Exploration of a European-centered strawberry diversity panel provides markers and candidate genes for the control of fruit quality traits

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Article Title

Exploration of a European-centered strawberry diversity panel provides markers and candidate genes for the control of fruit quality traits

Running title: GWAS of strawberry quality traits and candidate genes

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Abstract

Fruit quality traits are major breeding targets in cultivated strawberry (*Fragaria × ananassa*). Taking into account the requirements of both growers and consumers when selecting high quality cultivars is a real challenge. Here, we used a diversity panel enriched with unique European accessions and the 50K FanaSNP array to highlight the evolution of strawberry diversity over the past 160 years, investigate the molecular basis of 12 major fruit quality traits by Genome-Wide Association Studies (GWAS), and provide genetic markers for breeding. Results show that considerable improvements of key breeding targets including fruit weight, firmness, composition and appearance occurred simultaneously in European and American cultivars. Despite the high genetic diversity of our panel, we observed a drop in nucleotide diversity in certain chromosomal regions, revealing the impact of selection. GWAS identified 71 associations with 11 quality traits and, while validating known associations (firmness, sugar), highlighted the predominance of new QTL, demonstrating the value of using untapped genetic resources. Three of the six selective sweeps detected are related to glossiness or skin resistance, two little-studied traits important for fruit attractiveness and, potentially, postharvest shelf-life. Moreover, major QTL for firmness, glossiness, skin resistance and susceptibility to bruising are found within a low diversity region of chromosome 3D. Stringent search for candidate genes underlying QTL uncovered strong candidates for fruit color, firmness, sugar and acid composition, glossiness and skin resistance. Overall, our study provides a potential avenue for extending shelf-life without compromising flavor and color as well as the genetic markers needed to achieve this goal.

Keywords: *Fragaria* × *ananassa*, strawberry, diversity, fruit quality, GWAS, candidate genes

Introduction

Cultivated strawberry *(Fragaria* × *ananassa)*, the most widely consumed small fruit worldwide, results from spontaneous hybridization in botanical gardens in France in the 18th century between two octoploid (2n = 8x = 56) species (*F. chiloensis* and *F. virginiana*) imported from the New World[1]. Since then, cultivated strawberry has been continuously improved through the introgression of alleles from wild progenitors creating an admixed population of interspecific hybrid lineages[2,3,4]. Recurrent hybridization contributed to maintain genetic diversity in the domesticated populations[4]. However, lower genetic diversity and heterozygosity can be observed in highly structured populations, which nevertheless show considerably improved yield, fruit weight and firmness[5]. Current efforts, triggered by consumer demand for sweet and highly-flavored strawberries[6,7], are aimed at improving the sensory and nutritional quality traits of the fruit such as color[8] and flavor[9]. Another area for improvement is the extension of the storage period and the reduction of post-harvest rot, both of which are linked to fruit firmness[7] and little-explored fruit surface properties[10]. Several fruit quality traits can be easily manipulated using advanced technology, such as genome editing which has successfully been applied to create new alleles modifying various traits including fruit color, sweetness and aroma[7,11]. Other complex (e.g. fruit size) and/or little-studied (e.g. fruit glossiness) traits first require elucidation of their genetic architecture. Until recently, following initial studies[12,13], the dissection of the genetic control of complex fruit quality traits in *F.*× *ananassa* has mainly been achieved by mapping Quantitative Trait Locus (QTL) on genetic linkage maps of bi-parental[14,15,16] or multi-parental[17] populations. Causal genetic variants have been identified for several QTL, leading to the design of genetic markers for marker-assisted selection (MAS) of strawberry varieties with, for example, improved fruit color[8,18] and better aroma[9].

Complexity of the allo-octoploid genome of *F.*x *ananassa*, where up to eight homeo-allelic forms of the same gene can be found[19], has until recently hampered the mapping of QTLs on a given chromosome. Whole genome sequencing of *F.*x *ananassa*[1] and, more recently, its progenitors[20], showed that the four subgenomes of *F.* x *ananassa* are derived from the diploids *F. vesca* and *F. iinumae* and from two extinct species related to *F. iinumae*[20,21]. Genome sequence further enabled the design of a single nucleotide polymorphism (SNP) 50K array with selected chromosome-specific SNPs[22] allowing the high-resolution mapping of QTLs. A recent breakthrough has been the completion of haplotype-resolved genomes for five genotypes of *F.*× *ananassa*[9,23,24,25,26]. These developments make it possible to exploit strawberry diversity through genome wide association studies (GWAS), which scans the genome for significant associations between genetic markers and the trait studied[2]. It thus can help unveil beneficial alleles through the exploration and characterization of strawberry genetic resources, which display a wide genetic and phenotypic diversity[4,27,28,29]. So far, GWAS has been done on collections mostly centered on North American

strawberry populations[4,29], which enabled the discovery of major QTLs controlling fruit weight, firmness, sweetness and aroma[4,9,29,30,31]. It would certainly benefit from the exploration of other less well-characterized germplasm found in Europe[32], a historically active strawberry breeding center[6].

In this study, we explored by GWAS the genetic architecture of fruit quality in *F.*× *ananassa*. To this end, we used the unexploited genetic diversity found in traditional and modern European varieties, in comparison with the better described diversity of North American varieties and some Asian genotypes. Our results are consistent with recent insights into the evolution of modern strawberry varieties and detected major QTL recently described, for fruit firmness for example. Moreover, we detected new QTL for most of the 12 fruit quality traits studied and the underlying candidate genes (CG). An example of this are the QTL and strong CG for the little-explored glossy trait, which underpins the shiny appearance of all modern strawberry varieties and was found to co-localize with a skin resistance trait. Our results therefore highlight the richness of European collections as a source of genetic diversity for strawberry breeding.

Results

Population structure and genetic diversity of the diversity panel of cultivated strawberry

We analyzed a germplasm diversity panel comprising 223 accessions of cultivated strawberries (*F.*× *ananassa*) available at Invenio (South-West France) (Table S1). Unlike the main diversity panels studied to date, where the bulk of the panel was constituted by North American accessions[4,28], our panel was mostly composed of European accessions from several countries, with French cultivars being by far the most represented (96 accessions). In addition, the panel comprised representative cultivars from North America including California and Florida, Japan and other breeding programs around the world (Fig. 1A, Table S1). Many cultivars were released between 1990s and nowadays, but the panel also accurately covered the whole modern breeding period (>1950s) and the early stages of strawberry breeding, with cultivars reaching as far as the beginning of the 19th century (Fig. S1). Thirty-two accessions from this panel were common with those from the study by Horvath et al. (2011)[32]. Accessions from the diversity panel were genotyped with the 50K FanaSNP array[22]. A total of 38,120 SNPs was retained after filtering for minor allelic frequency (MAF) (< 5%) and missing data (> 3%).

To explore relationships among the 223 accessions, we first evaluated the population structure with STRUCTURE software. We identified three distinct genetic clusters (Fig. 1B, Table S1). Group 1 (G1) includes most of the older European cultivars and their more recent relatives, as well as some old American cultivars (Fig. 1B). This group is hereafter named the Heirloom & related group. Group 2 (G2) clusters essentially European, as well as 14 American cultivars mostly from North-East America (Maryland, New-York and Canada) and three out of five Japanese cultivars (Fig. 1B) and was therefore named the European mixed

group. California and Florida cultivars, together with other European ones, have been identified in group 3 (G3) hereafter named the American & European mixed group. A large amount of admixture (< 70%) was observed for each group, with 108 out of 223 accessions split across more than one group, with most of the admixture spread between G2 and G3 (Fig. 1B).

To further investigate population structure, we performed principal component analysis (PCA) of the 223 accessions using the 38,120 SNP markers (Fig. 1C). The first two dimensions (PC1, PC2) explained 8.2% and 4.7% of the structural variance, respectively. The three genetic groups were positioned at each vertex of the crescent shape. PC1 also reflected the temporal separation between G1 and the other two groups when cultivars were displayed according to year of release (Fig. 1D). The phylogenetic analysis (Fig. S2) was consistent with the structure (Fig. 1B) and PCA (Figs 1C, 1D) analyses.

Genome-wide comparisons of nucleotide diversity (π) between genetic groups revealed no clear loss of genetic diversity from G1 to G2 and G3 (Fig. 1E). At the chromosome level, the distribution of nucleotide diversity among groups was uneven, with several genomic regions associated with significant enrichment or loss. Of notice, some regions were associated with a sharp reduction in diversity in G2 and/or G3 compared with G1, for example the 23,233 kb to 29,635 kb region on chromosome 3D (Fig. 1F). Additional examples can be found on other chromosomes such as chromosomes 2C, 3B and 6B (Fig. S3). Progression towards the most recent American and European cultivars also translated in local augmentation in linkage disequilibrium, where LD (at $r^2 = 0.20$) increased from an average distance of 802,184 bp for G1 to 1,073,213 bp for G2 and 1,253,777 bp for G3 (Fig. 1G, Fig. S4).

We then combined the SNP data from our diversity panel with those from University of California Davis (UCD panel)[4] and United States Department of Agriculture (USDA panel)[27] to position our collection in relation with these studies. The PCA of the combined data revealed that the Invenio collection largely overlapped the two USA collections, with the exception of the extreme end of the PC1 corresponding to the UCD program and a small group of genotypes representing probable introgressions of wild accessions into the California panel (Fig. 1H, Fig. S5). The University of Florida (FL) program was less represented in the dataset, and closer to the UCD program on the PCA. Japanese and Asian varieties were located at the center of the crescent. In addition, the PCA highlighted the enrichment of our panel in unique 171 accessions not found in the UCD and USDA panel, thus emphasizing its potential to find new phenotypic diversity for fruit quality traits in cultivated strawberry (Fig. 1H, Fig. S6). Heterozygosity decreased in California and Florida cultivars in comparison to European and Asian cultivars. Interestingly, heterozygosity was significantly higher in cultivars and advanced lines of Invenio and in recent European cultivars (released after 1980) (Fig. 1I).

