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RESEARCH ARTICLE

Epistatic modifiers influence the expression of continual flowering in strawberry

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Society Impact Statement

Until the 1970s, the majority of commercial strawberry varieties produced a single bloom of flowers. However, continuously flowering, everbearing strawberries are now routinely cultivated and use is increasing. Indeed, introgression of the everbearing flowering trait can lead to economic benefits for growers through the production of a continual crop from the same plant. Genetically guided improvement has the power to streamline everbearing generation. As such, the genetic markers reported here can help to identify everbearing individuals at an early time point in the breeding process. Furthermore, these markers can help to improve the predictions of progeny segregation ratios.

Summary

- Previous work within the community led to the identification of a single dominant allele that controls the everbearing trait. However, frequent observations have indicated that crosses do not segregate in a Mendelian fashion, as would be expected for a trait controlled by a single dominant gene. Therefore, it was hypothesised that one or more unidentified epistatic alleles interact with the major gene.
- A genome-wide association study (GWAS) was conducted on 587 June bearers and 207 everbearers to assess the genetic components associated with flowering habit. The segregation ratios of parental strawberry lines with known phenotypes were used to validate the identified alleles.
- Three loci including the known major *FaPFRU* locus and two epistatic modifiers were identified. These modifiers function as enhancers of the everbearing trait in individuals containing a single copy of the *FaPFRU* everbearing allele and appear to be functionally redundant. Principally, heterozygous individuals required the presence of two modifying alleles in order to allow expression of the everbearing trait.
- Inclusion of the epistatic alleles improved the prediction of everbearing segregation ratios; beyond that of a single allele model, however, a large proportion of the

variation remained unexplained. Future work should identify the additional repressor and enhancer modifiers not identified here. Discovering the genetic components controlling the everbearing trait can enable genetic informed strawberry improvement.

KEYWORDS
epistasis, everbearing, flowering time, functional redundancy, genome-wide association study (GWAS), marker-assisted breeding

1 | INTRODUCTION

Plants that grow in non-seasonal environments are able to achieve high reproductive success through continuous flower production. However, plants that grow in temperate environments must flower within a restricted time period; flower too early and risk damage due to late frosts; flower too late and seed cannot be developed and dispersed before the onset of winter (Gaudinier & Blackman, 2020). To maximise the chance of reproductive success, a plant must also synchronise its life cycle with that of mutualistic organisms, both pollinators and dispersers (Krizek & Fletcher, 2005). As such, plants have evolved to modify the timing of flower emergence in response to different environments. Plants that grow in temperate environments need to tightly regulate flower production in order to achieve successful reproduction within a limited time frame. In order to regulate flowering time, temperate plants have evolved the ability to measure the season. Thus, temperate adapted plants use environmental cues that cycle reliably throughout the year, via the length of the day (photoperiod) and through a prolonged period of chilling where vernalisation generates a 'winter memory' (Andrés & Coupland, 2012; Bouché et al., 2017). Indeed, expansion of plants from tropical to temperate regions required the ability to control flowering time, and as such, 'winter memory' and photoperiodic responses have evolved numerous times, independently, across multiple angiosperm families (Bouché et al., 2017; Serrano-Bueno et al., 2017). By contrast, non-seasonal climates do not have annual fluctuations, and thus, there is no selection pressure on angiosperms to flower within a specific time frame.

Plants in the *Fragaria* genus are widely distributed across many different geographic locations and environments (Darrow, 1966; Liston et al., 2014), and both seasonal and continuous flowering habits have been reported in several species of the genus including woodland strawberry (*Fragaria vesca*), garden strawberry (*Fragaria* × *ananassa*), *Fragaria virginiana* and *Fragaria chiloensis* (Bringhurst & Voth, 1980; Brown & Wareing, 1965; Heide et al., 2013; Hummer et al., 2016). Garden strawberry is a 300-year-old hybrid of two wild allo-octoploid species, *F. virginiana* that grows in North America and *F. chiloensis* that is found in both North and South America (Darrow, 1966; Hardigan et al., 2018). The genome of garden strawberry has been sequenced, and several genotyping chips have been developed to facilitate genomics research and breeding (Bassil et al., 2015; Edger et al., 2019; Hardigan et al., 2020; Whitaker et al., 2020). Genomic synteny of octoploid species have been conserved during their evolution, and they have been shown to share same diploid ancestors; the role of *F. vesca* and *Fragaria iinumae* as ancestors have been confirmed by several studies, while the origin of the other two sub-genomes are still under debate (Edger et al., 2019; Feng et al., 2021; Hardigan et al., 2020; Liston et al., 2020).

Seasonally flowering strawberries, identified as short-day plants or June bearers, initiate flower buds in autumn, and the flowers emerge in the following spring. Flower induction requires short day conditions, when the photoperiod is less than about 14 h in length. However, temperature can modulate the short-day flowering response whereby strawberry plants are induced to flower in long photoperiods at cool temperatures (<15°C) (Guttridge, 1985; Heide et al., 2013; Koskela et al., 2016; Mookerjee et al., 2013; Rantanen et al., 2015). By contrast, strawberry genotypes that produce flowers continuously or repeatedly are termed 'everbearers'. Temperature and light periodicity also influence flowering time in everbearing strawberries, but after flower induction, everbearing strawberry plants will produce flowers in both short and long days (Bradford et al., 2010; Koskela et al., 2012). This continual flowering phenotype is of particular interest for strawberry breeders and growers due to the potential benefit of increased yield from a single planting for the duration of the growing season.

