A Genome-Wide Association Study of Apple Quality and Scab Resistance


Abstract

The apple (Malus × domestica Borkh.) is an economically and culturally important crop grown worldwide. Growers of this long-lived perennial must produce fruit of adequate quality while also combating abiotic and biotic stress. Traditional apple breeding can take up to 20 yr from initial cross to commercial release, but genomics-assisted breeding can help accelerate this process. To advance genomics-assisted breeding in apple, we performed genome-wide association studies (GWAS) and genomic prediction in a collection of 172 apple accessions by linking over 55,000 single nucleotide polymorphisms (SNPs) with 10 phenotypes collected over 2 yr. Genome-wide association studies revealed several known loci for skin color, harvest date and firmness at harvest. Several significant GWAS associations were detected for resistance to a major fungal pathogen, apple scab (Venturia inaequalis [Cke.] Wint.), but we demonstrate that these hits likely represent a single ancestral source. Using genomic prediction, we show that most phenotypes are sufficiently predictable using genome-wide SNPs to be candidates for genomic selection. Finally, we detect a signal for firmness retention after storage on chromosome 10 and show that it may not stem from variation in PG1, a gene repeatedly identified in bi-parental mapping studies and widely believed to underlie a major QTL for firmness on chromosome 10. We provide evidence that this major QTL is more likely due to variation in a neighboring ethylene response factor (ERF) gene. The present study showcases the superior mapping resolution of GWAS compared to bi-parental linkage mapping by identifying a novel candidate gene underlying a well-studied, major QTL involved in apple firmness.

Core Ideas

- Well-studied and novel associations detected using next-generation sequencing data and genome-wide association studies in apples.
- Several notable associations detected for apple scab using historical data.
- More studies in wider breeding material needed to assess suitability for marker-assisted selection.

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Abbreviations: ACO, 1-aminocyclopropane-1-carboxylate oxidase; CET, Cultivar Evaluation Trial; ERF, ethylene response factor; GBS, genotyping-by-sequencing; GDR, Genome Database for Rosaceae; GEBV, genomic estimated breeding values; GS, genomic selection; GWAS, genome-wide association studies; HWE, Hardy-Weinberg Equilibrium; IBD, identity-by-descent; LD, linkage disequilibrium; LRR, leucine-rich repeat; MAF, minor allele frequency; MAS, marker-assisted selection; PCA, principal components analysis; PG, polygalacturonase; QTL, quantitative trait locus; SNP, single nucleotide polymorphism; SSC, soluble solids content; TA, titratable acidity.

Plant Genome 11:170075
doi: 10.3835/plantgenome2017.08.0075

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Genomics technologies are increasingly being used to help breeders overcome the challenges involved in maintaining a safe and secure food supply while satisfying consumer demands. Apples are one of the most widely grown perennial fruit crops and some cultivars of apples have been propagated vegetatively for well over a hundred years, which leaves them frozen in genetic time and at the mercy of evolving pests and diseases (Myles, 2013). Con founding this problem is the fact that most international apple breeding programs are based on a narrow gene pool (Kumar et al., 2014a). Clearly there is a need for new cultivars to meet future climatic and consumer demands, but development of new apple cultivars is hampered by constraints at both the genetic (self-incompatibility and inbreeding depression), and management (large plant size and long juvenile period) levels (Brown and Maloney, 2003).

The goal of many apple breeding programs is to combine high fruit quality with disease resistance (Brown and Maloney, 2003). Molecular markers and marker-assisted selection (MAS) are currently used by apple breeders to achieve this goal: genotyping of greenhouse seedlings results in more desirable seedlings being planted in the field, thereby cutting down on cost and land requirements (Brown and Maloney, 2003). To that end, the release of an apple reference genome (Velasco et al., 2010) has greatly aided the efforts of MAS in apple breeding. Genetic marker data can be generated using several approaches, including SNP arrays (Bianco et al., 2014; Bianco et al., 2016), and SNP discovery using the next-generation sequencing genotyping-by-sequencing (GBS) approach (Elshire et al., 2011). One of the main advantages of GBS compared to SNP arrays is that one is not limited by a fixed set of SNPs when genotyping (Chagné, 2015). Another advantage is that the cost per data point for genotyping and whole genome sequencing continues to decline (Isik et al., 2015).

Quantitative trait locus (QTL) mapping in bi-parental populations has been the primary method to uncover markers linked to traits of interest. However, across studies there is limited crossover of markers, and even phenotyping protocols (Costa, 2015). Other challenges include the need for large populations of related individuals, and the accompanying limited number of recombination events (Soto-Cerda and Cloutier, 2012). An alternative is genome-wide association studies (GWAS), which allow for a higher resolution of causal loci in comparison to past mapping techniques (Ogura and Busch, 2015). Genome-wide association studies use diverse populations of unrelated individuals to explore genotype-phenotype relationships. Genome-wide association studies in apples can be particularly challenging due to apples’ heterozygous nature, their relatively recent whole genome duplication, and the rapid rate of linkage disequilibrium (LD) decay (Bianco et al., 2016). Nonetheless, GWAS have been used to explore several important fruit quality traits such as titratable acidity, soluble solids content, crispness, juiciness, flavor intensity, russetting, weight, skin color, harvest season, fruit firmness, and flesh mealliness (Kumar et al., 2013; Kumar et al., 2014b; Migicovsky et al., 2016; Moriya et al., 2017; Amyotte et al., 2017). Finally, genomic selection (GS) has recently emerged as another method to accelerate apple breeding. In GS, one attempts to predict the genetic value of potential selections using their genomic estimated breeding values (GEBVs) (Newell and Jannink, 2014). In GS, a training population is used to generate a predictive equation, then genotypic data from a population of non-phenotyped individuals (a selection population) is entered into the model, and GEBVs computed (Kumar et al., 2014a). It is thought that GS and calculated GEBVs may capture more genetic variation, as GS incorporates both minor and major marker effects (Newell and Jannink, 2014).

