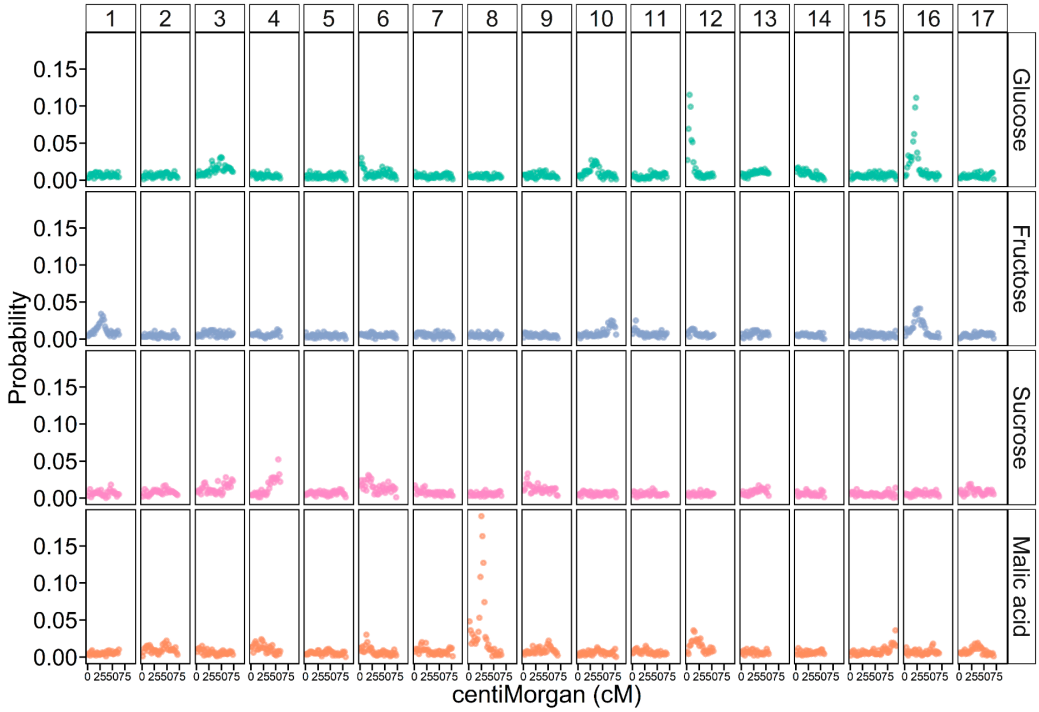

Figure 3. Apple fruit sugars and malic acid QTL identification via PBA. Manhattan plots are shown for each trait: glucose (green), fructose (blue), sucrose (pink), and malic acid (orange). The x-axis represents the genetic position of the center of each bin in centimorgan (cM), while the y-axis shows the probability, expressed as the number of times a 2-cM bin was included in a QTL model across the sampled simulations. Each chromosome is indicated with numbers on the top.

3.4. Estimation of the QTL Genotype

FlexQTL™ enabled the estimation of the QTL genotypes based on a bi-allelic model. Phenotypic performance for glucose, fructose, sucrose, and malic acid varied among the parental genotypes at the identified QTL (Figure 4). For glucose, QTLs located on LG12 and LG16 corresponded to differences across the six parental cultivars (Figure 4A). Parents carried either the 'QQ' or 'Qq' genotype alleles at these loci. 'Royal Gala' was homozygous ('QQ') at both LG12 and LG16 QTL, whereas 'Fuji' showed a heterozygous QTL genotypic allelic state ('Qq') for the LG12 and a homozygous state ('QQ') for LG16 (Figure 4A). For fructose, two QTLs were identified and mapped on LG1 and LG16. For the QTL