Everbearing is a relatively new trait in strawberry cultivation, and the majority of historical commercial production has focused on June bearers. The first documented introgression of the everbearing trait was from a wild *Fragaria virginiana* ssp. *glauca* from the Wasatch Mountains in 1955 (Bringhurst & Voth, 1980). It may be hypothesised that colder mountain conditions (<15°C) induced flowering, which removed the selection pressure to maintain the short-day flowering mechanism, and thus, everbearing plants evolved. Introgression of the everbearing trait into commercial strawberry cultivars has allowed continual fruit production to be extended across the season. The Wasatch source of everbearing has been used internationally and is thought to represent the main genetic source of everbearing in temperate cultivated strawberries (Faedi et al., 2002; Salinas et al., 2017; Simpson, 1993; Zurawicz & Masny, 2002). Before the commercialisation of everbearing strawberry varieties and the associated

improvement of fruit quality, it had been noted that significant refinement of everbearing genetics was required (Bringhurst & Voth, 1984).

A single major dominant locus *FaPFRU* was found to control both everbearing and runnering (clone plants produced from horizontal stems) in octoploid strawberries. Individuals containing the dominant allele(s) of this locus produced up to 20 times more inflorescences and up to 16 times fewer primary runners (Gaston et al., 2013). The *FaPFRU* locus was originally found on *LGIV-Fb2* between 0 and 8.7 cM, but the locus has since been fine mapped to a 1.1 Mb region (Perrotte, Gaston, et al., 2016). Furthermore, a simple sequence repeat (SSR) marker has been validated to colocalise with the *FaPFRU* locus across wider germplasm (Salinas et al., 2017). Verma, Zurn, et al. (2017) used single nucleotide polymorphism (SNP) markers to map the loci further and found a haploblock 1.5 Mb upstream from the original *FaPFRU* loci, and two copies of the identified recessive haploblock were required to produce a short-day variety (Verma, Zurn, et al., 2017). This marker has allowed the use of marker-assisted breeding to incorporate everbearing into high fruit quality lines without incurring phenotyping costs. Identifying a functional marker in the causative gene or allele would allow for highly accurate selection of the trait and negate the requirement for maintenance of non-desirable individuals. However, it has since been discovered that the 'single dominant gene' controlling the everbearing trait does not segregate in a straightforward Mendelian fashion in all biparental crosses (Lewers et al., 2019). Indeed, it was hypothesised that the allele might be controlled by at least two epistatic alleles that interact with *FaPFRU* to modify the expression of the everbearing trait (Lewers et al., 2019). Here, we provide evidence to confirm their original hypothesis and identify two new epistatic loci that are involved in the expression of continual flowering in strawberries.

2 | MATERIALS AND METHODS

2.1 | Plant material

The in-house NIAB strawberry genomic database was mined to study the genetic control of the everbearing trait using a genome-wide association study (GWAS). A total of 794 strawberry breeding lines and cultivars had genotypic data available with a known flowering habit status. The population represents modern released cultivars (post-1980s) and NIAB advanced breeding lines generated between 2000 and 2018 (Dataset S1).

2.2 | Phenotyping

Flowering habit was scored as a binary trait; genotypes were denoted as June bearers or everbearers (Dataset S1). Standard definitions for flowering habit were used across all experiments. June-bearing individuals give a single flush of flowers/fruit. Everbearers have multiple flushes of flowers/fruit with flower initiation obvious under long days and warm temperatures. Determination of flowering habit for each genotype was achieved through either literature searches or cultivar release information or determined based on the in-house phenotype databases. The initial in-house characterisation consisted of observing flower production in field conditions (with 0.55 m spacing between plants) or protected substrate culture over two consecutive growing seasons (April to October) at NIAB in East Malling (51° 17′ 24.202″ N 0° 26′50.918″ E). Phenotype observations were made in 2013–2017, 2019 and 2020. The presence of open flowers and fruiting was scored on a weekly basis over the season to confirm the June-bearing or everbearing nature of each of the genotypes.

2.3 | Genotyping

Genotyping data used for the GWAS analysis were generated as part of a previous project (Nellist et al., 2019), and an additional 501 genotypes were acquired through the Malling Fruits strawberry breeding programme. The parents of the validation germplasm were genotyped as part of this work; newly unfolded strawberry leaf material was sampled for DNA extraction using the QIAamp 96 DNA QIAcube HT Kit, with buffer volumes modified for isolation of total DNA from plant tissue, as per manufacturer's instructions. DNA quality was confirmed using a spectrophotometer (NanoDrop™ 2000), and DNA quantity was confirmed through Qubit analysis. All genotyping was performed on the Istraw 90 k or the Istraw 35 k axiom array (Dataset S1) (Bassil et al., 2015; Verma, Bassil, et al., 2017). The 28 chromosomes of octoploid strawberry are denoted by number (1–7), and sub-genome is denoted by letter (A–D) as detailed in van Dijk et al. (2014). The NIAB strawberry consensus map (Cockerton et al., 2018) was used to define marker positions. SNPs were anchored and positions scaled to the diploid *F. vesca* version 4 genome (Edger et al., 2018).

