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Research

Genetic determinism of phenological traits highly affected by climate change in *Prunus avium:* flowering date dissected into chilling and heat requirements

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Summary

- The present study investigated the genetic determinism of flowering date (FD), dissected into chilling (CR) and heat (HR) requirements. Elucidation of the genetic determinism of flowering traits is crucial to anticipate the increasing of ecological misalignment of adaptative traits with novel climate conditions in most temperate-fruit species.
- CR and HR were evaluated over 3 yr and FD over 5 yr in an intraspecific sweet cherry (*Prunus avium*) F_1 progeny, and FD over 6 yr in a different F_1 progeny.
- One quantitative trait locus (QTL) with major effect and high stability between years of evaluation was detected for CR and FD in the same region of linkage group (LG) 4. For HR, no stable QTL was detected. Candidate genes underlying the major QTL on LG4 were investigated and key genes were identified for CR and FD.
- Phenotypic dissection of FD and year repetitions allowed us to identify CR as the high heritable component of FD and a high genotype × environment interaction for HR. QTLs for CR reported in this study are the first described in this species. Our results provide a foundation for the identification of genes involved in CR and FD in sweet cherry which could be used to develop ideotypes adapted to future climatic conditions.

Introduction

Climate change is considered to be one of the main environmental problems of the 21st Century. According to the Intergovernmental Panel on Climate Change (IPCC) fourth assessment report, global average surface temperature increased by 0.74 ± 0.18°C in the last century and an increase of a further 1.1–6.0°C is projected to occur in this century (IPCC, 2007). This increase may put at risk fruit tree production in the coming decades in various temperate and subtropical regions throughout the world (Hanninen & Tanino, 2011; Luedeling, 2012). This risk is associated with the lack of adaptation of the dormancy/growth cycle to future climatic conditions, mainly related to a higher frequency of spring frost and insufficient chilling requirement fulfillment events (Campoy *et al.*, 2011a). In plants, the dormancy/growth cycle is synchronized to environmental and climatic conditions. Temperate tree species have developed a strategy to adapt to alternating well-differentiated seasons based on bud dormancy which helps to protect the bud from winter cold, ensuring that flowering occurs under optimal conditions. Lang (1987) proposed a physiological classification scheme for the different dormancy states considering the inputs and origins of dormancy-imposing cues. Ecodormancy corresponds to the growth inhibition found in late winter and spring and it is imposed by unfavourable temperatures. Paradormancy is equivalent to correlative inhibition or apical dominance, whereas deep dormancy defines endodormancy. In temperate fruit tree species, exposure to cold in winter (fulfillment of chilling requirements for overcoming endodormancy) followed by a warm period (fulfillment of heat requirements) in spring is essential for flowering. Thus, phenological traits such as chilling and heat requirements play a key role in flowering date and are crucial for the synchronization of flowering time with the environmental conditions. Consequently, climate warming during winter and spring is responsible for several disruptions already manifested in temperate fruit trees (Hanninen & Tanino, 2011; Luedeling, 2012). Advances in bud break and blooming dates are reported for many species, increasing the risk of frost damage (Cannell & Smith, 1986). Frost damage, in the USA, causes more economic losses than any other weather-related phenomenon (White & Haas, 1975). Moreover, climate warming will induce an incomplete dormancy release in many temperate and subtropical regions (Luedeling, 2012) that could lead to bud burst delay, low bud burst rate and lack of uniformity of leafing and bloom (Erez, 2000). This would lead to higher flower-bud drop and morphological abnormalities which could have important impacts on

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fruit production. This would be especially important in species with scarce low-chill commercial cultivars, such as sweet cherry (*Prunus avium* L.) and apricot (*Prunus armeniaca* L.).

Sweet cherry (*P. avium*), belonging to the *Prunus* genus within the Rosaceae family, is grown in temperate climates and enters into dormancy to persist through freezing temperatures during autumn and winter. However, as in most woody perennials, including poplar (*Populus* spp.), the most studied species for these traits, control of dormancy and flowering is not completely understood (Ruttink *et al.*, 2007; Cooke *et al.*, 2012). Some investigations have been directed towards the elucidation of the genetic control of flowering time. These studies have shown that chilling requirements have much stronger effects than heat requirements on flowering date in most *Prunus* species: sweet cherry (Alburquerque *et al.*, 2008), apricot (Campoy *et al.*, 2012), almond (*Prunus amygdalus* L. syn. *Prunus dulcis* M.) (Sanchez-Perez *et al.*, 2012), and peach *(Prunus persica* L.) (Okie & Blackburn, 2011).