The present study uses genome-wide SNP data generated using GBS to determine the ability of markers to predict fruit quality traits and scab resistance in a collection of diverse apple cultivars. We show that most traits are predictable using all markers and that several traits are controlled by a small enough number of loci of large effect that we detect the resulting SNP-trait association signal using GWAS. We also highlight some of the challenges when performing GWAS in apples.

Materials and Methods

The Cultivar Evaluation Trial (CET) population

Apple trees used for this study consisted of the Nova Scotia Fruit Growers’ Association Cultivar Evaluation Trial (CET) orchard, based at the Agriculture and Agri-Food Canada Kentville Research and Development Centre in Nova Scotia, Canada. The purpose of this orchard was to evaluate cultivars from international breeding programs for suitability to the local industry (see Supplemental Table S1 for a list of cultivars). Trees were planted annually (1996-2011) in a randomized completed block design, so that for a given year, all cultivars (N total = 172) were evenly distributed within a row. Therefore, trees varied in age, but all trees were physiologically mature at the time of data collection. All trees were grafted onto M.9 dwarfing rootstock, with trees planted 1 m apart, with 5 m between rows. One goal of the CET was to assess cultivars resistant to apple scab, and therefore roughly half of the cultivars were scab resistant, based on breeding records. Cultivars reported to be scab resistant were planted in a separate block from the cultivars reported to be scab susceptible. Trees were maintained according to standard horticultural practices for the area, including fungicide applications for scab susceptible cultivars, and no supplemental irrigation was applied. Trees were hand thinned in mid-July in 2013 to adjust crop load to commercial standards of one fruit per cluster with 10 to 15 cm between each fruit within the canopy. In 2014, trees were first chemically thinned using Sevin XLR at 5 L/ha \( \times \)ha \( ^1 \) when ‘Honeycrisp’ fruitlets were ~12 mm in diameter (mid-June), and hand thinning was completed to commercial standards in mid-July.

Phenotype Data Collection

In 2013 and 2014, fruit were harvested based on historical harvest dates, background skin color change,
fruit palatability (i.e., loss of bitter flavor), and seed color (Watkins, 2003). Harvest date was recorded as Julian day number. Forty fruit per cultivar were harvested, with fruit selected randomly from all replications of bearing trees within a cultivar. The maximum number of replicates per tree was 28 and the minimum number was two with a mean replicate number of six. Measurements were taken on either the same day or no more than two d after harvest. All measurements were conducted on 10 fruit per cultivar, except for soluble solids content (SSC) and titratable acidity (TA), which were completed on a composite juice sample from all 10 fruit. Fruit weight (g) was measured on individual fruit using a digital scale and the mean from 10 fruit was reported. Fruit skin color was scored, by eye, as the percentage of red blush covering the fruit surface. Firmness was measured in C Peak pounds (lb) on the red and green side of each fruit after a thin slice of skin was removed, using a fruit quality tester (Geo-Met Instruments, New Minas, NS, Canada), and then averaged across 20 measurements (median SD 2013 = 1.25, median SD 2014 = 1.21). Soluble solids content (%) was calculated using a hand-held digital refractometer (Atago Co., Tokyo). Titratable acidity was measured as mg malic acid×100 mL⁻¹ of juice using a 2 mL juice sample mixed in 40 mL of distilled water, and titrated using 0.1 M NaOH until the phenolphthalein color change was observed using an automated titrator (Brinkmann Model 350; Metrohm, Herisau, Switzerland).

Following the measurements taken at harvest, the remaining fruit were kept at 0.8 to 1°C in refrigerated air in stacked, perforated plastic bins covered in plastic sheets to retain moisture. After three mo of storage, fruit were removed, allowed to come to room temperature, and measured for post-storage characteristics. When possible, 10 fruit were chosen randomly for post-storage measurements including firmness, SSC, and TA. In 6 cases across both years where many fruit were rotten and not suitable for measurements, sample sizes were < 5. Changes in firmness, SSC, and TA were calculated by subtracting the measurements at harvest from the post-storage measurements. The correlation between all quantitative traits across years was examined using the cor.test (Pearson’s) function in R, and presented using the heatmap.2 function of the “gplots” package in R, after Bonferroni correction for multiple testing.

Genotype Calling

DNA extraction and sequencing via GBS (Elshire et al., 2011) using the restriction enzymes ApelKI and Pstl-EcoT221 were completed on 176 unique CET apple accessions. Libraries were sequenced with 100-bp, single-end reads on the HiSeq2000 (Illumina, San Diego, CA) by multiplexing 96 samples/lane. The 176 samples analyzed there were distributed across multiple lanes of sequencing that included samples from other populations of apples. Genotypes were called from .fastq files using TASSEL version 5.2.28 (Bradbury et al., 2007), and reference genome version 1.0p (https://www.rosaceae.org/species/malus/malus_x_domestica/genome_v1.0p). Quality filters included: i) a minimum depth of eight and max depth of 100 reads per site per cultivar, ii) sites deviating from Hardy-Weinberg Equilibrium (HWE) at P < 0.0001 were removed, iii) sites with more than 70% missing data were removed, iv) sites with fewer than 10 copies of the minor allele across all samples were removed, and v) only bi-allelic SNPs were retained. The HWE filter was implemented to remove SNPs with excess heterozygosity that are likely not “true SNPs” but are rather the result of paralogous genomic regions that are falsely represented as single copy unique regions in the reference genome. These filtering steps resulted in 31,809 SNPs and 43,650 SNPs from the ApelKI and PstI-EcoT221 data sets, respectively. The two resulting variant call format files were then merged in TASSEL, and samples with more than 70% missing data were removed. This resulted in 75,130 SNPs for 172 samples with an average of 32% missing data by individual and SNP (see Supplemental Fig. S1 for distribution of proportion of missing data by [A] cultivar and by [B] SNP). Genotypes were then imputed using LinkImpute v1.1 (Money et al., 2015) with the number of nearest neighbors (k) = 9 and number of SNPs (l) = 25, which resulted in an imputation accuracy of 93.4%. LD decay in the population was calculated using the –r2 command in PLINK (Purcell et al., 2007).