2.4 | Genetic analysis

Genome-wide association analysis was conducted in Plink (Purcell et al., 2007) as detailed on github https://github.com/harrisonlab/popgen/blob/master/snp/gwas_quantitative_pipeline.md. A total of 13,018 poly-high-resolution SNPs were used for the analysis presented here. SNPs were filtered to include only those with a minor allele frequency of greater than 0.05, and no greater than 20% missing values across the genotypes studied. Heterozygosity was calculated (heterozygosity = No. non-missing genotypes − No. homozygous genotypes/No. non-missing genotypes). Individuals with low heterozygosity were removed by examining the heterozygosity distribution across the genotyping set; individuals with less than 10% heterozygous alleles were excluded from the analysis; furthermore, a low heterozygosity rate and high missing data indicated possible technical issues with genotyping. The analysis was adjusted for population stratification through logistic regression using multidimensional scaling values as covariates (Setakis et al., 2006).

Epistatic interactions between the quantitative trait loci (QTL) were quantified through a binomial generalised linear model using the

R package lme4 (Bates et al., 2011), with the formula Flowering habit ~ A + B + C + A*B + B*C, where letters (A, B & C) denoted the three genetic loci, each locus was coded as continuous variables with 0, 1 or 2 copies of each allele and phenotype was coded as a factor. Data from 771 genotypes were used to assess epistatic interactions as 23 individuals had 'no call' for one of the three alleles. Pairwise interactions between A, A' and X were quantified through a binomial generalised linear model, with the formula following the format: Flowering habit ~ A + X + A * X. The location of SNPs was assessed using the *F. vesca* genome v.4.0, and gene annotations V4.0.2. were used to assess candidate flowering time genes that neighboured focal SNPs. Linkage disequilibrium (D) was calculated between A, X and A' using the equation: $D_{AB} = p_{AB} - p_A p_B$ to calculate D between neighbouring alleles (A and B) as detailed in (Slatkin, 2008), where p_{AB} is the frequency of individuals containing both alleles and $p_A p_B$ is the expected co-localisation of the two alleles by chance

Genotypic relationships were calculated through hierarchical cluster analysis of the Euclidean distance matrix, and these data were plotted as a dendrogram using the R packages dendextend and ggplot (Galili, 2015; Wickham, 2009). The number of coloured cluster groups (k) was set to three through using the 'Ward D2' hierarchical clustering method, which implements Ward's clustering criterion (Murtagh & Legendre, 2014).

2.5 | Determining the origin of SNPs

The origin of the SNPs of interest identified in this study were investigated by assessing the presence/absence in wild octoploids from the Americas using an existing genotype and phenotype dataset (Hardigan et al., 2018). A dendrogram was generated by hierarchical clustering for a subset of 108 wild accessions and 158 cultivated strawberries (developed before 1996) using 16,394 markers (Dataset S2). SNPs were quantified by Hardigan et al., 2018 using the Axiom IStraw35 array (Verma, Bassil, et al., 2017).

2.6 | Validation of the model using bi-parental crosses

Progeny from 156 crosses were screened for flowering habits; crosses had between 30 and 400 progeny. A total of 16,459 genotypes were assessed (Dataset S3). Two replicate plants were assessed per genotype. Trials were laid out as augmented designs with standard cultivars of known flowering habit across each block. The predicted percentage of everbearing individuals was calculated based on the genotype of the parents for one of three models: (1) the single allele model for the major allele (A), (2) the simple three-allele model where one modifier (Bb or Cc, treated as a single dominant allele) is required for heterozygous individuals for the major allele (Aa) require at least two of the modifiers (either BBcc, bbCC or BbCc) to exhibit the everbearing trait. For example, a parent with a genotype of AAbbcc crossed with a parent that has a genotype of aaBbCc would be predicted to produce 100% everbearing individuals for the single allele model, 75% everbearing individuals for the simple three-allele model and 25% everbearing individuals based on the complex three-allele model. Segregation ratios were calculated for each of the 730 possible genotype combinations based on the stipulations of the three models. A linear model ($Y = a + bX$, where a is the y-intercept and b is the slope) was generated between the observed and predicted percentage of everbearing individuals, and values were weighted by the total number of progeny in the population.

3 | RESULTS

3.1 | The three-gene model for everbearing trait

A total of 587 June-bearing and 207 everbearing strawberry varieties were analysed in a GWAS. Three highly significant loci were detected (Figure 1a). One large effect locus was identified on chromosome 4A with the most significant marker Affx-88857614 [A/G: 'A/a'] located at 29.5 Mb in *F. vesca* genome v4.0 (Edger et al., 2018). Hardigan et al. (2018) also identified this locus in different germplasm alongside a marker about 3.5 Mb downstream of our focal marker on the chromosome 4A, denoted hereafter as A'; this region is known to the community as the *FaPFRU* locus (Gaston et al., 2013). In our GWAS, A' (33.0 Mb) showed a much lower significance level than A. We identified an additional highly significant marker X (32.4 Mb) at the distal end of chromosome 4A near to A'. X delineated a 2.85-Mb marker gap between A and X (Figure 1b). Significant pairwise interaction was observed between A and A' ($z = -2.44$; $p = 0.015$), between A and X ($z = -5.129$; $p = 3.7 \times 10^{7}$) and between A' and X ($z = -2.07$; $p = 0.038$). No linkage disequilibrium was found between pairwise combinations of alleles A, A' and X.