on LG1, a full spectrum of QTL genotype allelic conformation was estimated for three cultivars: 'Royal Gala' ('qq'), 'Pinova' ('Qq') and 'Cripps Pink' ('QQ'), while for 'Golden Delicious', 'Fuji' and 'Delearly' any specific QTL genotypic conformation was estimated. A complete structure was instead computed for the QTL on LG16, where for 'Royal Gala', 'Fuji' and 'Delearly' a QTL homozygous genotype-'QQ' was estimated, while for 'Pinova' and 'Golden Delicious' a heterozygous conformation ('Qq') was defined. Conversely for 'Cripps Pink', a homozygous genotypic conformation for the q-allele was estimated ('qq') (Figure 4B). For sucrose, most of the parental cultivar exhibited either 'qq' or 'Qq' genotypes allele at the identified loci (Figure 4C). In contrast, malic acid variation was associated with the QTL on LG8, where 'Fuji' was the only cultivar distinguished by a 'QQ' genotype. 'Golden Delicious' and 'Royal Gala' were therefore characterized by a homozygous QTL genotype for the allele-q ('qq'), while 'Pinova' and 'Cripps Pink' were distinguished by a heterozygous allelic form ('Qq').

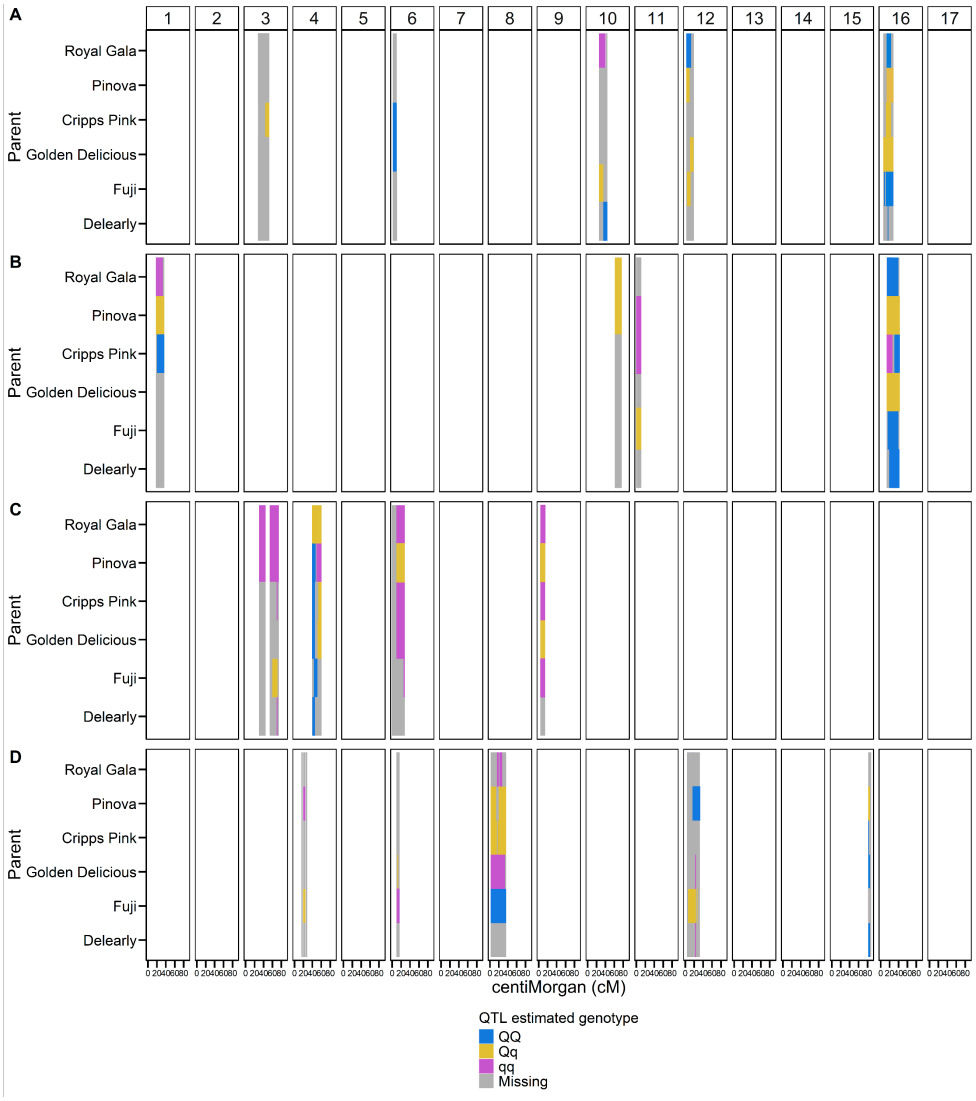

Figure 4. Posterior estimate of the QTL genotype probability of the parent cultivars. Tiles represent the estimated QTL genotypes: 'QQ' (blue), 'Qq' (yellow), 'qq' (purple), and missing (grey). Chromosomes are indicated by numbers on the top, while the y-axis shows the probability, expressed as the number of times a 2-centimorgan (cM), bin was included in a QTL model across the sampled simulations. Each row corresponds to one of the six parental cultivars: 'Golden Delicious', 'Fuji', 'Royal Gala', 'Cripps Pink', 'Pinova', and 'Delearly'. The several panels indicate the primary metabolites investigated, in particular (**A**): glucose, (**B**): fructose, (**C**): sucrose and (**D**): malic acid.

3.5. Functional Annotation of the Candidate Genes in the Identified QTL

Within QTL intervals a total of 172 potential candidate genes were founded (Table S1). A large proportion of the identified genes within the QTL regions were associated with ATP-related functions, including ATP binding, ATP hydrolysis, ATPase activity, and ATP-dependent transport processes (128 genes). For glucose-associated QTLs, 22 genes were identified