Genome-Wide Association Studies and Genomic Prediction for Fruit Quality

Genome-wide association studies were performed for fruit quality data in 2013 and 2014 using TASSEL version 5.2.28 for quantitative traits, and CARAT version 1.3 (Jiang et al., 2016) for binary traits, after removing SNPs that were not anchored to one of the 17 chromosomes, and filtering for a minor allele frequency (MAF) of 0.05. This resulted in 55,361 SNPs in 2013, 55,748 SNPs in 2014, and 55,147 SNPs for the GWAS for scab resistance. For all fruit quality traits analyzed using TASSEL, GWAS were performed using the WeightedMLM (PCA + K), except for scab resistance, which was run using just K to enable a meaningful comparison to the CARAT results. Quantitative data were transformed using the Box-Cox transformation only if transformation improved the normality of the residuals from the GWAS. The significance threshold was determined for GWAS run using 2013 data, 2014 data, and historical scab records separately, using the “simpleM” package in R (Gao et al., 2010), which calculates the effective number of independent tests (M_eff). The significance threshold was drawn as -log₁₀(a/M_eff) where a was set to 0.05. Candidate genes for each trait were identified as those within a 100 kilobase-pair (kb) window centered on a significant SNP using the Genome Database for Rosaceae (GDR) GBrowse tool (https://www.rosaceae.org). Genomic prediction accuracies were calculated using the x.val function of the “PopVar” R package, using rrBLUP for cross-validation with nFold = 5 and nFold.reps = 3 (Mohammadi et al., 2015). Finally, high resolution DNA melting was used to genotype a SNP in the PGI gene (G/T SNP at position 437 of GenBank accession L27743.1) described in Costa et al. (2010). The forward primer sequence used
was S'-TCCTTCATAACCGGACAC-3', and the reverse primer was S'-CTTGGCCTGATCAATTCCAT-3'.

Analysis of SNPs Associated with Apple Scab Resistance

Ninety-three SNPs significantly associated with apple scab were found on seven different chromosomes. Linkage disequilibrium between all pairs of scab-associated SNPs was calculated using the --r2 command in PLINK (Purcell et al., 2007). Because scab-associated SNPs within a chromosome were tightly clustered physically, it was assumed that these clusters of scab-associated SNPs likely each represented a single locus. To determine how many of the seven putative scab resistance loci each accession carried, the TASSEL WeightedMLM output was used to determine which allele was associated with scab resistance (see Supplemental Table S4). Next, the summed number of resistance genotypes (i.e., presence of the resistance allele in homozygous or heterozygous state) present within each accession at each of the seven putative loci was calculated. These values enabled us to determine unequivocally whether an accession carried the resistance allele at each of the seven loci.

Principal components analysis (PCA) was performed on 32,077 SNPs that had been LD-pruned (plink commands: indep-pairwise 10 0 0.5) and then filtered for MAF 0.05 using the prcomp function in R (R Core Team, 2016) after centering and scaling of the data. The pairwise identity-by-descent (IBD) was calculated in PLINK using the --geno command and \( \pi \). For the IBD analysis, we considered an accession ‘scab resistant’ if it was labeled as scab resistant in the orchard and also possessed all seven resistance loci. Similarly, an accession was considered ‘scab susceptible’ if it was labeled as scab susceptible in the orchard and carried no resistance loci according to our analyses. All pairwise IBD values within the scab resistant group (SR:SR) were compared to pairwise IBD values between scab resistant and scab susceptible cultivars (SR:SS), using the Mann – Whitney U test (W test statistic). The Cross Population Extended Haplotype Homozygosity (XP-EHH) statistic (Sabeti et al., 2007) was calculated between susceptible and resistant accessions using the program ‘selscan’ (Szpiech and Hernandez, 2014) from genotype data that had not been filtered for deviations from HWE (see above), but had been phased using ‘fastPHASE’ (Scheet and Stephens, 2006). Dotted lines in XP-EHH figures delinate the upper 1% quantile of SNPs genome-wide.

Results

Phenotype data

Correlations between all pairs of phenotypes over 2 yr were evaluated, and are displayed as a heatmap in Fig. 1 (see Supplemental Fig. S2 for numerical values, and Table S2 for phenotype data summary). For traits that were measured in both years, all showed strong positive correlations \((r \geq 0.7\) and \(p < 1 \times 10^{-15}\)) between the 2 yr of data except for SSC \((r = 0.39, p = 8.1 \times 10^{-5}\) and change in fruit firmness during storage \((r = 0.61, p = 1.5 \times 10^{-14}\)). In both years, we found that firmness at harvest was correlated with firmness after storage \((r > 0.75, p < 1 \times 10^{-15}\) for both 2013 and 2014). In the current study, harvest began in the first week of August and was completed in the first week of November and we found that harvest date was positively associated with firmness at harvest \((2013 \ (r = 0.48, p = 3.1 \times 10^{-11}); 2014 \ (r = 0.60, p = 1.1 \times 10^{-13})\) and after storage \((2013 \ (r = 0.51, p = 1.5 \times 10^{-9}); 2014 \ (r = 0.53, p = 3.6 \times 10^{-10})\)). In both years, harvest date was also positively correlated with SSC \((2013, r = 0.49, p = 1.5 \times 10^{-4}; 2014, r = 0.41, p = 1.5 \times 10^{-5}\)). In both years, fruit weight was correlated with SSC, suggesting that larger apples also had higher sugar content \((2013, r = 0.45, p = 4.5 \times 10^{-2}; 2014, r = 0.34, p = 0.002)\). Finally, in both years firmness after storage was positively correlated with change in firmness \((2013, r = 0.53, p = 1.6 \times 10^{-10}; 2014, r = 0.35, p = 0.002)\).