The majority of individuals (97.8%) which contained two recessive copies of the major locus (here denoted as 'aa') were June-bearers ($n = 506$; Dataset S1). The 17 everbearers that were 'aa' included the named cultivars 'Diamante', 'Elan', 'Portola' and 'Sierra'. All individuals containing two dominant versions of the major locus 'AA' were everbearers ($n = 59$). Of individuals containing a single copy of the major locus 'Aa', 64.7% were everbearers ($n = 130$) and 35.3% June bearers ($n = 71$). The whole distal portion of chromosome 4A was associated with the everbearing trait, where individuals containing both dominant upper (A) and lower portions (X, or A') gave rise to the greatest proportion of everbearing individuals (Figures S1 and S2, Dataset S4).

Two additional loci were identified on linkage group 5C ('B/b'; Affx-88866698 [T/C: 'B/b'] at 15.1Mbp) and 6A ('C/c'; Affx-88877135 [A/G: 'C/c'] at 5.3 Mbp). A significant epistatic interaction between the major locus A and the additional loci B ($p = 0.025$) and C

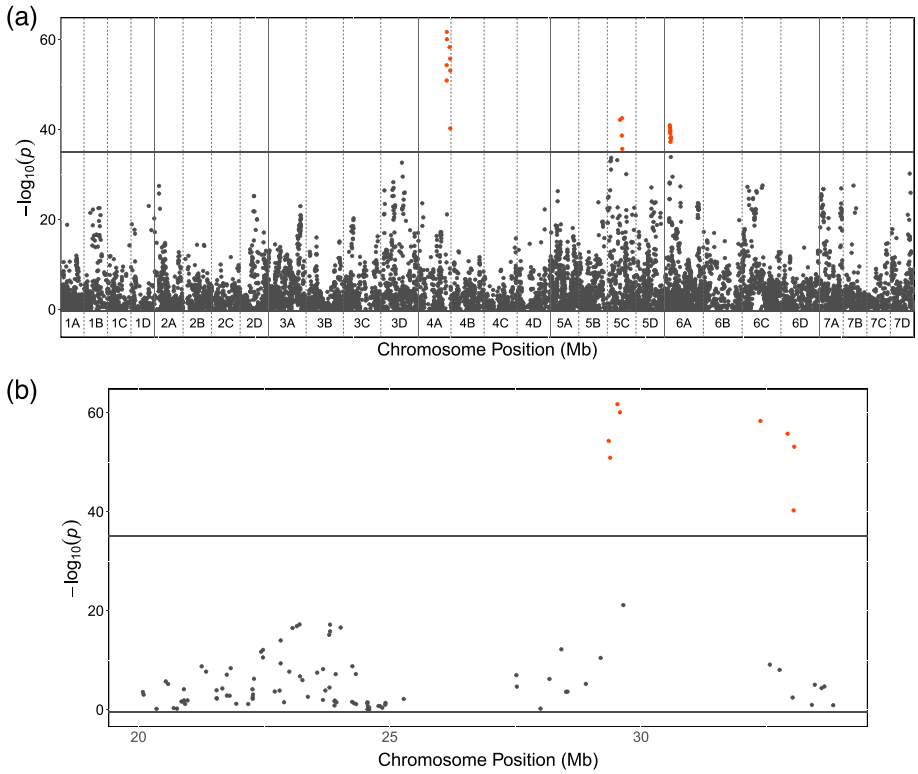

FIGURE 1 Manhattan plots showing the association of single nucleotide polymorphisms (SNPs) in the 794 accessions with flowering habit in octoploid strawberry. Marker positions are scaled to the *Fragaria vesca* version 4 genome (Edger et al., 2018). Grey horizontal bars represent the significance threshold. Red points represent markers passing the significance threshold. Grey points represent markers beneath the significance threshold. (a) Three significant quantitative trait loci (QTL) regions identified after genome-wide association analysis. Chromosomes are denoted by 1A–7D according to (van Dijk et al., 2014) where A–D represent the four sub-genomes and 1–7 represent chromosomes. (b) Regional plot, zoomed in on chromosome 4A (20 Mb onwards), depicting the most significant markers, denoted as A (Affx-88857614) and X (Affx-88857910) and the previously identified marker, denoted as A' (Affx-8851928) (Hardigan et al., 2018).

TABLE 1 Generalized linear model parameters for the effect of markers A (Affx-88857614), B (Affx-88866698) and C (Affx-88877135) alleles on *Fragaria* × *ananassa* flowering habit and pairwise interactions

	Estimate	Std. error	z value	*P* value	Significance
(Intercept)	73.8	15.0	4.9	9.4×10^{-7}	***
A	−17.2	3.2	−5.3	9.4×10^{-8}	***
B	−7.7	2.4	−3.2	0.002	**
C	−12.1	4.0	−3.0	0.003	**
AxB	1.2	0.5	2.2	0.025	*
AxC	2.7	0.8	3.3	0.001	**
BxC	0.5	0.6	0.9	0.362	NS

Note: Significance codes: 0 '***', 0.001 '**', 0.01 '*'.
Abbreviations: NS, non-significance; Std., standard.