across both haplomes. Out of these, 15 were assigned to the GO functional category of Biological Process, while 7 to Molecular Function. The largest number of these genes corresponded to carbohydrate metabolic processes, photosynthesis and sugar-binding activity (Table 2). Fructose-associated QTL on LG16 contained 12 genes, of which 10 were assigned to the GO functional category of Biological Process, and only 2 to Molecular Function and 20 ATP related-genes. A substantial proportion of these annotated genes corresponding to carbohydrate metabolism and enzymatic activities are involved in sugar interconversion (Table 2). In this QTL, genes encoding fructose-bisphosphate aldolase activity and glucose-6-phosphate dehydrogenase activity were also detected within the intervals with potential regulatory control of the carbon cycle (Table 2). Sucrose-associated QTL on LG4 included 10 candidate genes belong to two GO functional categories: Biological Process (8 genes) and Molecular Function (2 genes). These genes were related to carbohydrate metabolism, photosynthetic pathways, and transmembrane transport. The presence of genes annotated as carbohydrate transporters and proton-transporting ATPase components supported this possible involvement in sucrose translocation and compartmentalization (Table 2, Table S1). For malic acid, 3 genes assigned to the three GO functional categories were identified within the LG8 QTL interval and were predominantly annotated with tricarboxylic acid (TCA) cycle functions and oxidoreductase activity, including components associated with respiratory chain complexes II (Table 2, Table S1).

Table 2. Candidate genes functional annotation within the identified QTL associated with each fruit quality trait. The table shows for each trait (glucose, fructose, sucrose and malic acid) the chromosome, the haplome, the gene identifier according to the *Malus* × *domestica* 'Golden Delicious' GDT2T genome assembly, physical start and end positions (bp) of each gene, Gene Ontology (GO) category, and the corresponding functional annotation term. Physical coordinates refer to the GDT2T genome assembly and are given in base pairs. Candidate genes were identified based on their physiologically and biologically linked roles for each trait.