Genome-Wide Association Studies and Genomic Prediction for Fruit Quality

Genome-wide association studies were performed on nine fruit quality traits separately for each year and the results are presented in Supplemental Fig. S3 and Tables S3 and S4. Three noteworthy associations are highlighted in Fig. 2A-C. In both years, there were SNPs significantly associated with fruit skin color on the distal ends of chromosomes 1 and 9 (Fig. 2A). Although it did not exceed the significance threshold, a peak was observed on chromosome 3 associated with harvest date in 2013 (Fig. 2B). For change in firmness following storage in 2013, one significantly associated SNP was found near the center of chromosome 10 (Fig. 2C). This peak was not observed for GWAS using change in firmness data from 2014, despite a significant positive correlation between years for this trait \((r = 0.61, p = 1.548 \times 10^{-14})\).

When a 100 kilobasepair (kb) window centered on each significant SNP was analyzed, several candidate genes were identified (Supplemental Table S5). Two of the SNPs on chromosome 9 associated with fruit skin color (S9_32743361; S9_32743362) are located approximately 100 kb upstream of a MYB transcription factor (MDP0000259614). While the peak for harvest date (Fig. 2B) was not above the significance threshold, it was near the NAC5 gene (MDP0000868419) previously identified by Migicovsky et al. (2016) as a candidate gene for harvest date and fruit firmness. Within the 100 kb window surrounding the SNP significantly associated with change in firmness in 2013 (S10_21375724), there is an ERF (MDP0000855671) that is a candidate gene for this phenotype. Interestingly, PG1 (MDP0000326734), a well-studied gene involved in fruit texture (Costa, 2015), is found nearby but does not lie within this 100 kb window: it is found 500 kb from the most significant SNP. To determine whether the GWAS hit was in fact capturing an association with PG1, 98 cultivars were genotyped for a putatively functional SNP in PG1, which has been shown to predict firmness in several apple populations (Costa et al., 2010). The GWAS for change in
firmness was re-run including the PG1 SNP, for which genotypes were in Hardy-Weinberg equilibrium (CC = 26, CT = 44, TT = 28) and the minor allele frequency was 0.41. We found that while no SNPs exceeded the significance threshold, likely due to reduced power due to the population size, the PG1 SNP (S10_20843047; red dot on graph) showed no signal of association \( (p = 0.02561, \text{ at } M_{\text{eff}} \text{ significance threshold of } 1.539 \times 10^{-6}) \), while the SNP originally identified as the most significant still showed the strongest association (S10_21375724; indicated with a blue dot on the graph; Supplemental Fig. S4).

Finally, the ability to predict phenotypes using genomic prediction was evaluated by performing fivefold cross validation (Fig. 3). Predictability was measured as the correlation \( (r) \) between predicted and observed values, and ranged from 0.08 (SD = 0.11) for change in firmness (2014), to 0.72 (SD = 0.07) for scab resistance.

### Analysis of SNPs Associated with Apple Scab Resistance

The GWAS performed using TASSEL revealed 93 SNPs across seven chromosomes that were significantly associated with scab resistance (Fig. 4A). Several of these loci overlapped with signatures of selection for scab resistance as identified through an XP-EHH analysis (Fig. 4B). A Manhattan plot for each chromosome containing a locus associated with apple scab, together with the XP-EHH scan, are shown in Supplemental Fig. S6. For the SNPs associated with apple scab resistance (Fig. 4A), nearby predicted genes included several leucine-rich repeat (LRR) genes on chromosome 1 (MDP0000432882, MDP0000213589, MDP0000088815, MDP0000318241, MDP0000792424, MDP0000473154), 4 (MDP0000875229), 12 (MDP0000236942, MDP0000741253), and 15 (MDP0000282967) (Supplemental Table S5). A BLAST query suggested that in addition to LRR properties, several of these genes also shared similarity with the apple scab Rvi6 resistance gene or paralogs (HcrVf1-4). As apple scab resistance was recorded as a binary trait, TASSEL GWAS results were compared to another association mapping model designed for binary data (CARAT). The two methods gave similar results, and the \(-\log_{10} P\)-values were correlated \( (r = 0.75, p = < 2.2 \times 10^{-16}; \text{ Supplemental Fig. S5}) \).
The GWAS for scab resistance indicated that there were potentially seven independent loci on separate chromosomes associated with scab resistance (Fig. 4A). The LD between scab-associated SNPs was investigated to determine the degree to which the identified loci represented independent genetic sources of scab resistance. Figure 5A displays the correlations between all 93 scab-associated SNPs as a heatmap. The heatmap suggests significant LD not only within chromosomes, but also between chromosomes. Figure 5B shows the extent
of LD between SNPs on the same chromosome and between SNPs on different chromosomes. By comparing the \( r^2 \) values within and between chromosomes, it was found that intra-chromosomal LD was not significantly different from inter-chromosomal LD (Mann–Whitney U-test, \( W = 2 \times 10^6 \), \( p = 0.900 \)).

The TASSEL GWAS effect output file was used to determine which allele was associated with resistance at each of the 93 significant SNPs across the seven loci. A cultivar was deemed to possess resistance at a locus if it carried at least one copy of the resistance allele across the majority of SNPs on that chromosome. Each cultivar’s status across the seven resistance loci was displayed as a function of whether the cultivar was originally labeled as resistant or susceptible (Fig. 6A). In most cases, cultivars labeled as resistant had all seven resistance loci and those labeled as susceptible did not. However, of the 84 cultivars labeled as resistant, 15 had no resistance alleles.