($p = 0.001$) was observed, but no additive interaction was found for these components (Table 1 and Figure 2). The phenotypes of heterozygous genotypes for the major loci ('Aa') appear to be influenced by the presence of the two additional loci, indicating that they function as modifiers of *FaPFRU*. A single copy of B or C is sufficient to permit the everbearing genotype to occur in some individuals. However, the majority of individuals (87%) that did not contain one of the two modifying QTL (with the genotype 'Aabbcc') were June bearers. Also 'AaBbcc' and 'AabbCc' genotypes appear to divide into two flowering types with 43% and 50% of individuals exhibiting an everbearing flowering habit, respectively. There were no individuals with an 'AAbbcc' genotype; as such, it is not clear whether 'AA' alone is sufficient to produce an everbearing phenotype. Locus B does not neighbour any candidate genes known to play a role in the flowering response; by contrast, Locus C is located 100 kb away from a gene encoding the photoreceptor Phytochrome A.

3.2 | Phylogeny of the everbearing trait

We analysed the genetic relationships between individuals within the GWAS population by generating a dendrogram (Figure 3). The everbearing trait is mainly present in one of the three clades, indicating that many of the everbearing individuals appear to be highly related. This may be due to introgression from a single source, that is, the Wasatch source that was introduced to the University of California breeding programme and later widely used across the world (Bringhurst & Voth, 1980). Some individuals in this clade appear to

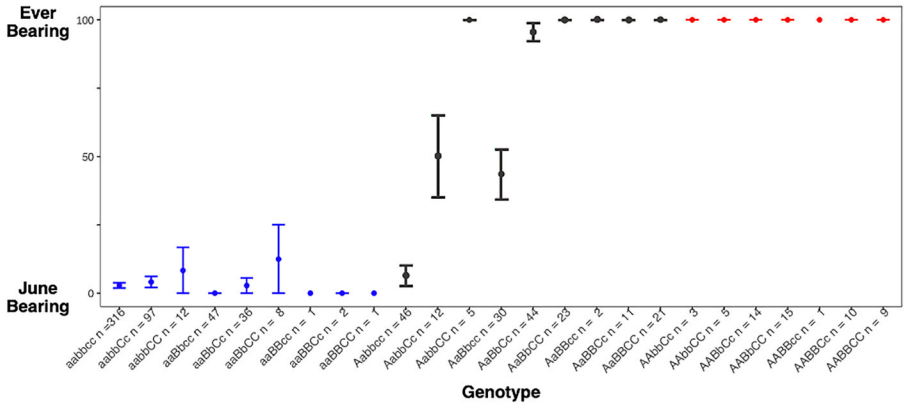

FIGURE 2 Three alleles interact to determine the flowering habit of strawberry cultivars. Flowering habits of each genotype represented in the population are shown. Points represent the percentage (%) of individuals exhibiting a June-bearing (0) or everbearing (1) phenotype. Error bars are standard errors. Loci resulting in the everbearing phenotype are denoted with a capital letter (A/B/C), and loci resulting in the June-bearing phenotype are denoted in a lower-case letter (a/b/c). Blue points represent homozygous recessive individuals for the major allele—aa. Grey points represent heterozygous individuals for the major allele—Aa. Red points represent homozygous dominant individuals for the major allele—AA. A total of 771 individuals are represented in this plot.

FIGURE 3 Everbearing individuals identified across all three clades of strawberry, the majority of everbearing individuals belong to a single clade. Dendrogram depicting the genetic relationship between the 794 individuals used in the genome-wide association study (GWAS) population: everbearing (black points) and June-bearing (no points) cultivars. Branch colours denote the three main clusters of genotypes based on the genotypic relationship across individuals. Numbers of individuals per clade were pink ($n = 97$), green ($n = 217$) and blue ($n = 480$).