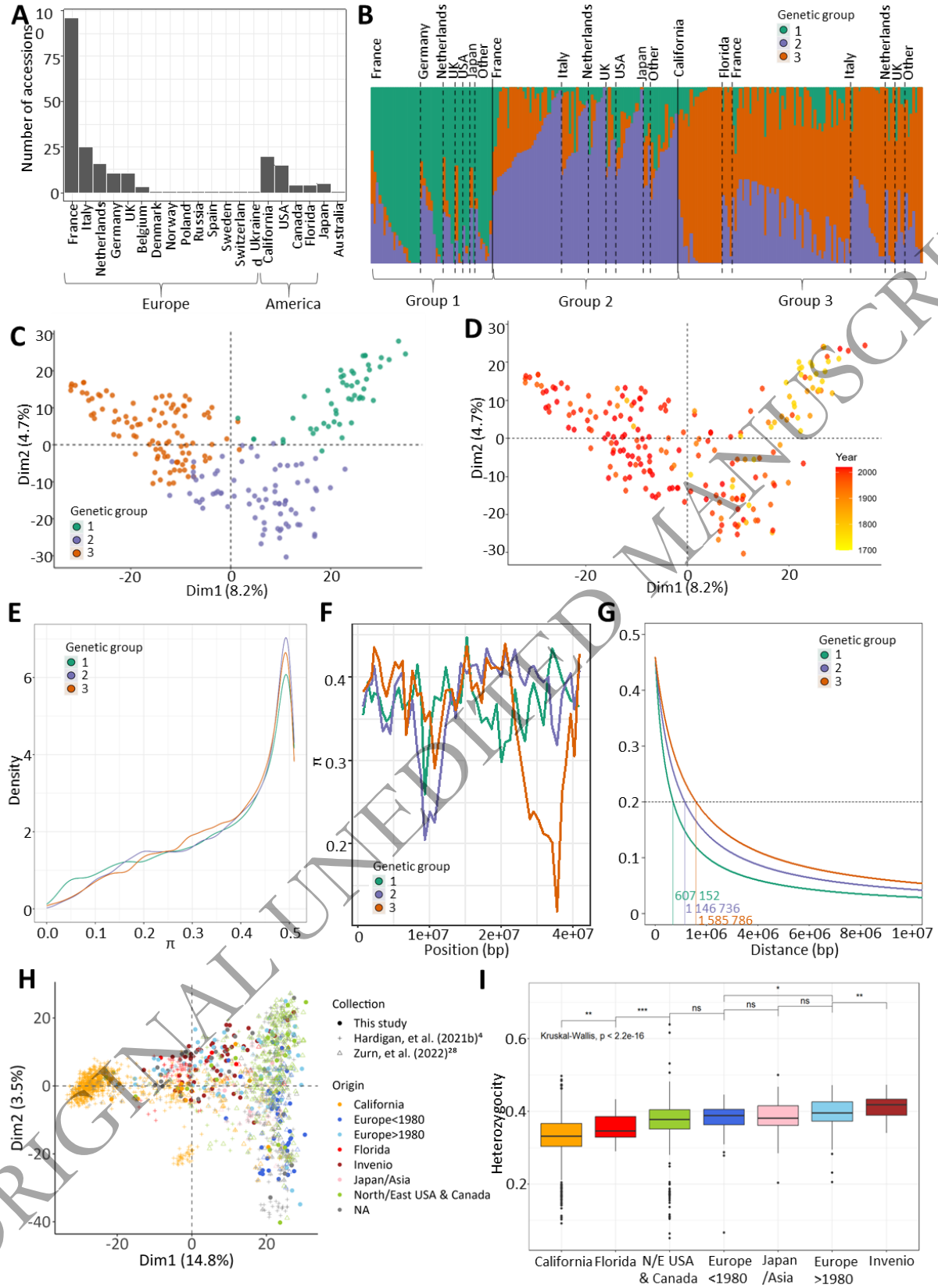

Figure 1. Genetic diversity of the panel. (A) Distribution of the geographical origin of the 223 accessions. (B) Structure barplot representing each genotype (bars) by its percentage of affiliation to each of the three genetic groups according to the STRUCTURE analysis. Individuals are sorted by genetic groups and

geographical origins. (C,D) Principal Component Analysis of 38,120 SNP markers. Each accession (dot) is colored by its genetic group (C) or year of release (D). (E) Nucleotide diversity (π) distributions in windows of 400 kb across each genetic group. (F) π chromosome-wide estimates for each genetic group for 400kb windows across the chromosome 3D. (G) Linkage disequilibrium (LD) decay along chromosome 1A. The dashed line represents the LD decay at r2=0.2. (H) Distribution of the Invenio panel (filled dots) among 1,569 genotypes (shaded dots) studied in Hardigan et al. (2021b)[4] and 539 genotypes studied in Zurn et al. (2022)[28] (shaded dots) with 3,215 SNP markers. Accessions are colored according to geographical/breeding origin. (I) Heterozygosity coefficients across different geographical/breeding origins when combining accessions from the diversity panel and 1,569 genotypes from Hardigan et al. (2021b)[4].
Genetic groups 1, 2 and 3 are colored in green, purple and orange, respectively. Groups 1, 2, 3: Heirloom & related, European mixed group and American & European mixed groups, respectively.

Fruit quality traits in the diversity panel

A total of 12 fruit quality traits were investigated in the panel of 223 accessions (Table 1). Traits were related to fruit weight (FW); fruit appearance (COL, skin color; UCOL, uniformity of skin color; UFS, uniformity of fruit shape; ACH, position and depth of achenes); firmness (FIRM); composition (TA, titratable acidity; TSS, total soluble solids (Brix units), and BA, the deduced ratio (Brix/TA)); and skin properties (GLOS, glossiness; SR, skin resistance; BRU, bruisedness). Analyses were carried out over two consecutive years, with the exception of the FIRM and SR traits, which were assessed over a single year (Table 1).
Most of the traits exhibited a considerable range of variation in the diversity panel, with coefficients of variation ranging from 3.7% for COL to 58.2% for skin resistance. For example, FW (average: 12.5 g) ranged from 1.8 and 32 g. (Table 1). Most traits showed a normal distribution, while SR, GLOS, UFS and BRU showed a skewed distribution (Fig. S6). Nine traits displayed high amount of genotypic variance associated with high broad sense heritability (H^2) ranging from 0.66 (ACH) to 0.98 (FIRM); H^2 of four traits, namely UFS (0.26), UCOL (0.43), BA (0.54) and BRU (0.57), was below 0.6 (Table 1). Few variations of H^2 between groups were observed for FW and FIRM, suggesting that phenotypic variability was equivalent between groups, whereas a strongest decrease in H^2 was observed for GLOS and BRU in G3 (Table 1). A significant interaction between genotype and environment was detected for all the traits for which repeated measurements were available over two years (Table 1), with the effect of environment being strongest for traits related to fruit composition (TSS, TA, BA).
To further explore the phenotypes of the diversity panel, we performed a PCA of the 223 accessions using a PCA biplot (Fig. 2A). PC scores revealed that the three genetic groups were distributed differently according to PC1 (39.2%) and PC2 (17.2%) in terms of fruit quality traits. G1 was distinct from G2 and G3 (Fig. 1B, Fig. S7A). Examination of the loadings of the traits on PC1 further showed that FW, appearance (UFS, UCOL), FIRM and skin properties (GLOS, SR and BRU) traits were responsible for the separation between G1 on one

side and G2 and G3 on the other side. TSS, TA and ACH had a very small contribution to the differentiation of the three subpopulations along PC1, and mostly contributed to PC2 and PC3, respectively (Fig. 2A, Fig. S7B).

Table 1. Summary statistics of the 12 fruit quality traits evaluated on the diversity panel in 2020 and 2021. n, number of accessions; CV, Coefficient of Variation; H^2, broad sense heritability; H^2G1, H^2G2, H^2G3, broad sense heritability within each genetic group; %GE, percentage of genotype-by-environment interactions in the total variance; r^2 structure, structuration of the trait as the coefficient of determination of the linear regression between the trait values and the genetic groups. 20-21 indicates the combined values across two years, 2020 and 2021. ns, not significant genotypic effect.

Trait	Year	n	Range	Mean	σ	CV	H^2	H^2 G1	H^2 G2	H^2 G3	%GE	r^2 structure
Fruit weight (FW, in g)	2020	169	1.8 - 30.9	12.4	5.4	43.6	0.96				-	-
	2021	169	2.0 - 32.0	12.6	5.6	44.5	0.92				-	-
	20-21	169	1.8 - 32.0	12.5	5.5	44.1	0.81	0.71	0.68	0.71	23.4	0.30
Shape uniformity (UFS)	2020	208	1 - 5	2.7	1.3	48.0	-				-	-
	2021	197	1 - 5	3.1	1.3	41.5	-				-	-
	20-21	208	1 - 5	2.9	1.3	44.9	0.26	ns	0.50	ns	-	0.05
Achene position (ACH)	2020	209	1 - 5	3.5	0.8	23.3	-				-	-
	2021	198	1 - 5	3.2	0.9	28.8	-				-	-
	20-21	209	1 - 5	3.4	0.9	26.2	0.66	0.71	0.67	0.60	-	0.08
Skin color (COL)	2020	201	1 - 7	1.2	4.507	3.7	-				-	-
	2021	209	1 - 7.5	1.2	4.535	3.8	-				-	-
	20-21	209	1 - 7.5	1.2	4.522	3.8	0.68	0.77	0.67	0.60	-	0.03
Color uniformity (UCOL)	2020	208	1 - 5	3.0	1.3	42.7	-				-	-
	2021	198	1 - 5	2.8	1.3	47.0	-				-	-
	20-21	208	1 - 5	2.9	1.3	44.9	0.43	ns	0.59	0.32	-	0.10
Firmness (FIRM, in kg/mm)	2020	-	-	-	-	-	-				-	-
	2021	210	1.04 - 0.07	0.433	0.2	40.3	0.98	0.94	0.97	0.95	-	0.44
	20-21	-	-	-	-	-	-				-	-
Titratable acidity (TA, in g / L)	2020	195	0.7 - 2.3	1.4	0.3	20.6	0.83				-	-
	2021	175	0.4 - 2.0	1.2	0.3	24.1	0.91				-	-
	20-21	195	0.4 - 2.3	1.3	0.3	23.1	0.70	ns	0.85	0.82	34.5	0.03
Total soluble solids (TSS, in Brix)	2020	207	3.6 - 11.0	7.1	1.4	19.7	0.92				-	-
	2021	184	3.2 - 11.8	6.9	1.5	22.1	0.94				-	-
	20-21	207	3.2 - 11.8	7.0	1.5	20.9	0.78	0.68	0.83	0.75	30.2	0.04
Brix/acidity ratio (BA)	2020	195	2.9 - 7.9	5.1	1.0	19.9	0.87				-	-
	2021	175	2.1 - 12	5.6	1.5	27.5	0.95				-	-
	20-21	195	2.1 - 12	5.3	1.3	24.8	0.54	ns	0.85	0.51	48.5	0.00
Glossiness (GLOS)	2020	195	1 - 5	3.5	1.2	35.8	-				-	-
	2021	182	1 - 5	3.5	1.1	31.8	-				-	-
	20-21	195	1 - 5	3.5	1.2	33.8	0.77	0.76	0.64	0.43	-	0.48
Skin resistance (SR)	2020	-	-	-	-	-	-				-	-
	2021	210	0 - 3	1.65	1.0	58.2	-				-	0.34
	20-21	-	-	-	-	-	-	-	-	-	-	-
Bruiseness (BRU)	2020	199	1 - 5	2.5	1.3	50.8	-				-	-
	2021	54	0 - 5	1.8	1.0	54.3	-				-	-
	20-21	199	0 - 5	1.9	1.1	55.7	0.57	0.52	0.45	ns	-	0.49

Correlation analysis of fruit quality trait data collected over 2020 and 2021 (Fig. 2B, Table S2) supported the relationships identified in the PCA biplot (Fig. 2A). GLOS, SR and BRU traits were positively and strongly correlated with each other and with FW and FIRM ($r = 0.51$ to 0.87), indicating the strong potential for

directional selection of these traits (Fig. 2A). UFS and UCOL were also highly correlated among them ($r = 0.64$) and, to a lesser extent ($r = 0.35$ and 0.26), with FW. TSS and TA were significantly correlated ($r = 0.45$) but, within groups, the correlation was only significant for G2 ($r = 0.51$) and G3 ($r = 0.47$) groups (Fig. S8). FW also demonstrated significant negative correlations with TSS ($r = -0.21$) and TA ($r = -0.15$) (Fig. 2B; Table S2). No or weak correlations were observed between ACH and other fruit quality traits.