Trait	Chromosome	Haplome	Gene Name	Gene Start Position	Gene End Position	Gene Ontology (GO)	Gene Functional Annotated Term
Glucose	12	1	GDT2T_hap112g00399	5146208	5146342	Biological Processes	photosynthesis
Glucose	12	1	GDT2T_hap112g00479	6208293	6211278	Molecular Function	carbohydrate binding
Glucose	16	1	GDT2T_hap116g00514	4331388	4335235	Molecular Function	asparagine synthase (glutamine-hydrolyzing) activity
Glucose	16	1	GDT2T_hap116g00549	4520886	4526041	Biological Processes	carbohydrate metabolic process
Glucose	16	1	GDT2T_hap116g00624	4954332	4959243	Molecular Function	carbohydrate binding

Glucose	16	1	GDT2T_hap116g00780	6244136	6245669	Biological Processes	carbohydrate metabolic process
Glucose	16	1	GDT2T_hap116g00803	6436556	6437857	Biological Processes	carbohydrate metabolic process
Glucose	16	1	GDT2T_hap116g00805	6443046	6462259	Biological Processes	carbohydrate metabolic process
Glucose	16	1	GDT2T_hap116g00839	6739858	6742500	Biological Processes	carbohydrate metabolic process
Glucose	16	1	GDT2T_hap116g00851	6827188	6831177	Biological Processes	carbohydrate metabolic process
Fructose	16	1	GDT2T_hap116g00816	6523820	6528762	Biological Processes	carbohydrate metabolic process
Fructose	16	1	GDT2T_hap116g00817	6537462	6540193	Biological Processes	carbohydrate metabolic process
Fructose	16	1	GDT2T_hap116g00832	6661993	6662217	Molecular Function	fructose-bisphosphate aldolase activity
Fructose	16	1	GDT2T_hap116g00852	6833603	6837934	Biological Processes	carbohydrate metabolic process
Fructose	16	1	GDT2T_hap116g00911	7405394	7406305	Biological Processes	photosynthesis
Fructose	16	1	GDT2T_hap116g00951	7735346	7738461	Biological Processes	glucose metabolic process
Sucrose	4	1	GDT2T_hap104g01679	27173216	27176775	Biological Processes	carbohydrate metabolic process
Sucrose	4	1	GDT2T_hap104g02128	31046707	31051693	Biological Processes	carbohydrate metabolic process
Sucrose	4	1	GDT2T_hap104g02131	31081254	31083125	Biological Processes	carbohydrate metabolic process
Sucrose	4	1	GDT2T_hap104g02131	31081254	31083125	Molecular Function	carbohydrate binding
Sucrose	4	1	GDT2T_hap104g02136	31107647	31109348	Molecular Function	carbohydrate transmembrane transporter activity
Malic Acid	8	1	GDT2T_hap108g00870	8252919	8254819	Biological Processes	tricarboxylic acid cycle

Malic Acid	8	1	GDT2T_hap108g00870	8252919	8254819	Cellular Component	respiratory chain complex II (succinate dehydrogenase)
Glucose	12	2	GDT2T_hap212g00406	5229868	5230002	Biological Processes	photosynthesis
Glucose	12	2	GDT2T_hap212g00472	6255653	6258616	Molecular Function	carbohydrate binding
Glucose	16	2	GDT2T_hap216g00542	4410096	4414640	Biological Processes	carbohydrate metabolic process
Glucose	16	2	GDT2T_hap216g00543	4418228	4421426	Molecular Function	UDP-glucose 6-dehydrogenase activity
Glucose	16	2	GDT2T_hap216g00625	4941572	4952960	Molecular Function	carbohydrate binding
Glucose	16	2	GDT2T_hap216g00815	6485853	6490795	Biological Processes	carbohydrate metabolic process
Glucose	16	2	GDT2T_hap216g00816	6499495	6502226	Biological Processes	carbohydrate metabolic process
Glucose	16	2	GDT2T_hap216g00818	6505789	6508677	Molecular Function	pyroglutamyl-peptidase activity
Glucose	16	2	GDT2T_hap216g00839	6701891	6704533	Biological Processes	carbohydrate metabolic process
Glucose	16	2	GDT2T_hap216g00851	6789220	6793210	Biological Processes	carbohydrate metabolic process
Glucose	16	2	GDT2T_hap216g00909	7370977	7371881	Biological Processes	photosynthesis
Glucose	16	2	GDT2T_hap216g00945	7674979	7678066	Biological Processes	glucose metabolic process
Fructose	16	2	GDT2T_hap216g00778	6230010	6231543	Biological Processes	carbohydrate metabolic process
Fructose	16	2	GDT2T_hap216g00802	6398283	6399890	Biological Processes	carbohydrate metabolic process
Fructose	16	2	GDT2T_hap216g00804	6405079	6424292	Biological Processes	carbohydrate metabolic process
Fructose	16	2	GDT2T_hap216g00832	6624026	6624250	Molecular Function	fructose-bisphosphate aldolase activity