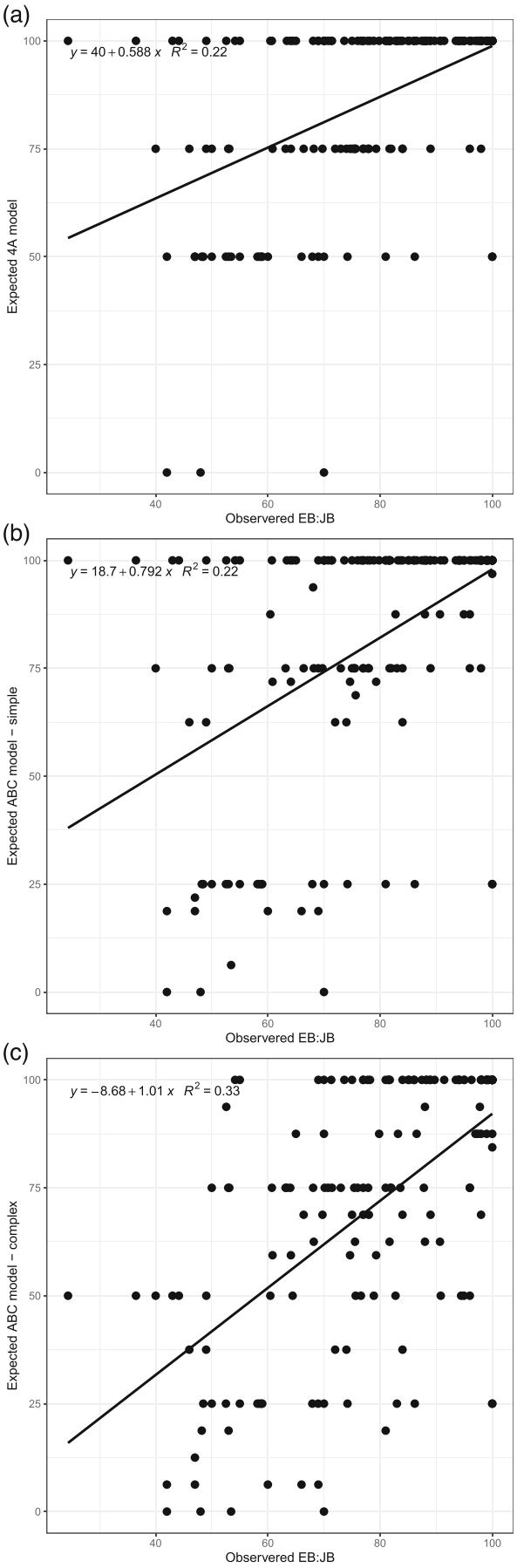

FIGURE 4 The complex three allele (ABC) model explains the most variation. Observed versus expected everbearing (EB): June-bearing (JB) *Fragaria* × *ananassa* segregation ratios in 156 validation crosses based on (a) the single allele model (4A), (b) the simple ABC model and (c) the complex ABC model, where A represents Affx-88857614, B Affx-88866698 and C Affx-88877135. R^2 values are adjusted R^2 values; thus, the values are not influenced by the different number of variables between the models.

have been selected for a June-bearing phenotype. However, everbearing individuals were also found across the length of the dendrogram in the other clades.

3.3 | Validation crosses

We generated 156 validation crosses and compared observed versus predicted segregation ratios using three different genetic models for the everbearing trait (Figure 4). The complex three-allele model (where heterozygous individuals for the major allele (Aa) require at least two of the modifiers, either BB, CC or BC, to be everbearing) has the best fit with the highest adjusted R^2 value of 0.33, indicating that the complex ABC model may provide a better predictive segregation ratio than the single allele model (where a single dominant A allele is sufficient) or the simple ABC model (where heterozygous Aa requires either dominant B or C). Furthermore, the equation of the complex model regression line is $y = -8.68 + 1.01x$, which is closer (relatively) to the perfect prediction model ($y = 1x$) than the equations of other models tested (Figure 4). Nonetheless, the model does not explain all the variation in segregation ratios, and it is clear there may be more unidentified alleles or other factors influencing expression of the trait.

3.4 | Potential origin of the alleles

All four alleles identified in this study were found in *F. virginiana* ssp. *virginiana* accessions, whereas only three of the four alleles were identified in *F. virginiana* ssp. *glauca*, the accessions representing the original source of everbearing trait from the Wasatch mountains (Bringhurst & Voth, 1980), and in *F. chiloensis* ssp. *pacifica* (Table 2). Analysis of the origin of the A, A', B and C alleles using the data from Hardigan et al. (2018) revealed that the alleles are restricted to and segregating within *F. chiloensis* ssp. *pacifica*, *F. virginiana* ssp. *glauca* and *F. virginiana* ssp. *virginiana*, despite there being multiple other subspecies and populations of both *F. virginiana* and *F. chiloensis* in the dataset (Table 2). However, sample numbers for some subspecies are low (e.g. *F. virginiana* ssp. *platypetala* $n = 2$ *and F. chiloensis* ssp. *chiloensis* $n = 4$), and thus, it is possible that the presence of the alleles in these subspecies was not captured due to the small sample. Phylogenetic analysis clearly shows that the everbearing trait stems from two discrete pools of individuals, represented by *F. chiloensis* ssp. *pacifica* and *F. virginiana* (Figure S3, Dataset S3).

TABLE 2 Presence of identified alleles in wild octoploid *Fragaria* spp.

Species	Subspecies	Number	Flowering habit[a]	A	A'	B	C
F. chiloensis	*pacifica*	$n = 18$	1/13 clones successive blooming	y	y	y	-
F. chiloensis	*chiloensis*	$n = 4$	Seasonal	-	-	-	-
F. chiloensis	*patagonica*	$n = 34$	Seasonal or long successive blooming	-	-	-	-
F. chiloensis	*lucida*	$n = 12$	Seasonal		-	-	-
F. virginiana	*platypetala*	$n = 2$	Seasonal or successive blooming	-	-	-	-
F. virginiana	*glauca*[b]	$n = 8$	Successive blooming tendencies	y	y	-	y
F. virginiana	*virginiana*	$n = 20$	Successive blooming tendencies	y	y	y	-

Notes: A represents Affx-88857614, A' Affx-8851928, B Affx-88866698 and C Affx-88877135. 'y' denotes yes the allele is present, a '-' indicates absences of the allele.
[a]Based on Hummer et al., 2016.
[b]Original Wasatch source: Bringhurst et al., 1988.

4 | DISCUSSION

We show for the first time that complex epistatic interactions across unlinked loci influence expression of the everbearing flowering trait in strawberries. We also provide a model that can be used to increase the efficiency of incorporating the Wasatch source of everbearing into strawberry breeding material.