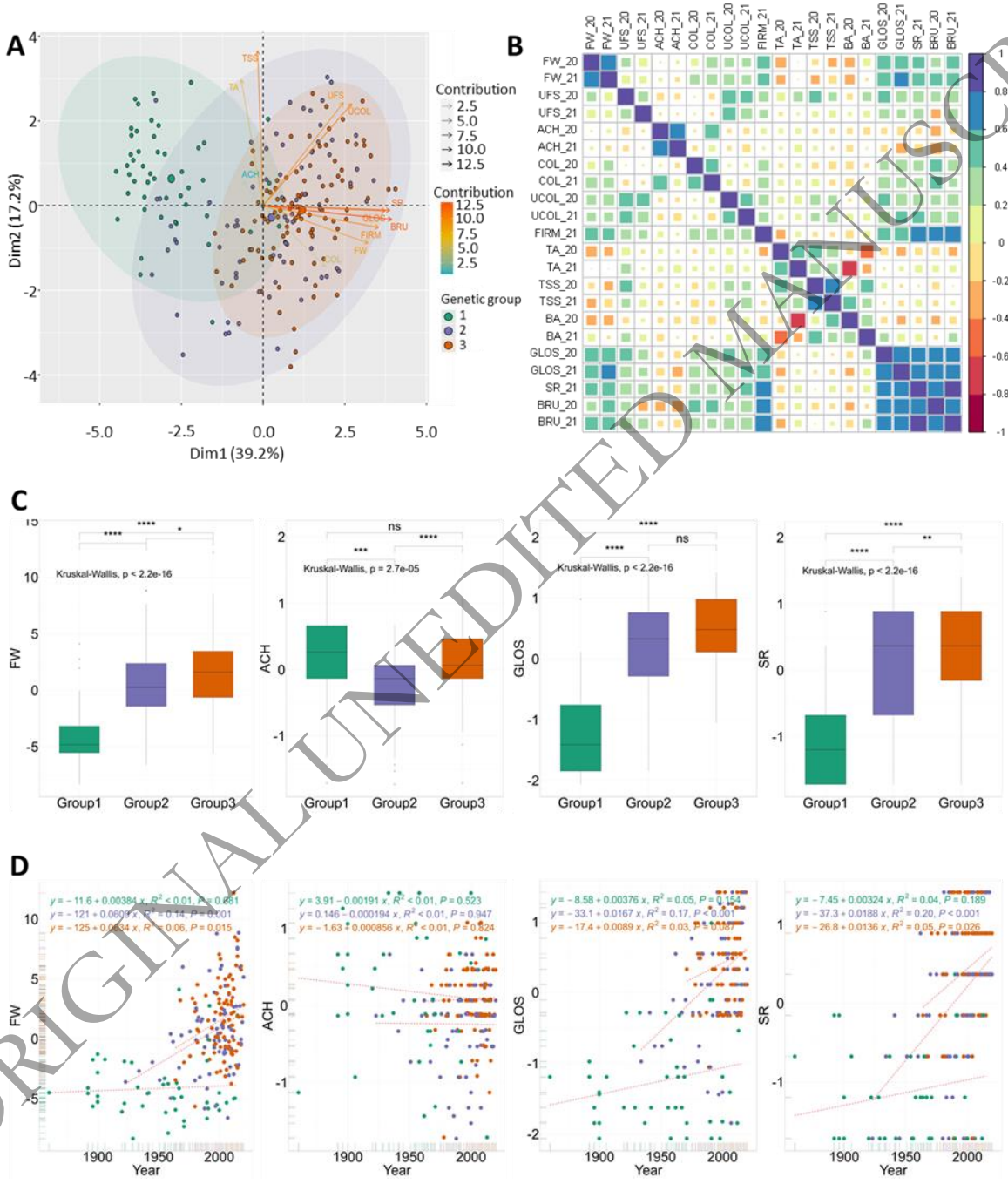

Figure 2. Phenotypic variations across the three genetic groups of the panel. (A) Principal Component Analysis of the 2-year BLUP values for 11 traits. (B) Correlations between the 12 traits for each year. (C) Comparisons of 2-year BLUP values for FW, ACH, GLOS and SR among genetic groups. (D) Genetic gains for

FW, ACH, GLOS and SR among genetic groups. Genetic groups 1, 2 and 3 are colored in green, purple and orange, respectively. Groups 1, 2, 3: Heirloom & related, European mixed group and American & European mixed groups, respectively. FW, fruit weight; UFS, uniformity of fruit shape; COL, skin color; UCOL, uniformity of skin color; ACH, position and depth of achenes; FIRM, firmness; TA, titratable acidity; TSS, total soluble solids; GLOS, glossiness; SR, skin resistance; BRU, bruisedness.

Most fruit quality traits have undergone significant phenotypic changes over time, as old varieties have evolved into modern cultivars (Figs 2C, 2D, Fig. S9). Phenotypic values of all fruit quality traits, except BA and COL, were significantly different between the three genetic groups. For example, FW considerably increased during the modern breeding phase, as reflected mainly in trends within G2 and G3 (Figs 2C, 2D). G1 was associated with low FW, dull, soft, low SR and easily wounded skin with uneven color and shape, whereas G3 exhibited the highest values for these traits (Fig. 2C, Fig. S9). G2 was equivalent to G3 for UFS and UCOL, TSS and TA, and GLOS; and was in the average of G1 and G3 for FW, FIRM, SR and BRU. Cultivars from G2 displayed more outcropped achenes than the others (Fig. 2C, Fig. S9).

These changes are linked to significant genetic gains over time for most fruit quality traits, with the exception of ACH and BA (Fig. 2D, Fig. S10). For G2 and G3, the traits most affected along the breeding cycles were fruit appearance (GLOS, Fig. 2D; UFS and UCOL, Fig. S10), fruit resilience to transport and postharvest storage (SR, Fig. 2D; BRU and FIRM, Fig. S10) and fruit weight (FW, Fig. 2D). These traits are important for consumers, retailers and growers respectively. A negative, non-significant trend was however observed for TSS and TA, whose values were lower in G2 and G3 than in G1 (Fig. S9). Remarkably, regardless of the TSS and TA reduction in modern varieties compared to old varieties, no significant differences for BA values were observed between groups (Fig. S9).

While positive and time-dependent genetic gains were observed within G2 and G3, genetic gains were usually low or inexistent within G1. For FW, for example, several recent varieties of the G1 showed the same trait values as old ones. The absence of major improvements for such traits in modern cultivars belonging to G1 is probably due to a low selection pressure, as the main selection objective was to produce ornamental plants with pink flowers ('Frel' and 'Toscana') or white fruits ('Anablanca', 'Blanche_du_Morvan', 'F_Eure_et_Loire').

GWAS of fruit quality traits

To reveal the genetic architecture of fruit quality in strawberry, we performed GWAS on the 12 fruit quality traits assessed in the 223 accessions of the strawberry diversity panel using genome-wide SNP markers from the 50K FanaSNP array[22]. The structuration of the population (Figs 2B, 2C) was considered by fitting both kinship and structure as cofactors for GWAS analysis. Detailed Manhattan plots for all 12 traits are

shown in Figs 3, 4, Fig. S11. The 71 significant associations with SNP markers are distributed on 51 chromosomal regions spread on 23 chromosomes (Table S3).

Fruit weight and appearance (FW, UFS, COL, UCOL, ACH).

Three significant SNP were identified for FW on chromosome 1B (19,119,571 bp, p-value 6.74E-09), 5B (17,045,086 bp, p-value 1.60E-06) and a highly significant SNP on chromosome 2D (15,565,564 bp, p-value 3.27E-12) (Fig. 3A, Table S3). The minor allele of AX-184592155 had a phenotypic variance explained (PVE) of 11.8% with an effect of 1.8g on FW (Fig. 3B, Table S3). Sixteen unique significant SNPs were identified for appearance traits, eight for UFS, three for ACH and five for COL (Fig. 3A, Fig. S11, Table S3). No signal was detected for UCOL.