Fructose	16	2	GDT2T_hap216g00852	6795636	6799967	Biological Processes	carbohydrate metabolic process
Fructose	16	2	GDT2T_hap216g01048	8812255	8815766	Biological Processes	glucose metabolic process
Fructose	16	2	GDT2T_hap216g01048	8812255	8815766	Molecular Function	glucose-6-phosphate dehydrogenase activity
Sucrose	4	2	GDT2T_hap204g01634	27332616	27334832	Biological Processes	carbohydrate metabolic process
Sucrose	4	2	GDT2T_hap204g01635	27337975	27340270	Biological Processes	carbohydrate metabolic process
Sucrose	4	2	GDT2T_hap204g01650	27429481	27431491	Biological Processes	photosynthesis
Sucrose	4	2	GDT2T_hap204g01825	28791210	28794548	Biological Processes	photosynthesis
Sucrose	4	2	GDT2T_hap204g02080	31042121	31046842	Biological Processes	carbohydrate metabolic process
Malic Acid	8	2	GDT2T_hap208g00603	5519431	5523610	Molecular Function	cytokinin dehydrogenase activity
Malic Acid	8	2	GDT2T_hap208g00869	8575934	8577776	Biological Processes	tricarboxylic acid cycle
Malic Acid	8	2	GDT2T_hap208g00869	8575934	8577776	Cellular Component	respiratory chain complex II (succinate dehydrogenase)

4. Discussion

Fruit quality is a major factor that determines fruit marketability, and the ratio between sweetness and acidity is critical for consumer appreciation [15,50] In this study, a comprehensive investigation on six families obtained by crossing a set of high-quality commercial cultivars was performed. The phenotypic analyses showed trait-specific patterns of variability. Traits with larger means, like fructose and sucrose, exhibited high absolute variance but low CV^2, indicating stability relative to their average values, whereas traits with small means, like malic acid and glucose, showed low absolute variance but high CV^2, indicating high relative variability (Figure 1). These results highlight the advantage of using the CV^2 for comparing relative variability across different traits. Variance decomposition further indicated that glucose and sucrose retained mostly genetic components, whereas malic acid was strongly affected by year, suggesting that acidity is particularly sensitive to seasonal conditions (Figure 2B). Nevertheless, the estimated H^2 values indicated that stable genotypic differences contributed to all traits, with malic acid showing the highest H^2 despite the large year effect. Overall, these findings are consistent with previous studies showing that apple sugar and acid traits are quantitatively inherited but mostly regulated by both genetic and environmental components [18,21,24,51,52].