4.1 | The three-allele model and epistatic interaction controlling the everbearing trait

The major effect QTL, *FaPFRU*, controlling the everbearing trait in cultivated strawberry was previously identified on chromosome 4A (Gaston et al., 2013; Perrotte, Gaston, et al., 2016) and has been utilised extensively by breeding companies. It was believed that a single dominant *FaPFRU* allele was sufficient to achieve the everbearing trait; however, spurious segregation patterns have been observed in breeding populations (Lewers et al., 2019). Lewers et al. (2019) predicted the hypothetical existence of epistatic enhancer and repressor modulators involved in the expression of the everbearing trait. Our GWAS has revealed three loci associated with the everbearing trait including a major SNP on chromosome 4A, here named A, and two potential epistatic enhancers in chromosomes 5C and 6A, named B and C, respectively.

The major locus A, on chromosome 4A, is located close to a previously identified region, *FaPFRU* (Hardigan et al., 2018). The previously identified significant SNP named A' in our study was 3.5 Mb away from A on chromosome 4A and suggested that recombination is strongly suppressed between these two loci in everbearing genotypes, but not in June bearers. However, an additional SNP called X, located between A and A', showed a stronger association with the everbearing trait than A' in our germplasm. Considering the highly significant interaction between the two loci A and X found in this study, it is likely that *FaPFRU* is located between these markers on chromosome 4A. However, the lack of SNP markers in the area prevents a more accurate detection of the locus in our dataset. Previously, *FaPFRU* was mapped between the markers bx089 and bx215 that delimited a QTL region of ~1.1 Mb, corresponding to the genomic fragment between 29,738,228 and 30,924,902 bp in *F. vesca* chromosome 4 (Perrotte, Gaston, et al., 2016), located just 200 kb downstream of the marker A. Nonetheless, here we have identified eight genotypes with putative recombinations in the region that are potentially useful for the identification of causative alleles (Dataset S5).

All individuals containing two copies of the major allele 'AA' were everbearers. Also, the majority of individuals (98%) that were heterozygotes for the major allele 'Aa' but contained two copies of 'B' or 'C' modifier alleles (either BB or CC, or heterozygous for both loci) were everbearers. The majority of individuals (88%) that did not contain a modifier were June bearers and individuals containing a single modifier segregated for flowering habit. Based on these findings, we suggest that B and C are functionally redundant quantitative enhancers of *FaPFRU* that are involved in the expression of the everbearing trait, at least in individuals that are heterozygous for dominant *FaPFRU* alleles. Furthermore, the segregation of June-bearing and everbearing phenotypes in the genotypes 'AaBbcc' and 'AabbCc' led us to hypothesise that there could be a fourth undetected QTL 'D', but further work is needed to test this hypothesis.

Through predicting the segregation ratios of progeny in our validation crosses, the complex three-gene model with epistatic interactions showed the best fit between expected and observed phenotypes. This supports the importance of the two epistatic modifiers identified here. Nonetheless, the R^2 value of the model was 0.33, indicating that other loci are likely to be involved in the expression of the everbearing trait, at least within the studied germplasm. The studied germplasm contains 651 breeding lines that are temperate Northwest European accessions. Greater relevance for commercially available material can be found in the 129 cultivars that were included in the analysis. Nonetheless, we propose the origin of the epistatic alleles may be traced back to wild octoploid accessions (see following text).

The 17 everbearers that did not contain a copy of the major allele A may have undergone a recombination between the focal maker Affx-88857614 and the causative allele (Dataset S6). Parents of the recombinant everbearers include those which are represented in two or three pedigrees such as 'Malling Pearl' (AaBbCc), FA807 (AaBbCC),

FA801 (genotype unknown) and HT18 (genotype unknown). Alternatively, these individuals may contain other 'non-Wasatch' sources of the everbearing trait. Indeed, other sources of the everbearing trait have been introgressed into breeding germplasm and described. For example, a QTL on linkage group 3C was identified to influence the intensity of late season flowering (Perrotte, Guédon, et al., 2016), and a significant locus for everbearing trait was identified on chromosome 3 in the pre-1990 breeding material that lacked the dominant *FaPFRU* locus (Hardigan et al., 2018). It is possible that some of the everbearing but homozygous recessive individuals 'aa' identified in this study may contain these sources of continual flowering.

4.2 | Molecular control of perpetual flowering

Previous studies have identified candidate genes for *FaPFRU* locus including *FLOWERING LOCUS T2* (*FaFT2*) and *PHYTOCHROME B ACTIVATION TAGGED SUPPRESSOR 1* (*BAS1/CYP72B1*) (Hardigan et al., 2018; Perrotte, Gaston, et al., 2016). The role that *FaPFRU* and *BAS1/CYP72B1* play in the control of the everbearing trait remains to be shown, although recent work has provided evidence that FT2 is a florigen with a role as a mobile flower inducing signal (Gaston et al., 2021). The mechanism controlling perpetual flowering has been extensively characterised in the diploid woodland strawberry (*F. vesca* L.), and many interacting components have been described (Koskela et al., 2012; Kurokura et al., 2017; Mouhu et al., 2013; Rantanen et al., 2014). None of these genes are located close to the *FaPFRU* loci, nor the two other loci identified here, but we have found a gene encoding a Phytochrome A photoreceptor less than 100 kb away from the most significant marker on chromosome 6A in *F. vesca* genome.