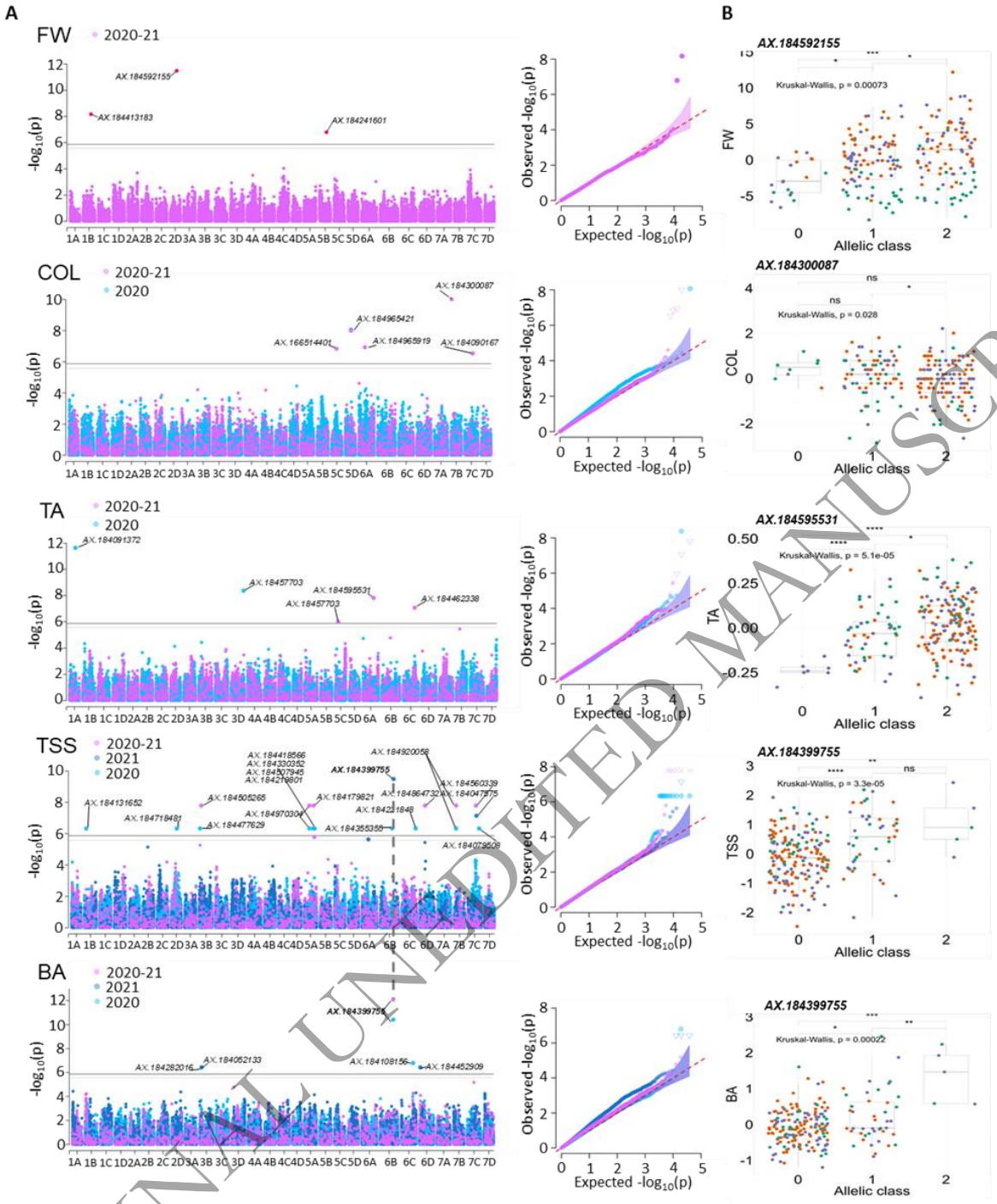

Figure 3. Genome wide association study of FW, COL, TA, TSS and BA. (A) Manhattan and Q-Q plots for yearly and 2-year BLUP values. (B) Effect of the most significant SNP marker. Genetic groups 1, 2 and 3 are colored in green, purple and orange, respectively. Groups 1, 2, 3: Heirloom & related, European mixed group and American & European mixed groups, respectively. FW, fruit weight; COL, skin color; TA, titratable acidity; TSS, total soluble solids; BA, brix acidity ratio. Marker classes are as follows: 0=AA genotype, 1=AB, and 2=BB genotype according to the Axiom™ Strawberry FanaSNP 50k.

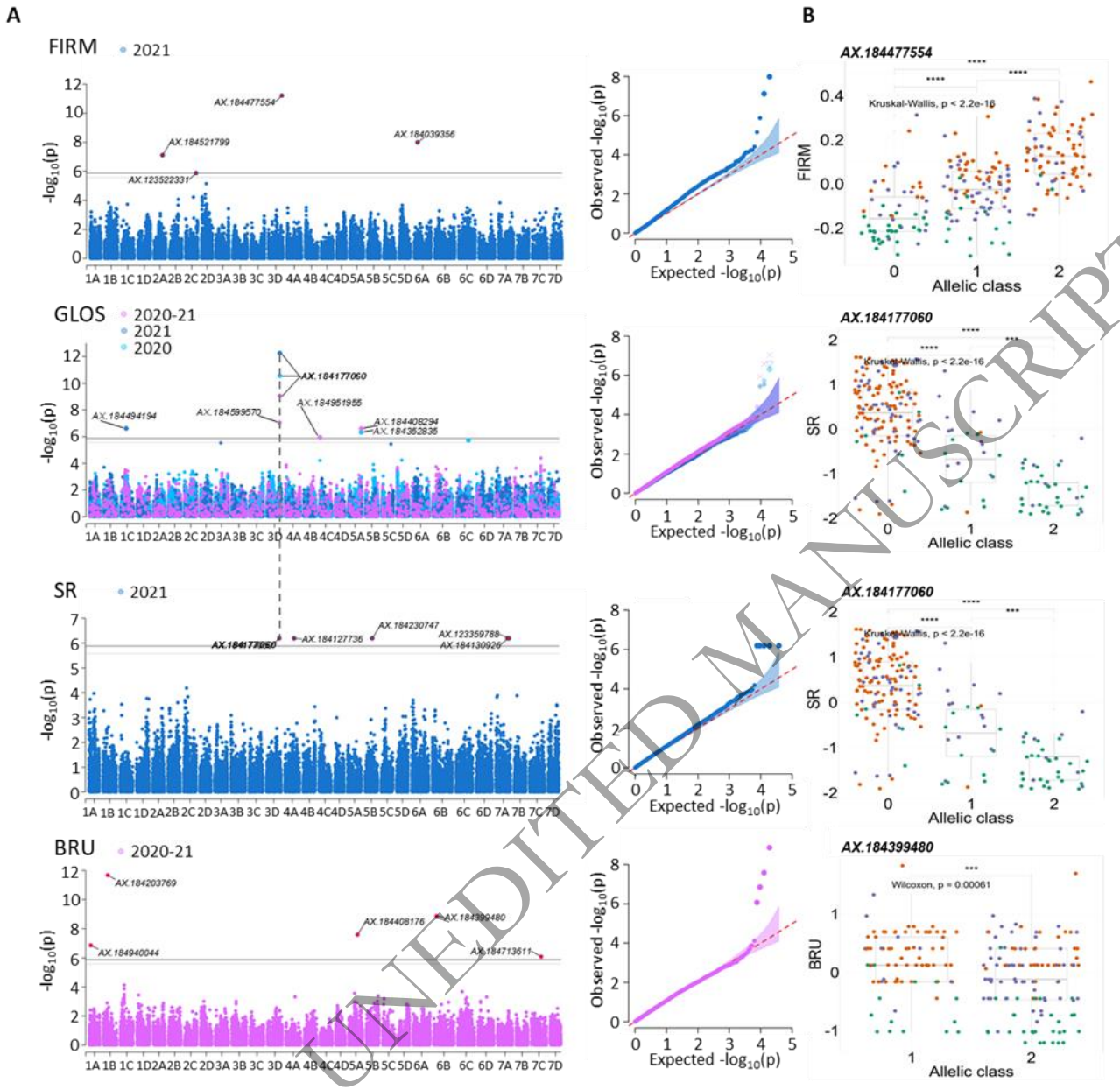

Figure 4. Genome wide association study of FIRM, GLOS, SR and BRU. (A) Manhattan and Q-Q plots for yearly and 2-year BLUP values. (B) Effect of the most significant SNP marker. Genetic groups 1, 2 and 3 are colored in green, purple and orange, respectively. Groups 1, 2, 3: Heirloom & related, European mixed group and American & European mixed groups, respectively. FIRM, firmness; GLOS, glossiness; SR, skin resistance; BRU, bruisedness. Marker classes are as follows: 0=AA genotype, 1=AB, and 2=BB genotype according to the Axiom™ Strawberry FanaSNP 50k.

Fruit composition (TA, TSS, BA).

Twenty-seven unique significant SNPs were detected for fruit composition traits, five for TA, 18 for TSS and five for BA (Fig. 3A, Table S3). The SNP AX-184595531 detected for TA on the 2020-2021 combined values (25,621,066 bp, p-value 1.55E-08) was particularly notable for its PVE of 35.5%. Only seven cultivars, all belonging to G2, were unfavorable homozygous for this marker (Fig. 3B). SNPs AX-184091372 on

chromosome 1A (13,540,517 bp, p-value 2.30E-12, PVE 17.1%) and AX-184457703 on chromosome 3D (25,621,066 bp, p-value 4.22E-09, PVE 17.9%) were also of particular interest for their PVE and impacting effect on TA in 2021. AX-184399755 was the highest effect SNP for TSS in 2021 on chromosome 6B (31,578,303 bp, p-value 3.24E- 10, PVE 19%) (Fig. 3B). It was also highly significant for BA in 2020 (p-value 3.84E- 11, PVE 46.1%) and 2020-2021 combined values (p-value 7.88E- 13, PVE 65.8%) (Fig. 3B). Only five cultivars were favorable homozygous for this marker. Interestingly, the SNP markers associated with the TSS QTL on 6B (AX-184399755) and 6D (AX-184864732), and 7B (AX-184920058) and 7C (AX-184079508), were found very close on the diploid *F. vesca* reference genome (*F. vesca* v4.0.a1[33]), at 606,797 bp (Fvb6) and 449,220 bp (Fvb7) from each other respectively.

Fruit firmness and skin properties (FIRM, GLOS, SR, BRU).

Four significant SNPs were detected for FIRM, six for GLOS, five for SR and five for BRU (Fig. 4A, Table S3). The chromosome 3D was of particular interest for these traits as it comprises one highly significant SNP for FIRM (29,275,014 bp, p-value 6.07E- 12, PVE 11.2%) and the highly significant SNP AX-184177060 (27,845,440 bp) common to both GLOS (p-value 9.01E- 10, PVE 26.2% on combined values) and SR (p-value 6.45E- 07, PVE 8.4%) (Fig. 4B). The latter SNP was detected systematically in 2020, 2021 and 2020-2021 for GLOS with PVE ranging from 26.2% to 28.7%, with a negative effect of the minor allele (-0.7 to -0.8 on 1 to 5 scale). MAF of this SNP was highly reduced towards G3, indicating strong selection of the favorable allele (Fig. 4B). SNPs for BRU were detected for the 2020-2021 combined values only.

Selective sweep signals during strawberry improvement

We identified markers under selection during strawberry improvement in light of genome scans based on Mahalanobis distance across the diversity panel (Fig. 5A) and nucleotide diversity throughout the genome for all of the accessions in the diversity panel (Fig. 1F, Fig. S3). Six significant associations of SNP markers with fruit quality QTL were detected, including one for UFS, one for FIRM, one for TSS, two for GLOS and one for SR (Fig. 5A, Table S4). The AX-184177060 marker associated with GLOS and SR (chromosome 3D), the AX-184477554 associated with FIRM (chromosome 3D), and the AX-184864732 (chromosome 6D) associated with TSS, are found in chromosomal regions displaying a drastic reduction in nucleotide diversity in modern genotypes (Fig. 1F, Fig. S3, Table S4). For example, in the case of the SNP marker AX-184177060 associated with the SR and GLOS QTL, the favorable allele is over-represented in the most recent accessions (average year of release 2000), whereas cultivars heterozygous or unfavorably homozygous for the marker were released around 1967 and 1947 respectively. This finding supports the fact that the favorable allele has been selected over time.