Fig. 4. The (A) GWAS and (B) Cross Population Extended Haplotype Homozygosity (XP-EHH) test results for the historical scab resistance data. In the top panel, the solid line represents the \(-\log_{10}(\alpha/M_{\text{all}})\) value significance threshold. In the bottom panel, the dotted line represents the upper 1% quantile of XP-EHH values. The precise genomic coordinates and the names of the genes falling within the associated region are listed in Supplemental Table S5.

Fig. 5. A comparison of linkage disequilibrium (LD) represented by \( r^2 \) of the 93 SNPs that produced significant GWAS results for historical scab resistance. (A) A heatmap of LD (\( r^2 \)) between SNPs, where darker blue colors represent stronger positive correlations between SNPs. (B) A comparison of \( r^2 \) among SNPs on the same chromosome (green = intra-chromosomal \( r^2 \)), and SNPs on different chromosomes (blue = inter-chromosomal \( r^2 \)). The intra- and inter-chromosomal \( r^2 \) values were similar, and not significantly different (\( W = 2 \times 10^6 \), \( p = 0.900 \)).
Similarly, of the 88 cultivars labeled as susceptible, three possessed all seven resistance loci.

If there was predominantly one genetic source of scab resistance in the population studied here and it was introgressed relatively recently from a single ancestor, it would be expected that scab resistant cultivars would be more closely related to each other than they are to scab susceptible cultivars. To investigate this, IBD was calculated for
all pairwise comparisons between scab resistant cultivars (SR:SR), and all pairwise comparisons between resistant and susceptible cultivars (SR:SS). It was found that IBD for SR:SR was significantly higher than for SR:SS (Fig. 6B) (Mann–Whitney U-test, \( W = 906080, p = 0.003 \)), although the mean IBD values were similar (SR:SR = 0.102, SR:SS = 0.095). To further investigate the relationships among all cultivars as a function of both their genetically-inferred and their labeled scab resistance status, each cultivar was labeled according to both of these statuses and displayed on a PCA plot generated from the genome-wide genotype matrix (Fig. 6C). Weak correlations between the number of resistance alleles and the two major axes of genetic variation were found (PC1: \( r = 0.18, p = 0.02; \) PC2: \( r = 0.22, p = 0.004 \)), as well as slight differences in PC1 or PC2 scores between cultivars labeled as susceptible and those labeled as resistant (PC1: \( W = 5000, p = 0.003 \); PC2: \( W = 4000, p = 0.02 \)).

**Discussion**

The development of new tree fruit cultivars is an expensive and time-consuming endeavor, but it is believed that new genomic technologies will help with its advancement by elucidating the genetic basis of commercially important traits and enabling MAS (Ishik et al., 2015; Edge-Garza et al., 2015). Arguably the most crucial steps toward establishing robust genotype-phenotype associations that lead to MAS are the design of mapping populations and the collection of high-quality phenotypic data (Ogura and Busch, 2015). Phenotypic data collection for robust GWAS in tree fruit is challenging, as it can be prohibitively expensive to evaluate sufficiently large populations, across years and locations to control for non-genetic factors (Soto-Cerda and Cloutier, 2012; Myles, 2013). In this study, care was taken to limit such effects and this is evident from the strong correlations observed within traits across years (Fig. 1). However, GWAS results were not always consistent across years (Fig. 2B, 2C; Supplemental Fig. S3). It should be noted that apple maggot (Rhagoletis pomonella [Walsh]) pressure was greater in 2013, and fruit received a chemical thinning spray in 2014, followed by hand thinning after fruitlet drop, as opposed to solely hand thinning in 2013. In addition, because replicate samples were not randomly distributed throughout the orchard, we were unable to control for spatial effects within the orchard. To what degree these potential confounding effects influenced downstream analyses remain unknown. Change in firmness after storage measurements were significantly correlated across years (\( r = 0.614, p = 1.548 \times 10^{-15} \)), but the absence of a GWAS peak in 2014 that was clearly present in 2013 together with a dramatic difference in genomic prediction accuracies for this trait between years highlight the sensitivity of this type of analysis to between-year variation. Replicating phenotype measurements across clonal trees in addition to collecting composite fruit samples likely would have better captured phenotype variability in the CET population (Brown, 2012), but was cost prohibitive. Attempts here to provide post-hoc explanations for the observed discrepancies in GWAS results between years highlight the importance of consistent data collection in single location, multi-year trials.

In addition to between-year correlations, several noteworthy correlations between phenotypes were observed (Fig. 1). We found that late apples have higher sugar content, are firmer, and tend to be among the firm apples after storage. Early apples are generally lower in sugar and tend to be softer at harvest and after storage. Apple size was also associated with sugar content: larger apples were found to have higher SSC. Finally, firmness after storage was positively correlated with change in firmness, suggesting that apples that had high firmness after storage experienced less softening during storage. Previous work also found that later harvested apples are firmer and had higher sugar content (Nybom et al., 2013; Migicovsky et al., 2016) and these observations suggest that breeders selecting for firmness and firmness retention may also be inadvertently selecting for late maturing apples with high SSC.

Despite the absence of significant SNP-trait associations for most traits, several noteworthy associations were detected (Fig. 2). Apple fruit skin color – a well-studied trait – served as a benchmark to assess this study’s robustness, and a convincing peak was detected on chromosome 9 for fruit skin color in both years (Fig. 2A). This peak highlights a genomic region that harbors an R2R3 MYB transcription factor (MdMYB1 or MdMYB4; MDP0000259614) known to regulate apple fruit skin color (Takos et al., 2006; Ban et al., 2007; Gardner et al., 2014; Kumar et al., 2014b; Migicovsky et al., 2016; Amyotte et al., 2017). The strength of the association found here, and its detection in several other studies, suggests that this locus plays a key role in determining fruit skin color across diverse apple germplasm. Our ability to detect this major effect locus demonstrates that the data presented here are of sufficient quality and quantity to uncover loci at least of large effect.