This combined genetic and environmental control provides an interesting framework for interpreting these traits in the context of apple domestication and breeding history, where selection has progressively shaped the balance between sweetness and acidity. The domestication of cultivated apple (*Malus* × *domestica*) has been shaped by long-term animal and human selection, followed by guided hybridization and modern breeding [53]. During this process, fruit size, flavor, and overall quality composition were progressively modified [53,54]. In particular, sugars and organic acids have represented major targets of selection because they directly influence perceived sweetness, acidity, and the final eating quality of fruit [23,54]. Previous comparative analyses between wild and cultivated apple accessions indicated that domestication strongly affected acidity-related loci, including the *Ma1* malate transporter region, whereas total sugar content was less clearly differentiated between wild and cultivated material [17,55]. This suggests that early selection may have primarily reduced excessive acidity and increased fruit size, while the fine adjustment of sweetness and sugar composition has been largely driven by more recent breeding efforts [17]. Sucrose and fructose are two sugars playing a major role in differentiating wild *Malus* spp. and *Malus* × *domestica* accessions [56], since they are characterized by a high level of perceived sweetness. In the present study, the relatively low CV^2 values observed for fructose and sucrose are consistent with the use of elite commercial parents, in which these high-sweetness sugars may already have been partially stabilized by breeding. This restricted parental diversity also explains the weak family separation observed in the PCA and may have reduced the power to detect large-effect sugar QTLs. Furthermore, the relatively low proportion of phenotypic variance explained by most QTLs is likely related to the genetic structure of the families used in this study. The populations were generated from crosses among elite apple cultivars that had already undergone selection for fruit quality traits, including sugar and acid content. As a result, the phenotypic variability available for these traits was narrower than that typically observed in studies based on crosses involving wild or old accessions [13,17,53,56] or on highly diverse germplasm collections [14,23,24,57,58]. Although this reduced variability may have limited the statistical power to detect large-effect QTLs in our study, it also reflects the level of variation that is most relevant to modern breeding programs using pre-selected elite material [57,58].

Even though the phenotypic data were collected over two seasons, additional years and environments would strengthen the assessment of QTL stability. Seasonal variability, tree physiological status, crop load, and climatic conditions can influence sugar and organic acid accumulation in perennial fruit crops [51,52,59]. Therefore, the QTLs reported here should be interpreted as supported by repeated observations in the present material, but further validation across years, locations, and independent populations will be required before routine marker-assisted deployment.

Within this context, the following QTL results provide a first indication of the genomic regions contributing to sugar and organic acid variation in elite apple breeding germplasm, the PBA identified QTLs on LGs 1, 4, 8, 12, and 16, with different levels of statistical support depending on the trait. The glucose QTLs on LG12 and LG16 showed the strongest evidence among sugar traits, whereas the fructose QTLs on LG1 and LG16 and the sucrose QTL on LG4 showed lower BF (Table 1; Figure 3). These results are in line with previous reports showing that apple sweetness-related traits are controlled by multiple loci with moderate to small effects [18,20,21,25,53]. For example, QTLs for individual sugars and soluble solids content have been detected across several linkage groups, including regions associated with fructose, glucose, sucrose, and sorbitol accumulation [18], while sensory sweetness and acidity QTLs have also been reported in multiple pedigreed families [20]. More recently, integrated transcriptomic, QTL, and genomic analyses identified several sugar- and acid-related QTLs and regulatory candidates involved in carbon

metabolism [21]. Several candidate genes previously reported in genomic regions associated with sugar and acid traits further support our findings. On chromosomes 4 and 16, genes involved in sugar transport and metabolism, including *MdSOT2* and members of the *MdSWEET* family (*MdSWEET2e*, *MdSWEET9b*, and *MdSWEET15a*), have been implicated in fruit sugar accumulation [21,60–63]. The partial overlap between our LG16 sugar signal and previously reported soluble solids or sugar-associated loci suggests that this genomic region may contribute to variation in hexose accumulation across different germplasm backgrounds [14,18,21]. In addition, Larsen et al. [58] also reported a fructose-associated locus on chromosome 1, consistent with the LG1 fructose QTL detected in the present study.

Similarly, the malic acid QTL detected on LG8 showed the strongest statistical support in this study and is particularly relevant because acidity-related loci on LG8 have been repeatedly reported in apple. Major QTLs for titratable acidity have been described on LG16 (*Ma*/*Ma1*) and LG8 (*Ma3*), and subsequent studies further validated the contribution of LG8 to acidity variation in multi-family and pedigree-based germplasm [14,16,19,24]. In our analysis, the LG8 QTL showed high posterior QTL probability and strong Bayesian evidence, whereas its estimated percentage of PVE was extremely small and rounded to 0% in Table 1. This apparent discrepancy is not unexpected, because the posterior probability reflects the evidence supporting the presence of a segregating QTL, while the PVE reflects the magnitude of its phenotypic effect. The six families analyzed here were generated from elite breeding parents selected for fruit quality, resulting in limited segregation for malic acid content. Moreover, no significant QTL was detected at the major acidity locus on LG16, suggesting that the principal determinant of fruit acidity showed little or no segregation in these populations. Therefore, the LG8 signal likely represents a minor-effect acidity locus with detectable statistical support but negligible contribution to the overall phenotypic variance in the present elite germplasm.