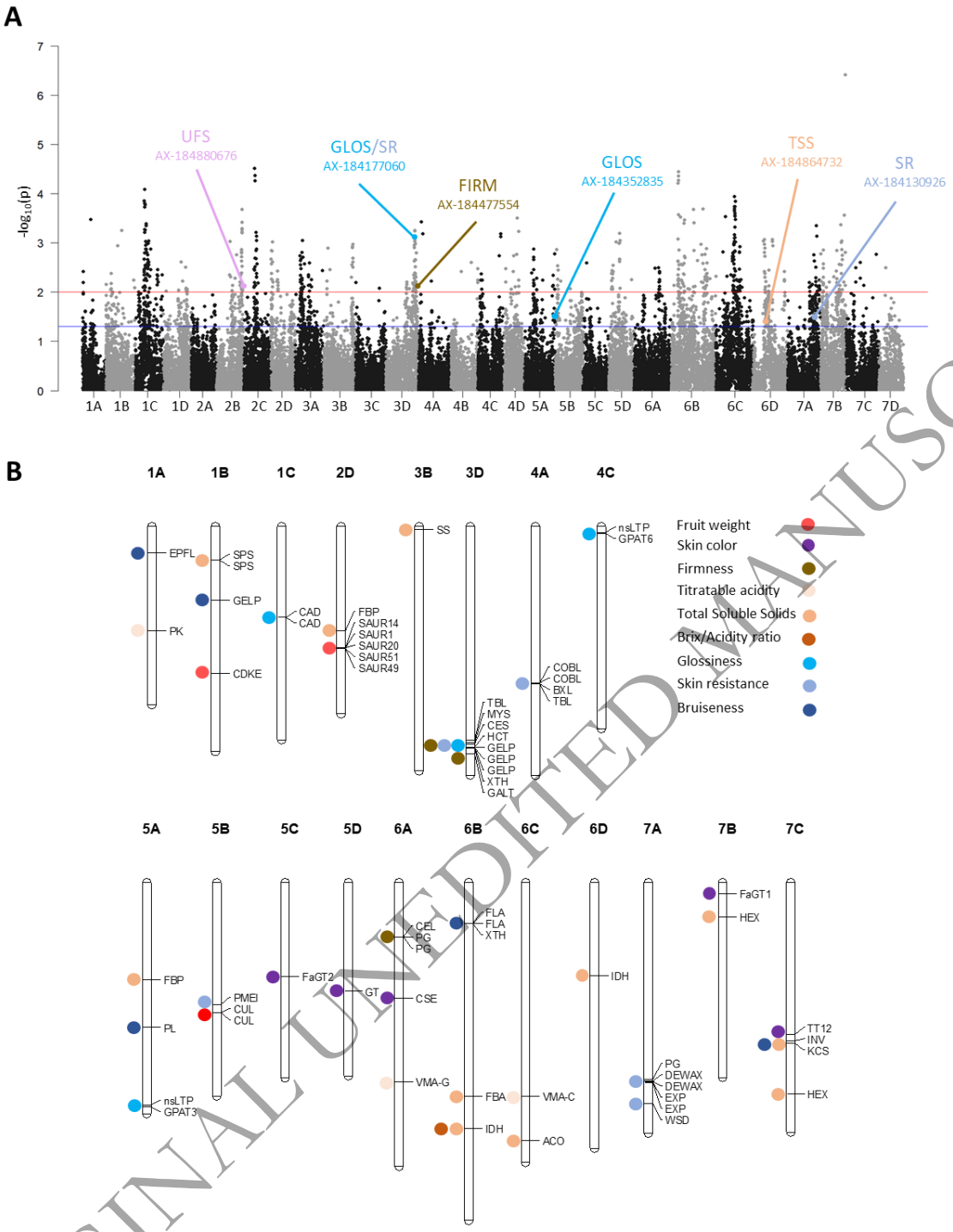

Figure 5. Selective sweeps and candidate genes. (A) Selective sweeps as a Manhattan plot of p-values of the genome scan based on Mahalanobis distance. Red and blue lines indicate thresholds at 0.01 and 0.05, respectively. Only significant (p-value < 0.05) SNP markers associated with fruit quality QTL are represented. UFS, uniformity of fruit shape; GLO, glossiness; SR, skin resistance; FIRM, firmness; TSS, Total soluble solids. (B) Physical mapping of the candidate genes underlying the GWAS of nine traits on the Camarosa genome. Full names and abbreviations of candidate genes are given in Table 2.

Candidate genes were identified for 37 QTL controlling 9 fruit quality traits

Candidate genes (CG) underlying fruit quality QTL were identified within a window of ~400 kb surrounding the QTL marker. This value, which corresponds to the short-range LD found in the California cultivars of *F.* × *ananassa*[4], is stringent compared to the average LD calculated on the 28 linkage-groups in our diversity panel, which is 932 kb. In chromosomal regions harboring strong QTL of interest and displaying low genetic diversity and high LD, i.e. the 3D region extending from 23,233 to 29,635 kb (Fig. 1F), we considered much larger intervals based on LD estimates (up to ~1,382 kb) for 3B and 3D. We excluded two traits (UFS and ACH) from CG analysis because the molecular pathways underlying these traits are far from being deciphered in strawberry. No QTL was detected for UCOL. In total, we identified 64 candidate loci for 37 SNP markers associated with the nine fruit quality traits (Fig. 5B). Table 2 provides names, abbreviations and positions on Camarosa and Royal Royce genomes of these 64 CG. Their possible functions are indicated in Table S5.

Table 2. Candidate genes underlying nine fruit quality traits. PVE, Phenotypic variance explained (%). Position Camarosa and Position Royal Royce: physical positions on Camarosa and Royal Royce reference genomes. Protein encoded by the candidate gene (CG): annotation in the Camarosa genome. CG Position Camarosa and *F.x ananassa* identity: physical position and identity in the Camarosa genome. Published GWAS/QTL: QTL detected with Affymetrix strawberry arrays on the same chromosomal regions as in this study. *, CG found outside ~400 kb intervals around SNP markers.