Fruit texture is an important trait for consumers, and there has been a focus on breeding firm apple cultivars that maintain their firmness during long-term storage. The literature has highlighted the possible involvement of several genes for fruit texture including PGI (Longhi et al., 2012; Longhi et al., 2013; Nybom et al., 2013; Costa, 2015), NOR (Longhi et al., 2012), RIN (Longhi et al., 2012), Pel (Longhi et al., 2012), ACS1 (Oraguzie et al., 2004; Costa et al., 2005; Wang et al., 2009; Longhi et al., 2012; Nybom et al., 2013), ACS3 (Wang et al., 2009), ACO1 (Costa et al., 2005; Nybom et al., 2013), and Exp7 (Costa et al., 2008; Nybom et al., 2013). In this study, a strong GWAS hit was found specifically for change in firmness after storage on chromosome 10 in 1yr (Fig. 2C). Several previous studies have identified QTL for firmness-related traits on chromosome 10, and two candidate genes underlying these QTL are polygalacturonase (PG) and 1-aminocyclopropane-1-carboxylate oxidase (ACO) (Kumar et al., 2013; Costa, 2015; Moriya et al., 2017; Amyotte et al., 2017). It is believed that PG is associated with firmness through depolymerization of cell wall pectin (Kumar et al., 2013), while ACO is thought to affect apple shelf-life through the conversion of 1-aminocyclopropane-1-carboxylic acid to
ethylene (Costa et al., 2005). In our study, the most significantly associated SNP with change in fruit firmness was located ~500 kb downstream of \( PG \) and 15.5 Mb upstream of \( ACO \). In diverse apple collections like the one studied here, LD decays rapidly, often within 100 bp (Migicovsky et al., 2016; see Supplemental Fig. S7). Therefore, \( ACO \) can be excluded as the cause of the signal detected, as it is located too far downstream. A lack of recombination around the GWAS peak could conceivably result in LD extending up to 500 kb, therefore variation in the known firmness-related gene \( PG \) could perhaps be the cause of the signal observed here. A SNP in the first exon of \( PG \) currently used for marker-assisted breeding (Costa et al., 2010) was genotyped in a subset of the CET population and was not found to predict loss of firmness (Supplemental Fig. S4). Therefore, it was concluded that neither \( ACO \) nor \( PG \) underlie the observed GWAS peak for loss of firmness during storage in this study.

Interestingly, 7,670 bp upstream of the top GWAS hit for loss of firmness is a gene predicted to be an ERF (MDP0000855671) (Fig. 2C). Ethylene is a gaseous plant ripening hormone and growth regulator, known to be involved in apple softening (Johnston et al., 2002). In apples, a burst of ethylene is generated as maturing fruit approach the climacteric phase, which promotes ripening (Greene, 2003). Ethylene response factors are members of the AP2/ERF family of transcription factors (Zhuang et al., 2011) and it has been shown that two \( MdERFs \) (\( MdERF1 \) [MDP0000128979] and \( MdERF2 \) [MDP0000226115]) are expressed in ripening fruit and that, when treated with an antagonist of ethylene receptors (1-methylcyclopropene [1-MCP]), their expression is reduced (Wang et al., 2007). Li et al. (2016) found that \( MdERF2 \) suppressed \( MdACS1 \) transcription through multiple mechanisms including binding to its promoter, and as a result negatively affected biosynthesis of ethylene and fruit ripening. The link between ethylene biosynthesis and fruit softening is well-established (Tacken et al., 2010) and we hypothesize that genetic variation in \( ERF \) on chromosome 10 could influence firmness loss in apples during storage. Moreover, our observation that the widely-used \( PGI \) marker does not predict firmness loss in the present study, but is located nearby our observed association signal, could suggest that firmness-related QTL signals near \( PGI \) found from bi-parental mapping populations (Costa, 2015) may in fact stem from variation in \( ERF \). In this scenario, the improved mapping resolution offered by GWAS compared to bi-parental linkage mapping might have enabled the refinement of the location of the causal locus. Future work in larger germplasm collections will help clarify the genesis of the signal on chromosome 10 associated with loss of firmness.

We observed several convincing non-significant GWAS peaks, such as the association between SNPs on the distal end of chromosome 3 and harvest date (Fig. 2B), firmness at harvest in 2013, and firmness after storage in 2013 (Supplemental Fig. S3). All three correspond to the association between variation in the NAC5 transcription factor, harvest date, and firmness (Migicovsky et al., 2016). The lack of statistical significance for these associations in the present study could be due to narrow phenotypic data variability, reduced robustness due to sample size, or a significance threshold that is too conservative. Regardless of the reason for the lack of statistical significance of this signal, our results point to the same genomic region as reported previously involving NAC5 in harvest date and fruit firmness, although they were not significant here.

Our observation of SNPs falling short of our chosen significance threshold for harvest date and firmness, despite overlapping with known loci underlying these traits highlights the difficulty of achieving sufficiently robust GWAS in apples. The number of accessions (\( N = 172 \)) and the number of markers (~55,000 SNPs) evaluated here are both modest, especially in light of recent research suggesting that millions of SNPs may be required for well-powered GWAS in diverse apple populations due to rapid LD decay (Migicovsky et al., 2016). The composition of this population may have also limited our ability to detect QTL, as cultivars were chosen to assess their commercial suitability for the growing region, and therefore may not have encompassed trait variation available in wider breeding material. Finally, composite fruit samples were collected due to labor shortages, therefore it is possible that replication would have better captured phenotypic variation, by correcting for factors such as tree position within the orchard. Future GWAS in apple with larger sample sizes and more genetic diversity will help clarify the contributions of these factors to identifying genotype-phenotype associations.