Functional annotation of the QTL intervals provided additional biological support for the QTL mapped regions. Sugar-associated intervals contained candidate genes annotated for carbohydrate metabolism, sugar binding, sugar transport, photosynthesis, and enzymatic processes involved in sugar interconversion (Tables 2 and S1). These annotations are consistent with the metabolic relationship among glucose, fructose, and sucrose and support the hypothesis that variation in fruit sugar content may be influenced by both metabolic conversion and transport processes. For malic acid, candidate genes within the LG8 interval were mainly associated with the tricarboxylic acid cycle, oxidoreductase activity, and respiratory chain complex II, supporting the link between organic acid accumulation, mitochondrial metabolism, and redox regulation [64,65]. Although the number of genes within the malic acid interval was smaller than in sugar-related intervals, their functional annotation is coherent with malate biosynthesis and turnover pathways. (Table 2, Table S1). The integration of functional annotation revealed potential genes involved in carbohydrate metabolism and energy-related pathways within trait-associated QTLs regions. The concentration of biologically associated functional categories within these QTL highlights these genes as potential regulators of sugar and organic acid variation.

The estimated QTL genotypes further highlighted how allelic variation at the mapped loci may contribute to differences in fruit sugar and organic acid composition among the parental cultivars (Figure 4). For glucose, QTLs on LGs 12 and 16 explained variation among parental cultivars, where favorable alleles were primarily present as homozygous ('QQ') or heterozygous ('Qq') genotypes. 'Royal Gala' carried the favorable homozygous genotype at both loci, whereas 'Fuji' was homozygous only at LG16. A comparable pattern was observed for fructose at LG1 and LG16. These results are consistent with previous reports identifying major-effect QTLs controlling hexose accumulation and sweetness-related traits in apple, supporting the presence of stable loci with additive ef-

fects on sugar metabolism [14,24]. In contrast, sucrose-associated loci exhibited a different genetic architecture. Most parental cultivars carried either the recessive ('qq') or heterozygous ('Qq') genotype at the detected QTLs (Figure 4C), indicating limited fixation of favorable alleles. This suggests that sucrose accumulation may be controlled by a more complex architecture involving minor-effect loci, environmental sensitivity, or limited allelic diversity in the material analyzed. Malic acid showed clearer parental differentiation at the LG8 QTL, where 'Fuji' carried the favorable 'QQ' genotype, 'Golden Delicious' carried the alternative 'qq' genotype, and 'Pinova' and 'Cripps Pink' were heterozygous. This pattern is consistent with prior reports describing major and background-dependent acidity loci in apple germplasm [14,19,23,24]. These results suggest that, although major QTLs contribute to phenotypic variation, additional polygenic effects, epistatic interactions, and environmental influences are likely to shape the final trait expression. Such genetic architecture aligns with previous studies reporting variable heritability and complex regulation for acid and sucrose traits in apple germplasm [12,24,57].

In conclusion, our study highlights the genetic controls associated with fruit flavor in apple, identifying major loci associated to these traits and identified in high-value elite plant materials. These results, although requiring additional validation, provide a set of loci potentially useful to breeders to exploit the genetic variability in high quality varieties and to improve fruit quality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae12070832/s1. Table S1: Candidate genes functional annotation within the identified QTL associated with each fruit quality trait. The table shows for each trait (glucose, fructose, sucrose and malic acid), the chromosome, the haplome, the gene identifier according to the Malus × domestica 'Golden Delicious' GDT2T genome assembly, physical start and end positions (bp) of each gene, Gene Ontology (GO) category, and the corresponding functional annotation term. Physical coordinates refer to the GDT2T genome assembly and are given in base pairs.

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