Trait	Chr	PVE	SNP marker	Position Camarosa	Position Royal Royce	Protein encoded by the Candidate Gene (CG)	CG Abbreviation	CG Position Camarosa	F.x ananassa identity	TAIR Arabidopsis homolog	Published Strawberry GWAS/QTL a
Fruit weight (FW)	1B	5.5	AX-184413183	19 119 571	15 971 709	cyclin-dependent kinase E-1	*CDKE*	18 842 135	FxaC_2g34880	AT5G63610.1	
	2D	11.8	AX-184592155	15 565 564	8 801 569	small auxin upregulated RNA 14	*SAUR14*	15 581 781	FxaC_8g28530	AT4G38840.1	
						small auxin upregulated RNA 1	*SAUR1*	15 594 644	FxaC_8g28550	AT4G34770.1	
						small auxin upregulated RNA 20	*SAUR20*	15 611 587	FxaC_8g28590	AT5G18020.1	
						small auxin upregulated RNA 51	*SAUR51*	15 687 585	FxaC_8g28690	AT1G75580.1	
						small auxin upregulated RNA 49	*SAUR49*	15 697 093	FxaC_8g28700	AT4G34750.2	
	5B	3.6	AX-184241601	17 045 086	10 918 733	cullin	*CUL*	16 733 799	FxaC_18g25221	AT4G02570.4	
						cullin	*CUL*	16 708 079	FxaC_18g25150	AT4G02570.4	
Skin color (COL)	5C	1.7	AX-166514401	11 987 143	--	anthocyanidin 3-O-glucosyltransferase	*FaGT2*	12 158 604	FxaC_19g22400	AT5G17050.1	
	5D	3.8-23.4	AX-184965421	14022053	13542145	flavonoid 3-O-glycosyltransferase	*GT*	14049628	FxaC_20g25430	AT5G54010.1	
	6A	2.6	AX-184965919	14 476 176	21 070 121	caffeoylshikimate esterase	*CSE*	14 923 586	FxaC_21g30560	AT1G52760.1	
	7B	3.00986	AX-184300087	1386945	23020879	anthocyanidin 3-O-glucosyltransferase	*FaGT1*	1422660	FxaC_26g03070	AT5G17050.1	
	7C	9.9	AX-184090167	19 438 028	13 528 856	TT12-like MATE transporter	*TT12*	19 581 584	FxaC_27g28060	AT4G00350.1	
Firmness (FIRM)	3D	11.2	AX-184477554	29 275 014	2 454 715	AGP galactosyltransferase	*GALT*	29 300 471	FxaC_12g45150	AT4G21060.2	
						endotransglucosylase/hydrolase	*XTH*	28 520 127	FxaC_12g43560	AT3G23730.1	
						* cellulose synhase	*CES*	27 994 587	FxaC_12g42810	AT4G18780.1	
	6A	6.3	AX-184039356	7 277 130	27 533 782	cellulase 1	*CEL*	7 038 578	FxaC_21g15730	AT1G70710.1	Cockerton et al. (2021); Hardigan et al. (2021b); Fan et al. (2024); Muñoz et al.
						polygalacturonase	*PG*	7 048 245	FxaC_21g15750	AT3G07820.1	
						polygalacturonase	*PG*	7 054 511	FxaC_21g15770	AT3G07820.1	
Titratable acidity (TA)	1A	17.1	AX-184091372	13 540 517	13 469 152	pyruvate kinase	*PK*	13 355 376	FxaC_1g27460	AT2G36580.1	
	6A	35.3	AX-184595531	25 621 066	9 172 070	V-type proton ATPase subunit G	*VMA-G*	25 704 934	FxaC_21g49700	AT3G01390.4	
	6C	6.8	AX-184462338	27 458 040	--	V-type proton ATPase subunit C	*VMA-C*	27 580 375	FxaC_23g44340	AT1G12840.1	
TSS (Brix)	1B	3.6	AX-184131652	4 174 690	1 302 571	sucrose-phosphate synthase	*SPS*	4 316 703	FxaC_2g09440	AT5G20280.1	
						sucrose-phosphate synthase	*SPS*	4 319 488	FxaC_2g09441	AT5G20280.1	
	2D	1.41107	AX-184718481	13298291	--	fructose-1,6-bisphosphatase, cytosolic	*FBP*	13361786	FxaC_8g25210	AT1G43670.1	
	3B	0.7	AX-184477629	1 541 393	1 842 542	* starch synthase	*SS*	453 429	FxaC_10g00830	AT4G18240.1	Fan et al. (2024)
	5A	0.9-2.1	AX-184970304	12400711	10544768	fructose-1,6-bisphosphatase, cytosolic	*FBP*	12546445	FxaC_17g26250	AT1G43670.1	
	6B	1.40326	AX-184355355	27647943	10765128	fructose-bisphosphate aldolase	*FBA*	27600407	FxaC_22g44640	AT3G52930.1	
	6B	18.9621	AX-184399755	31578303	9217798	isocitrate dehydrogenase [NAD]	*IDH*	31758475	FxaC_22g50900	AT5G03290.1	
	6C	0.58182	AX-184221848	33293635	29374007	aconitase	*ACO*	33266087	FxaC_23g56470	AT2G05710.1	
	6D	4.8	AX-184864732	12013420	9512125	isocitrate dehydrogenase [NAD]	*IDH*	11337718	FxaC_24g21140	AT5G03290.1	
	7B	1.6-4.0	AX-184920058	4515979	19984301	hexose carrier protein 6	*HEX*	4480262	FxaC_26g09610	AT5G61520.1	
	7C	7.71803	AX-184047575	20267768	--	alkaline/neutral invertase	*INV*	20384455	FxaC_27g29380	AT4G09510.1	
	7C	0.5	AX-184079508	26901718	13298291	hexose carrier protein 6	*HEX*	27292700	FxaC_26g09610	AT5G61520.1	
BA ratio	6B	65.8	AX-184399755	31 578 303	9 217 798	isocitrate dehydrogenase [NAD]	*IDH*	31 758 475	FxaC_22g50900	AT5G03290.1	
Glossiness (GLOS)	1C	17.8	AX-184494194	11 470 545	10 966 861	cinnamyl-alcohol dehydrogenase	*CAD*	11 709 567	FxaC_3g21930	AT4G39330.1	
						cinnamyl-alcohol dehydrogenase	*CAD*	11 726 197	FxaC_3g21970	AT4G37970.1	
	3D	4.7-28.7	AX-184599570-AX-184177060	26 901 693-27 845 440	3 815 908-4 775 280	MYB-SHAQKYF	*MYS*	27802931	FxaC_12g42500	AT2G38300.1	
						trichome birefringence-like 38	*TBL*	27558105	FxaC_12g42010	AT1G29050.1	
						* hydroxycinnamoyl-CoA shikimate/quinate	*HCT*	28432367	FxaC_12g43430	AT5G48930.1	
						* GDSL esterase/lipase	*GELP*	28471370	FxaC_12g43470	AT1G29670.1	
						* GDSL esterase/lipase	*GELP*	28475004	FxaC_12g43480	AT1G29670.1	
						* GDSL esterase/lipase	*GELP*	28478770	FxaC_12g43490	AT1G29670.1	
	4C	3.4	AX-184951955	727143	26073661	non specific Lipid Transport Protein	*nsLTP*	756662	FxaC_15g01440	AT2G37870.1	
						glycerol-3-phosphate acyltransferase	*GPAT6*	901347	FxaC_15g01830	AT2G38110.1	
	5A	4.1-11.1	AX-184408294-AX-184352835	28658239-28239789	24966878-25341723	non specific Lipid Transport Protein	*nsLTP*	28693018	FxaC_17g54360	AT5G64080.1	
						glycerol-3-phosphate acyltransferase	*GPAT3*	28952583	FxaC_17g54920	AT4G01950.1	
Skin resistance (SR)	3D	8.4	AX-184177060	27 845 440	3 815 908	MYB-SHAQKYF 1	*MYS*	27 802 931	FxaC_12g42500	AT2G38300.1	
						trichome birefringence-like 38	*TBL*	27 558 105	FxaC_12g42010	AT1G29050.1	
						shikimate/quinate hydroxycinnamoyltransferase	*HCT*	28 432 367	FxaC_12g43430	AT5G48930.1	
						* GDSL esterase/lipase	*GELP*	28 471 370	FxaC_12g43470	AT1G29670.1	
						* GDSL esterase/lipase	*GELP*	28 475 004	FxaC_12g43480	AT1G29670.1	
						* GDSL esterase/lipase	*GELP*	28 478 770	FxaC_12g43490	AT1G29670.1	
	4A	5.4	AX-184127736	20012930	16433479	COBRA-like	*COBL*	20088406	FxaC_13g38300	AT4G16120.1	
						COBRA-like	*COBL*	20089498	FxaC_13g38301	AT4G16120.1	
						beta-D-xylosidase	*BXL*	20219525	FxaC_13g38530	AT5G49360.1	
						trichome birefringence-like 43	*TBL*	20238095	FxaC_13g38560	AT2G30900.1	
	5B	3.6	AX-184230747	15853776	12173853	pectin methylesterase inhibitor	*PMEI*	15657184	D=FxaC_18g2343(	AT5.1G38610	
	7A	7.3	AX-184130926	25616815	18667225	polygalacturonase	*PG*	25398299	FxaC_25g46610	AT2G43890.1	
						expansin B2	*EXP*	25744435	FxaC_25g47360	AT1G65680.1	
						expansin B2	*EXP*	25766632	FxaC_25g47430	AT1G65680.1	
						decrease was biosynthesis 2	*DEWAX*	25608061	FxaC_25g47130	AT5G07580.1	
						decrease was biosynthesis 2	*DEWAX*	25647466	FxaC_25g47200	AT5G07580.1	
	7A	1.6	AX-123359788	28581204	--	wax ester synthase	*WSD*	28523042	FxaC_25g53661	AT3G49210.1	
Bruiseness (BRU)	1A	6.91851	AX-184940044	3531536	--	epidermal patterning factor	*EPFL*	3396016	FxaC_1g08100	AT3G13898.1	
	1B	6.88666	AX-184203769	9439391	7276991	GDSL lipase	*GELP*	9476517	FxaC_2g20370	AT2G23540.1	
	5A	4.38749	AX-184408176	18814640	16414924	pectate lyase	*PL*	18712472	FxaC_17g36850	AT5G09280.1	
	6B	5.48905	AX-184399480	5731875	30532752	fasciclin-like arabinogalactan protein	*FLA*	5305159	FxaC_22g11190	AT5G06920.1	
						fasciclin-like arabinogalactan protein	*FLA*	5332088	FxaC_22g11250	AT5G06920.1	
						xyloglucan endotransglucosylase/hydrolase	*XTH*	5351858	FxaC_22g11330	AT4G03210.1	
	7C	2.8	AX-184713611	20943204	15149370	3-ketoacyl-CoA synthase 1-like	*KCS*	20714519	FxaC_27g29940	AT1G01120.1	

[a], Publications: Natarajan et al. (2020)[41], Alarfaj et al. (2021)[40], Cockerton et al. (2021)[17], Hardigan et al. (2021b)[4], Rey-Serra et al. (2021)[15], Fan et al. (2023)[29], Fan et al. (2024)[31], Muñoz et al. (2024)[3]

Discussion

Genetic and phenotypic shifts in modern strawberry breeding programs

Our study sheds light on the genetic and phenotypic shifts that occurred over the last 160 years of strawberry breeding by analyzing 223 accessions comprising original old and modern European breeding material. According to our analyses, old strawberry cultivars, which here consist mainly of European cultivars selected before 1950 and included in the Heirloom & related group (G1), are clearly separated from other genetic resources (Fig. 1C), in agreement with earlier studies[32] confirmed in recent papers[4,34]. For over half a century[35], breeding programs in Western and Southern Europe have made extensive use of California cultivars and, more recently, of Florida cultivars, which are underrepresented in our study, as progenitors. As a consequence, our results show the clustering of most European recent cultivars in an American & European mixed group (G3). The European mixed group (G2), which includes other European cultivars, is likely related to the group previously named Cosmopolitan[4]. European cultivars were also separated from US cultivars in the Zurn et al. (2022) study[28] due to the large number of American accessions.

The overall nucleotide diversity is well-conserved among the genetic groups of our panel (Fig. 1E). In contrast, a significant erosion of genetic diversity was observed in highly structured populations[4,5,29]. We found a more nuanced picture by examining nucleotide diversity at the chromosome level, since it drops dramatically in regions potentially subject to selection pressure (Fig. 1F, Fig. S3). The decrease in both LD and heterozygosity specifically observed in the most recent American cultivars[4] is likely explained by the gradual differentiation of California and Florida populations. In contrast, cultivars and advanced lines of Invenio as well as the recent European cultivars released after 1980 display higher heterozygosity values (Fig. 1I). One possible explanation for the high genetic diversity retained in European accessions is that European breeders had to cope with a wide range of breeding targets due to the diversity of cultural practices, markets and consumer preferences found in Europe[6,36]. High quality strawberry varieties released in Europe therefore had to meet the requirements of both high cultivar performance, e.g. high fruit yield, as in California cultivars[5] and high sensory fruit quality, e.g. high flavor[6].

Remarkably, recent studies have shown that despite a loss in genetic diversity, increases in both genetic gain and phenotypic variation were observed in highly structured populations such as those of the California breeding programs[5]. In these programs, breeding efforts rapidly led to the improvement of fruit weight and fruit firmness[5,27,29], which participated to the so-called California green revolution[5]. European breeding programs have benefited from these efforts, as modern American cultivars appear in the pedigree

of prominent European cultivars[3]. Consistently, our results indicate a similar trend towards improved fruit size and firmness, as well as skin glossiness and resistance, in recent European germplasm (Fig. 2C, Fig. S9). Interestingly, we found that TSS and TA values decreased over time in G1 but that the BA ratio kept the same value (Figs S9, S10), in agreement with Feldmann et al. (2024)[5] who even observed an increase in BA levels, which could partly counterbalance the decrease in fruit sweetness. Antagonism between yield and firmness, on one side, and TSS and TA, on the other side, was previously reported[5,27,31].

Novel markers for the selection of fruit quality traits

GWAS is a powerful tool for the detection of SNP markers linked to different traits in strawberry[9,17,29,31,37,38]. Here, based on a large diversity panel, we detected 71 marker-associations to major fruit quality breeding targets. Some of the marker/QTL associations detected confirm published results and, consequently, validate our findings in a different genetic context. In comparison with the GWAS/QTL published data[4,15,17,29,31,39,40,41] obtained using the Affymetrix strawberry arrays, only two common fruit quality QTL located on the same chromosomal regions were detected here (Table 2). Our AX-184039356 marker linked to FIRM on 6A is very close to those previously described for fruit firmness[4,17,31,39]. Likewise, our AX-184477629 marker linked to TSS on 3B is in the same chromosomal region as the SSC1 QTL controlling soluble solids content[31]. In contrast, several previously reported QTL such as a FW QTL on 5B[29,41], a TSS QTL on 5A[41], a TA QTL on 5A[15] have been found on the same chromosomes but in different regions. The genetic diversity of unique European accessions included in our study allowed us to reveal new QTL and associated SNP markers, even for well-studied fruit quality traits such as FW and TSS.