In *F. vesca*, a mutation in the gene encoding the floral repressor TERMINAL FLOWER1 (TFL1) leads to a perpetual flowering phenotype (Koskela et al., 2012), demonstrating that FvTFL1 functions as a floral repressor in this species. Because *FaTFL1* is not located close to an everbearing QTL in cultivated strawberries, we hypothesise that regulators of the everbearing trait either function as suppressors of *FaTFL1* or bypass its repressor function. Three other genes, *CONSTANS* (*FvCO*), another putative florigen gene *FvFT1* and *SUPPRESSOR OF THE OVEREXPRESSION OF CONSTANS1* (*FvSOC1*), control photoperiodic flowering in *F. vesca* (Koskela et al., 2012, 2016; Rantanen et al., 2014, 2015). If FaPFRU functioned as a repressor of *FaTFL1*, the long day-activated photoperiodic pathway genes *FaCO*, *FaFT1* and *FaSOC1* could be involved in rapid activation of flowering in long days, and in short days, normal flower induction could take place after gradual downregulation of *FaTFL1* (Koskela et al., 2016). This hypothetical model could explain the relatively weak photoperiodic responses of everbearing cultivars that are also commonly called as day-neutral plants (Hardigan et al., 2018; Hytönen & Kurokura, 2020). By contrast, continual flowering can be interrupted by high temperatures through an unknown mechanism (Bradford et al., 2010; Wagstaffe & Battey, 2006). It is clear that multiple regulators interact to modulate flowering habits in strawberries according to environmental signals (Koskela et al., 2016; Rantanen et al., 2014, 2015), but additional studies are needed to establish how known regulators and unknown loci are intertwined to control everbearing in cultivated strawberries. Moreover, studies should explore the function of the modifiers in different strawberry production areas, as previous studies have shown that expression of the everbearing trait is influenced by the local environment (Castro et al., 2015; Weebadde et al., 2007). This knowledge would enable the fine-tuning of fruit production in everbearing cultivars within different environments.

4.3 | The origin of the everbearing trait

Markers associated with everbearing alleles were found in the wild everbearing octoploid species *F. chiloensis* ssp. *pacifica*, *F. virginiana* ssp. *glauca* and *F. virginiana* ssp. *virginiana*. These findings are consistent with the reported origin of the everbearing phenotype in the studied cultivated strawberry having been sourced from *F. virginiana* ssp. *glauca* (Hancock & Simpson, 1995; Sakin et al., 1997). However, the alleles were also found in a *F. chiloensis* ssp., which could indicate that the everbearing loci arose prior to speciation of *F. chiloensis* and *F. virginiana*. Alternatively, it is possible that gene flow has occurred between the two species and selection has fixed the trait, as *F. chiloensis* ssp. *pacifica* is a northerly subspecies with a similar latitudinal range to *F. virginiana* spp. *glauca*, on the west coast of North America (Hummer et al., 2011).

4.4 | Trade-off between flowering and runner formation

Vegetative propagation of strawberry cultivars requires the production of runners from axillary buds. However, there is a long-standing observation that runner production negatively correlates with flower production (Serçe & Hancock, 2003) This trade-off between flower truss and runner production is particularly clear in everbearing strawberries, because the activation of continuous flowering by FaPFRU causes strong suppression of runner production (Gaston et al., 2013). It has been shown that there is no difference between the number of runners produced by cultivars homozygous or heterozygous for the dominant *FaPFRU* allele (Perrotte, Guédon, et al., 2016). However, as seen in our work, the phenotype of heterozygous 'Aa' is influenced by modifiers B and C, and therefore, their possible effects on runner production should be studied. Ideally, introducing stronger environmental regulation of flowering in everbearing strawberries would allow growers and nurseries to switch between the production of runners and flowers by changing the growing conditions.

4.5 | Applying the three-allele model

The three-allele model can be used to inform parental selection in order to increase the frequency of everbearing progeny. Where everbearing is an essential trait for a new variety, the use of a cost-effective genotyping platform such as KASP marker screening

(Semagn et al., 2014) could be used to minimise the costs associated with maintaining non-everbearing accessions and grubbing undesirable progeny. As a minimum, breeders using Northwest European germplasm should consider screening for the major 'A' locus in parental lines, as this will help to increase the chances of achieving 100% everbearing progeny. However, breeders using American germplasm should continue to use pre-existing markers that have been identified and validated in related germplasm (Perrotte, Gaston, et al., 2016; Salinas et al., 2017; Verma, Zurn, et al., 2017). Where crossing with Northwest European germplasm will generate heterozygous individuals (Aa), then screening for the B and C alleles could be used to inform parental selection and thus increase the frequency of everbearing individuals.

5 | CONCLUSIONS

The continuous flowering trait may be partially explained by the three-allele model detailed in this study—one large effect allele *FaPFRU* and two modifier alleles, B and C. The modifier alleles are functionally redundant such that two modifiers (either 'CC', 'BB' or 'BC') are required to produce the everbearing flowering habit in individuals containing a single copy of the dominant *FaPFRU* allele. Our results go some way towards explaining why breeders have been observing non-Mendelian segregation in what was once believed to be a monogenic trait.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author states that there is no conflict of interests regarding the publication of this work.

AUTHOR CONTRIBUTIONS

HMC, CN, TH, MS and RJH: Conceived and designed experiments. MS, HMC and RJH: Conducted quantitative genetics analysis and marker analysis. CN, TH, KH and AW: Performed experiments. SL: Performed DNA extraction for genotyping. HMC, CN, TH and RJH: Wrote the manuscript with contributions from all authors.

DATA AVAILABILITY STATEMENT

Data will be made available upon reasonable request through contacting the corresponding author.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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