Genomic prediction was also performed to investigate the potential utility of the entire marker data set to predict phenotypic variation using a genomic selection model (Fig. 3). Genomic selection is in its infancy in tree fruit, but work by Kumar et al. (2012) generated prediction accuracies for fruit quality traits ranging from 0.70 to 0.90 in a population derived from seven full-sib families. In this study, genomic prediction accuracies ranged from 0.08 to 0.72, which fell within the range of values from another highly diverse collection of apples (Migicovsky et al., 2016). The lower prediction accuracies observed here in a diverse collection compared to those from Kumar et al. (2012) highlight the effects of population structure on genomic prediction accuracies. With 1120 seedlings derived from six common parents, LD decayed 100 to 1000 times more slowly in the apple population assessed by Kumar et al. (2012) compared to levels observed in a diverse collection of apples similar to the one studied here (Migicovsky et al., 2016). As such, genotyped markers are more likely to be in strong LD with causal variants in Kumar et al. (2012), which results in higher prediction accuracies. The advantage of genomic analyses of diverse collections is the ability to uncover variants that would otherwise be missed in populations derived from a small number of founders. Although the prediction accuracies are likely to be lower, the ability to mine useful alleles from diverse populations nevertheless holds great promise in contributing to future apple improvement.
Several breeding programs focus on the development of elite apple cultivars that contain one or more sources of scab resistance. Scab resistance is generally derived from wild Malus germplasm, and then introgressed by crossing with elite germplasm. Several strong signals of association for scab resistance were detected here using GWAS (Fig. 4A; Supplemental Fig. S5). Two of these loci, on chromosomes 1 and 15, also overlap with strong signatures of positive selection detected using the XP-EHH statistic (Fig. 4B; Supplemental Fig. S6). This result suggests that resistance alleles at these loci have recently and rapidly risen to high frequency due to positive selection. Thus, by selecting for scab resistance over multiple generations, apple breeders have shaped the apple genome by reducing genetic diversity and creating extended homozygosity at the loci underlying scab resistance. The GWAS for scab resistance produced associations on seven different chromosomes. There are about a dozen known sources of resistance from various Malus species that have been genetically mapped (Bus et al., 2011). In this study it was presumed unlikely that seven independent sources of resistance were detected. Therefore, we attempted to determine if the GWAS signals might represent the presence of a single causal locus that, due to reference genome misassembly, appeared across multiple regions of the genome. To investigate whether the result was consistent with one or multiple QTL, an analysis of inter- and intra-chromosomal LD was conducted for SNPs associated with scab resistance (Fig. 5A & B). No difference was observed between intra- and inter-chromosomal \( r^2 \) values (\( W = 2 \times 10^6, p = 0.900 \)), and thus it was concluded that the GWAS hits across seven chromosomes likely represent a single causal locus.

A cursory investigation of the pedigrees of the cultivars studied here supports our conclusion of a single locus for scab resistance, and it was determined that the source of resistance is most likely derived from Malus floribunda 821 – the most common source of scab resistance in modern apple breeding (Paticchi et al., 2009; Cova et al., 2015). The strongest and most extended GWAS signal observed was located on chromosome 1, where the M. floribunda 821 resistance locus (Rvi6) is located (Bus et al., 2011). The strongest signal of selection also overlapped with this locus (Fig. 4B, Supplemental Fig. S6). Therefore, we conclude that the seven observed GWAS hits for scab resistance likely represent the signal from Rvi6 alone. Previous studies found that 13.7% and 18.3% of SNPs were assigned to chromosomes that conflicted with their locations according to the reference genome used here (Antanaviciute et al., 2012; Gardner et al., 2014). In agreement with these observations, 28% of SNPs associated with scab resistance in the present study were located on chromosomes other than chromosome 1 and their appearance as independent GWAS hits could be due to reference genome misassembly. It could also be that some or all of the seven GWAS hits capture previously unknown resistance loci associated with M. floribunda 821 resistance introgression, but our data do not enable us to test this less parsimonious explanation. With new releases of the reference genome in the future, conclusions could be drawn with more certainty as to whether these results capture one or more introgression events.

Rvi6 resistance from Malus floribunda 821 has been widely used in breeding programs around the world (Brown, 2012), but new pathogen strains have emerged that can overcome this form of resistance (Cova et al., 2015). This has necessitated the exploration of new sources of resistance and subsequent pyramiding into new cultivars (Paticchi et al., 2009). Although the associations presented here likely stem from introgression of a single source of resistance, the GWAS results were compared to the recorded resistance of cultivars by determining how many alleles at the seven loci were carried by each cultivar. In general, cultivars labeled as resistant carried all seven loci, while cultivars labeled as susceptible carried none (Fig. 6A), with several exceptions. For example, 15 cultivars labeled as resistant did not carry any of the resistance alleles (Fig. 6A). Breeding records were examined and it was noted that several of these cultivars were descended from wild Malus species other than M. floribunda 821. For example, ‘Murray’ and ‘Rouville’ carry Rvi5 scab resistance (Paticchi et al., 2009), derived from Malus micromalus 24538–38 and M. atrosanguinea 804, which maps to chromosome 17 (Cova et al., 2015). Thus, several sources of scab resistance likely exist in the population studied here, but it is likely that only Rvi6 resistance is present at a sufficiently high frequency for GWAS mapping. Similarly, three cultivars labeled as susceptible carried all seven resistance alleles. It is hypothesized that these cultivars were mislabeled as susceptible when introduced into the collection, but in fact carry Rvi6 resistance. These conflicting results will be used to verify and thus improve the curation of this diverse collection of apples.