In contrast to these well-studied traits, few studies have unveiled the genetic architecture of skin associated traits such as fruit glossiness[17,42] which is, alongside color, one of the most prominent traits for fruit attractiveness to the consumer[43]. Remarkably, of the six associations found among the selective sweeps detected, two were found for GLOS and one for SR (Fig. 5A). Furthermore, by highlighting a ~6,400 kb region on chromosome 3D linked to glossiness, skin resistance and firmness, our results shed a new light on a genomic region under strong breeding pressure (Figs 1F, 5A). Remarkably, among the six associations found among candidate selective sweeps, two were found for GLOS and one for SR. This chromosomal region has thus probably played a crucial role in improving the attractiveness and post-harvest qualities of strawberries, a feature that is receiving increasing attention in strawberry breeding programs. Information on the position of SNP markers on both Camarosa and Royal Royce genomes will facilitate new studies on fruit quality traits, thus contributing to validate these markers for MAS.

Candidate genes

Fruit weight and appearance

Fruit weight (FW) and shape are complex traits. Underlying genes of previously unknown functions have been identified by map-based cloning in species such as tomato[44] and corresponding CG have been detected in several crops[45]. Translation of these findings to strawberry may however prove difficult because of the different ontogenic origin of strawberry, which is an accessory fruit derived from the flower receptacle and not from the ovary. Indeed, our GWAS study did not detect any known gene families linked to fruit weight and shape, but highlighted for FW QTL several CG (*CDKE,* a cluster of five *SAUR*, *CUL*) involved in cell division and expansion processes and their regulation (Table S5).

Red-colored anthocyanins, which give strawberries their attractive bright red appearance, are flavonoids derived from the phenylpropanoid pathway. In cultivated strawberry, allelic variants of the master regulator *MYB10* belonging to the MBW complex have been shown to be responsible for the white skin-color and red flesh-color[8,11]. In our GWAS study, we did not detect any previously known color QTL nor CG linked to the MBW complex, probably because white fruit genotypes and flesh-color trait were under-represented in our analysis. However, our diversity panel has enabled us to reveal new skin color QTLs and identify strong CG involved in the successive steps leading to anthocyanin accumulation in strawberry[11]: (i) anthocyanin biosynthesis; (ii) formation of stabilized anthocyanidin-glucosides; and (iii) transport of anthocyanidin-glucosides for storage in the vacuole. Color CG, which deserve further study, include a gene (*CSE*) encoding shikimate esterase, an enzyme involved in lignin pathway that may compete with anthocyanin biosynthesis for common substrates; several genes encoding glycosyltransferases (*GT*), among which the strawberry FaGT1 enzyme that has been shown to generate anthocyanidin 3-O-glucosides[46] and its homolog FaGT2; and a gene encoding a vacuolar flavonoid/H+-antiporter (*TT12*) that can actively transport cyanidin-3-O-glucoside to the vacuole[47].

Fruit firmness and composition

Breakdown of the cell wall (CW) is the main mechanism responsible for fruit softening during ripening. CW is mainly constituted by a cellulose-hemicellulose network immersed in a pectin matrix. Strawberry fruit softening involves the pectin-degrading enzymes polygalacturonase (PG) and pectate lyase (PL)[48]. The down-regulation of *PL*[49] and of *PG*[50] influences fruit firmness and/or shelf life of strawberry. Many additional proteins are involved in CW modifications e.g. pectin methylesterase (PME) and its inhibitors (PMEI) that control cell adhesion and elasticity through pectin esterification, enzymes of the xyloglucan endotransglycosylase/hydrolase (XTH) family involved in hemicellulose remodelling, cellulases (CEL) that degrade cellulose, and expansins (EXP) that promote CW loosening. Other enzymes such as cellulose synthase (CES) or proteins with ill-defined roles such as arabinogalactan-proteins (AGPs) likely play a role in CW structure and properties. Therefore, considerable variations in fruit firmness can be expected by

modulating the activity of enzymes encoded by CG underlying the 3D QTL (*GALT, XTH, CES*) and 6A (*CEL, PG*) QTL. *XTH* and *CES* are strong candidates located at 750 to 1,280 kb from the AX-184477554 marker in the well-conserved 3D region while *CEL* and *PG* underly the 6D FIRM QTL previously detected[4].

The sugar/acid balance is central for consumers perception of fruit quality[19] and the sugar/acid ratio has been widely adopted as a breeding target[5]. The major soluble sugars that accumulate during fruit ripening are glucose, fructose and sucrose, the concentration of which depends on the cultivar[51]. The major organic acids are malate and especially citrate, which is the predominant organic acid[48]. Their concentrations are stable or decrease during fruit ripening. Fruit sweetness is usually assessed in refractometer (Brix units), which measures total soluble solids (TSS), including sugars and organic acids. Fruit acidity is assessed by TA, to which citrate contributes most in strawberry. The accumulation in strawberry of soluble sugars and organic acids depends on synthesis in the leaf (source) and long-distance transport of photoassimilates (sucrose, inositol) to the fruit (sink). Photosynthetic sugars are further metabolized in the fruit to produce soluble sugars and organic acids that are then stored in the vacuoles[52]. Our GWAS study identified several CG implicated in the metabolism of sugars, either in the leaves or in the fruit, including *SPS* (1B QTL), *FBP* (2D and 5A QTL), *SS* (3B QTL), *FBA* (6B QTL), and *INV* (7C QTL). The starch synthase (*SS)* is located more than 1 Mb apart from the 3B QTL marker but has been recently identified as a CG for a TSS QTL[31]. The neutral invertase (*INV*), which underlies the major 7C TSS QTL (PVE 7.7%), is a strong candidate that has been shown to be crucial for glucose and fructose accumulation during ripening in tomato[53] while a cell wall invertase is responsible for a major TSS QTL in this species[54]. Another strong candidate is the hexose transporter (*HEX*) (7B and 7C QTL), which could transport glucose and fructose across the tonoplast, as suggested in grape berries[55]. Furthermore, the *HEX* gene may underlie two possible TSS homoeo-QTL located on chromosomes 7B and 7C, respectively.

Two CG underlying TSS QTL encode enzymes involved in the tricarboxylic acid (TCA) cycle, notably isocitrate dehydrogenase [NAD] (IDH) (6B QTL) and aconitase (ACO) (6C QTL). TCA is the central metabolic cycle that uses substrates from the glycolysis to produce energy. It fulfills major roles in the fruit, among which the metabolism of citric acid[56]. While aconitase has been shown to contribute to the regulation of acidity in the citrate-accumulating lemon[57], we did not detect any TA QTL corresponding to the 6C Brix QTL. Interestingly, *IDH* underlies strong shared QTL for TSS (PVE=19.0) and BA (PVE=65.8) on chromosome 6B. The implication of IDH a significant contributor to the TCA cycle, in the sugar/acid balance of strawberry, therefore merits further studies. Moreover, *IDH* is also located at ~ 675 kb from the TSS QTL on chromosome 6D, indicating that it could underlie two TSS homoeo-QTL located on chromosome 6B and 6D, respectively. As previously suggested[13], the detection of homoeo-QTL could depend on environmental conditions, which vary according to the year of study.

The CG underlying the 1A TA QTL encodes pyruvate kinase (PK) a crucial enzyme for gluconeogenesis which has already been demonstrated to regulate citric acid metabolism during strawberry fruit ripening[56]. Two additional CG for the TA QTL located on 6A (PVE 35.3%) and 6C (PVE 6.8%) encode subunits of the V-type proton ATPase (*VMA-G* and *VMA-C*), respectively. Both are strong candidates for the control of fruit acidity, as they are part of a protein complex whose role is to generate a proton gradient across the tonoplast, which is essential to drive the storage of organic acids in the vacuole of fleshy fruits[58].

Skin properties

The outermost wall of the fruit is composed of the cuticle, the epidermis and several layers of sub-epidermal cells[59]. This ill-defined tissue, also called fruit skin[60], acts as a barrier against water-loss and pathogens and provides protection against mechanical injuries[61]. Its properties depend on epidermal and sub-epidermal cell patterning (cell size and shape) and on the composition and structure of CW and cuticle. To date, the cuticle has been poorly studied in strawberry, except for its composition[62]. Recent studies, in particular in the tomato model, furthered our understanding of the synthesis of cuticle components (wax and cutin polyester, phenolics) and explored the complex interactions between cutin polyesters, CW polysaccharides and phenolics, and their possible contribution to cuticle properties[59].

Fruit glossiness is an environment-sensitive trait linked to wax and cutin accumulation on the fruit surface but also to epidermal cell patterning[63]. Among CG identified for GLOS QTL are genes involved in phenylpropanoid pathways (*CAD* in 1C QTL), epidermal patterning (*TBL* in 3D QTL), regulation of wax biosynthesis (MYS, 3D QTL), lipid and cutin biosynthesis (GPAT6, 4C QTL; GPAT3, 5A QTL) and possibly transport of cutin precursors (nsLTP, 4C and 5A)[61,64]. In addition to the *MYS* gene, a transcription factor involved through *DEWAX* in the regulation of the ECERIFERUM1 (CER1) enzyme involved in the biosynthesis of wax alkanes[65], this region harbors, within ~700 kb of 3D QTL markers, the phenolic pathway *HCT* gene that is essential for cuticle formation[66] and, close-by, three *GELP* genes. Several members of the large *GELP* family have been demonstrated to play crucial roles in cutin polymerization (cutin synthase[67]) and in assembly-disassembly of the related polyester suberin[68]. Examination at the Tomato eFP Browser (http://bar.utoronto.ca) and TEA-SGN (https://tea.solgenomics.net) databases of the expression of the three closest tomato homologs (*Solyc03g005900, Solyc02g071610, Solyc02g071620*) of the 3D GLOS QTL-linked *GELP* genes indicate that they are strongly expressed in the young fruit, when the cutin synthesis rate is the highest[63]. Furthermore, in the tomato pericarp, their expression is restricted to the outer and inner epidermis. These findings strongly suggest that, in cultivated strawberry, a cluster of genes with likely roles in cuticle formation and structure has been selected in modern varieties for its impact on fruit cuticle-related traits, including GLOS.

Remarkably, we found that the major skin resistance (SR) QTL, which estimates the fragility of the fruit surface to peel off when a mechanical stress is applied, is shared with the GLOS QTL on 3D. The major 3D FIRM QTL (PVE 11.2%) was also found nearby (at ~1,400 kb). Since the FIRM trait was estimated by measuring the force needed to punch a hole in the fruit surface (penetrometer), it can be linked to the properties of the fruit skin. Interestingly, connections between fruit firmness and the cuticle have recently been demonstrated in tomato where changes in cuticle composition and properties are responsible for a major firmness QTL[69]. Altogether, these results suggest that in the 3D conserved region, modifications of fruit surface properties, either due to changes in epidermal cell patterning and/or in cell wall and cuticle properties, have been selected in modern strawberry varieties for their effect on both fruit glossiness, resistance to mechanical damages, and possibly firmness. Other candidates linked to either epidermis patterning (*TBL* on 4A), cell wall modifications (*COBL* and *BXL* on 4A, *PMEI* on 5C, *PG* and *EXP* on 7A) and cuticle formation (*DEWAX*, a target of MYS, and *WSD* on 7A) underly the additional SR QTL detected.

In contrast, none of the QTL detected for fruit bruisedness (BRU), a trait assessed visually, were found to co-localize with either GLOS, SR or FIRM QTL while all these traits are strongly correlated, indicating that the underlying mechanisms are probably different or that the corresponding QTL are below the detection threshold. CW-related GC that may affect cell wall properties (*PL* on 5A, *XTH* on 6B) or cell adhesion of sub-epidermal cells (*FLA* on 6B[70]) merit further investigation, as fruit susceptibility to bruising is essential for post-harvest handling and defense against fruit decay.

Conclusion

In summary, the exploration of untapped genetic resources, including European cultivars spanning 160 years of breeding, has revealed considerable changes in recent decades in the genetic and phenotypic diversity of cultivated strawberry. American cultivars have had a major impact on recent European breeding programs and, therefore, on modern strawberry varieties in Europe. However, our findings also revealed that a considerable, and previously undescribed, genetic diversity can be harnessed for improving fruit quality through breeding. Our study also highlights the contribution of fruit surface traits (glossiness, skin resistance, bruisedness) to the development of modern varieties. The strong CGs underlying the main QTL detected for these little studied traits warrant further investigations. This can be done, for example, through additional association studies or functional analyses. From a more applied perspective, the genetic markers highlighted will be used for the selection of improved strawberry varieties with high fruit quality.

Materials and methods

Plant materials and experimental design

A total of 223 accessions from the historical germplasm collection of Invenio was chosen to constitute the diversity panel. The trial took place in a soilless system, at Douville in the South-West of France (45° 1.2831' N; 0° 37.0198' E, France). The crop management was the one used for commercial semi-early cultivated strawberry in France. The trial was organized in a randomized complete block design of two blocks of four biological replicates each in a 288 m² glass greenhouse in 2020 and 2021. Planting of tray plants occurred around the 15th of December of the previous year.

Sample preparation and phenotyping

Fruits were harvested once per season and evaluated for 12 fruit quality traits: FW, fruit weight; UFS, uniformity of fruit shape; COL, skin color; UCOL, uniformity of skin color; ACH, position of achenes; FIRM, firmness; TA, titratable acidity; TSS, total soluble solids; BA, TSS/TA ratio; GLOS, glossiness; SR, skin resistance; BRU, bruisedness. FW was evaluated as the mean weight of harvested fruits after discarding immature and overripe fruits. UFS, UCOL, ACH as well as GLOS and BRU were visually assessed on 1-5 scales (Table 1) as a single note on a whole strawberry tray (>10 red ripe fruits). COL was evaluated on 4-5 red ripe fruits a 1-8 scale based on the strawberry color chart from Ctifl (http://www.ctifl.fr/Pages/Kiosque/DetailsOuvrage.aspx?IdType=3&idouvrage=833). FIRM was evaluated on six fruits from each accession with an FTA-GS15 (Güss) penetrometer (5 mm diameter) at 3 mm depth (5 mm/s speed, 0.06kg release threshold). SR was evaluated on three fruits per accession on a 1-5 scale by applying an ascending pressure with the extremity of the thumb on the fruit surface. Bruisedness, which represents the susceptibility of the fruit to mechanical damages, was evaluated by visual inspection of the fruits 4 h after harvest. Analyses were performed for two consecutive years except for FIRM and SR traits which were evaluated a single year in 2021. TA and TSS were evaluated from a homogenized pool of a minimum of 10 fruits with a pH-metric titration with sodium hydroxide of 10 g fruit puree and an Atago Handheld (PAL-1) Digital Pocket Refractometer (Atago, Saitama, Japan), respectively.

Statistical analysis

Best Unbiased Linear Predictors (BLUPs) for the diversity panel were calculated using a linear mixed model (LMM) from the lme4 R package[71]:

$$yijkl = \mu + \underline{Gi} + Bk + Yl + \underline{(G:Y)}il + \varepsilon ikl$$

where Y/E represented the fixed effects of year/environments; B the fixed effect of blocks; G the random genotypic effect, with G ~N(0, $\sigma g^2 I$); GxY/E the random genotype x year/environment effects, with GxY/E ~N(0, $\sigma Y/E^2 I$); ε the residual term, with e ~N(0, $\sigma e^2 I$).

Variance components for these effects were estimated using restricted maximum likelihood (REML). Broad sense heritability was estimated as follows:

$$H^2 = \frac{\sigma^2 G}{\sigma^2 G + \frac{\sigma^2 G{:}Y}{nyear} + \frac{\sigma^2 e}{nyear\ x\ nrep.year}}$$

where genotype (G) variance at the numerator. Random variance components involving year (Y) were divided by the mean number of years (*nyear*). Other random variance components involving block effects or residuals were divided by the mean number of years times the mean number of replicates per year (*nrep.year*).

Pearson correlation between different traits were calculated using 'cor' function and visualized by 'corrplot' v. 0.92 R package. PCA on all traits was performed using the prcomp function from R core and visualized with fviz_pca function from factoextra v.1.0.7 package or ggplot2 package. The impact of the structure on each variable was assessed by simple regression of the genetic groups on their respective phenotypes.

Genotyping

DNA was extracted from young leaves with a CTAB method adapted from Sánchez- Sevilla *et al.*, (2015)[34]. Samples were genotyped using Affymetrix® 50K FanaSNP array[22] in the 'Gentyane' genotyping platform (Clermont-Auvergne-Rhône-Alpes, INRAE, France). SNP calling was processed through Axiom™ Analysis Suite software (v5.1.1.1; Thermo Fisher Scientific, Inc.) following the best practices of the software documentation. Accessions with missing data higher than 3% were removed from analysis. Markers presenting more than 5% of missing data and minor allele frequencies of less than 5% were filtered out.

Structure and genetic diversity analysis

We performed a structure population analysis using STRUCTURE (v2.3.4[72]) with 5 runs for a range of K = 2 to 10 with 38,120 markers. The burn-in period length was set to 10,000 and 20,000 Markov Chain Monte Carlo (MCMC). The best fitting K was identified with STRUCTURE HARVESTER[73]. Plots were performed using the ggplot2 v.3.3.6 package[74]. PCA analyses were performed with PCA function from factorMinerR v.2.7 package[75]. Additionally, we included genotypes from Hardigan et al., (2021b)[4] and Zurn et al. (2022)[28] to perform PCA using the prcomp function from R core and visualize with fviz_pca_ind function from factoextra v.1.0.7 package or ggplot2 package. We conducted a ML tree with the 233 accessions using IQ-TREE v.2.1.3[76] with 1000 bootstrap and the TVMe+ASC+R3 model suitable for SNP arrays. Linkage disequilibrium for each chromosome and genetic group was computed using the LDcorSV v.1.3.3 package[77]. Nucleotide diversity among each genetic group was calculated using TASSEL[78]. Finally, we performed principal component analysis-based genomes scans to detect markers under selection using the pcadapt package[79], implementing the pcadapt function with K = 3. Output from genome scans were then compared with nucleotide diversity profiles to search for selective sweeps.

632

633 **Genome-Wide Association Study**

634 The association mapping was performed using GAPIT v.3[80] using the Camarosa genome physical positions[1]
635 with the Bayesian-information and linkage-disequilibrium iteratively nested keyway (BLINK) model[81]. In
636 order to control for confounding effects, the structure was implemented for each trait in two different
637 ways by 1) adding the previously calculated structure parameters as covariates or 2) fitting directly principal
638 components from the principal component analysis using the PCA.total argument. Best models were
639 selected based on genomic inflation factors, λ. The kinship was determined from the SNP data using the
640 VanRaden mean algorithm. The analysis was performed on yearly and across two years Best Linear
641 Unbiased Predictors (BLUPs), using a 5% Bonferroni threshold. Manhattan and Quantile-Quantile plots
642 were plotted using the CMplot R package. Allelic effects for each significant marker were plotted on
643 adjusted means using the ggplot2 R package.

644

645 **Candidate gene mining**

646 Candidate genes (CG) underlying fruit quality QTL were identified in intervals of ~400 kb around the QTL
647 marker. This value, which corresponds to the short-range LD found in California cultivars of *F.* × *ananassa*[4],
648 is stringent compared to the average LD of 932 kb calculated in our diversity panel. In chromosomal regions
649 harboring strong QTL of interest and displaying low genetic diversity and high LD, larger intervals (up to
650 1,382 kb) were considered. The annotations of genes located within the QTL interval were retrieved from
651 *F.*× *ananassa* cv. Camarosa reference genome assembly v1.0[1] and cv. Royal Royce haplotype-resolved
652 genome v1.0 assembly[23]. Based on the authors' expertise in fruit biology, QTL intervals were first inspected
653 manually for genes belonging to categories possibly related to fruit quality traits, e.g. enzymes, transporters
654 and regulators of anthocyanin biosynthesis for fruit color[11,14,18], enzymes and regulators of wax and cutin
655 biosynthesis pathways for glossiness and skin resistance[59,61,63,64,67], and enzymes of primary metabolism,
656 organic acid transporters and proton pumps for titratable acidity[13,51,82,83,84]. Their function in plant and fruit
657 was then investigated by exploiting the Arabidopsis database (https://www.arabidopsis.org/) with
658 corresponding TAIR accession numbers; the relevant literature, especially that relating to strawberry and
659 other fleshy fruit species of the *Rosaceae* family; and gene expression patterns in strawberry fruit using *F.*
660 *vesca* eFP browser[85]. For skin-associated traits, patterns of gene expression in plant organs and fruit cell
661 types were further investigated in tomato, the fleshy fruit and cuticle model, using SGN-TEA
662 (https://tea.solgenomics.net/) and Tomato eFP browser
663 (http://bar.utoronto.ca/efp2/Tomato/Tomato_eFPBrowser2.html).

664

665 **Conflict of interest statement**

666 The authors declare that there are no conflicts of interest.

Author's contributions

BD and AlP conceived and designed the experiments. AlP conducted hands-on experiments and data collection. AuP, JoP and JuP contributed to data collection. AlP, PRS, BD and CR analyzed the data. AlP, BD and CR wrote the original draft. All authors read and approved the final manuscript.

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Data availability

All relevant data generated or analyzed are included in the manuscript and the supporting materials.

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