Although the majority of scab resistant cultivars in our study likely derive ancestry from M. floribunda 821, samples labeled as resistant with all seven resistance loci were found to be only slightly more closely related to each other than they were to cultivars labeled as susceptible with no resistance loci (Fig. 6B). This observation was corroborated by PCA results, in which a cultivar’s resistance status is a poor predictor of its position along the two primary axes of genetic differentiation (Fig. 6C). Thus, while the effects of scab resistance breeding have left a statistical signature in the form of reduced diversity among resistant cultivars and genetic differentiation between resistant and susceptible cultivars, the signal is relatively weak, suggesting that the number of generations since introgression has been sufficient to shuffle the Rvi6 locus into a diversity of genetic backgrounds. Inferring the number of generations since Rvi6 introgression requires more in depth analyses, however, since the IBD estimates used here are unlikely to be able to detect relatedness beyond three or four generations. Finally, it is worth noting that the scab resistant and susceptible cultivars were planted in separate blocks and thus reported disease resistance status is confounded with location in the orchard. We argue that the strong GWAS signal detected here for scab resistance is unlikely to be an artifact of this confounding effect, however this does

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highlight the limitations that established populations often impose on the design of genetic mapping studies.

Apples are an ideal candidate for MAS due to their long juvenile periods, large size, and the expense associated with their maintenance (Myles, 2013), but MAS has only recently been applied to breeding programs (Kumar et al., 2014a). The present study demonstrates that GWAS holds promise for detecting novel loci associated with commercially relevant traits while simultaneously highlighting the challenges with performing genetic mapping in relatively small but diverse apple collections with rapid LD decay. Several traits appear to be heritable and thus predictable using genomic prediction and are thus good candidates for genomic selection in cases where genetic mapping does not reveal single large effect markers that can be used for MAS. The extent to which the marker-trait associations discovered here are transferable to the advanced breeding material currently used by apple breeders worldwide will require further investigation. Overall, we provide the foundation for the future development of markers for both MAS and genomic selection, which will enable breeders to more rapidly and cost-effectively generate new apple cultivars that achieve commercial success.

Supplemental Information

Supplemental Fig. S1. A graphical representation of the proportion of missing data by (A) cultivar, and (B) SNP.

Supplemental Fig. S2. Numerical r values and P-values corresponding to the CET phenotype data heatmap presented in Fig. 1. NS = indicates P-value > 0.05, * = 0.01 > P-value < 0.05, ** = P-value < 0.01.

Supplemental Fig. S3. The GWAS results for each trait studied in the CET in 2013 and 2014. Each plot also contains a histogram of the trait value, a Manhattan plot of the GWAS results, a Q-Q plot of the observed and expected -log_{10} P-values, and a histogram of the residual values for the GWAS for each trait from TASSEL.

Supplemental Fig. S4. A preliminary examination of SNPs adjacent to polygalacturonase (PGI) and an ethylene-responsive (ERF) transcription factor and their GWAS results. The blue dot represents the SNP (S10_21375724) closest to the ERF transcription factor (MDP0000855671) and the red dot (S10_20843047) represents PGI (MDP0000326734). Even when the PGI marker data were inserted into the GWAS, SNP S10_21375724 still produced the association with the smallest P-value. The solid line represents the -log_{10} (p) value significance threshold. No SNPs exceeded the significance threshold, likely due to the small sample size (N = 98).

Supplemental Fig. S5. The scab TASSEL (A), and CARAT (B) association mapping results, and a -log_{10}(p) comparison of the two (C). In the top and bottom plot, the solid line represents the -log_{10}(p) value significance threshold. Panel (C) is a comparison of the P-values for TASSEL (x-axis) and CARAT (y-axis), which displayed a positive, significant correlation (r = 0.745, p < 2.2 × 10^{-16}).

Supplemental Fig. S6. A comparison of scab TASSEL GWAS results, and Cross Population Extended Haplotype Homozygosity (XP-EHH) test results across chromosomes 1, 4, 5, 7, 12, 14, and 15. In the top panel, the solid line represents the -log_{10}(p) value significance threshold. In the bottom panel, the dotted line represents the upper 1% quantile of XP-EHH values.

Supplemental Fig. S7. A graphical representation of LD decay in the CET population using comparisons over inter-SNP distances of (A) 1 Mb, and (B) 500 bp. The fitted line was calculated on all SNP data using the LOESS method. Dots represent a binning value of 10 bp windows.

Supplemental Table S1. A list of cultivars present in the CET population, their historical scab status (scab susceptible = SS, scab resistant = SR), and their country of origin as recorded by the Nova Scotia Fruit Growers’ Association research staff.

Supplemental Table S2. A table summarizing the phenotype data for harvest date and fruit quality in the CET in 2013 and 2014. Phenotype data that were transformed using the Box-Cox transformation for GWAS are indicated accordingly.

Supplemental Table S3. A table summarizing the significant 2013, 2014, and scab resistance GWAS results for the CET population.

Supplemental Table S4. A table summarizing the significant 2013, 2014, and scab resistance GWAS marker effect results for the CET population.

Supplemental Table S5. A table summarizing the results for each SNP that produced a significant GWAS result from Genome Database for Rosaceae (GDR) (https://www.rosaceae.org) and NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi), within a ± 100 kb window centered on each SNP.

Conflict of Interest Disclosure

The authors declare that there is no conflict of interest.

Acknowledgments

This research was supported in part by funding from the Canada Research Chairs program (SM), the National Sciences and Engineering Research Council of Canada (SM, KM) and A-Base funding (NOI-1238) from Agriculture and Agri-Food Canada (JS). Technical support from Marina Myra, Peter Harrison, Tim Hughes, and Leslie Campbell-Palmer is greatly appreciated. The authors gratefully acknowledge the Nova Scotia Fruit Growers’ Association for establishing and maintaining the apple collection studied here.

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