Flowering date is considered to be quantitatively inherited in most fruit tree species (Anderson & Seeley, 1993). Substantial progress has been made in identifying quantitative trait loci (QTLs) for this trait in *Prunus* species: almond (Silva *et al.*, 2005; Sanchez-Perez *et al.*, 2012), apricot (Campoy *et al.*, 2011b; Dirlewanger *et al.*, 2012), sour cherry (*Prunus cerasus* L.) (Wang *et al.*, 2000), sweet cherry (Dirlewanger *et al.*, 2012) and peach (Dirlewanger *et al.*, 2012). Recently, the dissection of flowering date into chilling and heat requirements has been reported in peach (Fan *et al.*, 2010) and in apricot (Olukolu *et al.*, 2009). The colocalization of flowering date and chilling requirements together with the low heritability of heat requirements suggests that flowering date might be more strongly associated with chilling requirements than heat requirements. However, the effect of the interaction between environmental conditions and genetic control is still to be determined and requires time-consuming phenotyping to dissect flowering date into chilling and heat requirements.

The main objective of this study was to dissect the genetic determinism of flowering date into chilling and heat requirements and to analyse their interaction with environmental conditions. To achieve this objective, phenotyping was performed under highly contrasted climatic conditions. The aim of this study was: to investigate the effect of environmental conditions on the genetic control of these traits, to compare the genomic regions involved in flowering date in two different genetic backgrounds, and to identify candidate genes by *in silico* mapping using the peach genome sequence annotation (Verde *et al.*, 2013). This work provides further information on the interaction and genetic basis of these phenology traits and its findings will allow increased efficiency of breeding strategies focused on adaptation to the future climatic conditions of a specific region.

Materials and Methods

Plant material

Two intraspecific sweet cherry (*Prunus avium* L.) F_1 progenies from a cross-pollination of heterozygous parents were analysed. One of these progenies, called R × L, was derived from a cross between a German cultivar, 'Regina', and an American cultivar, 'Lapins', and consisted of 124 individuals. 'Lapins' was one of the first self-fertile commercialized cultivars. The other F_1 progeny, called R × G, was derived from a cross between the cultivar 'Regina' and the American cultivar 'Garnet', and included 117 individuals. 'Regina', 'Lapins' and 'Garnet' were used because they differ for various traits and especially flowering date. These progenies were cultivated in the fruit tree experimental field of the Institut National de la Recherche Agronomique (INRA)-Bordeaux research centre in Toulenne, near Bordeaux in France. The mature progenies and parents were grown on deep loamy soil on the bank of the Garonne River at 15 m above sea level (latitude 44.57 N, longitude 0.28 W), in a mild winter region with a yearly average rainfall of 825 mm.

Phenotypic trait assessment

Phenotyping was carried out on each parent and their progenies. Chilling requirements were evaluated during three consecutive years (2010–2012) on the 117 individuals of the R × G progeny following a forcing protocol widely used in temperate fruit trees (Campoy *et al.*, 2011a). Hourly temperatures were collected in the field from 1 October until the flowering date using an automatic data-logger (Ebro®; Ebro Electronic, Ingolstadt, Germany) in order to determine chilling and heat accumulation in the experimental field conditions. Three 1-yr-old fruiting branches for each individual were randomly collected once a week from 1 November until chilling requirements were achieved. At least a total of 30 flower buds were collected across the three branches for each sampling date. The bases of the branches were placed in water in a growth chamber (at 25°C, under white fluorescent tubes with a 16 h : 8 h, light : dark photoperiod) to force floral bud break (Mazzitelli *et al.*, 2007; Ruiz *et al.*, 2007; Alburquerque *et al.*, 2008; Sanchez-Perez *et al.*, 2012). After 7 d, the phenological stage of the flower buds was observed. The date of dormancy release was established when 30% of flower buds were at advanced Baggiolini stage B–C or at phenological growth stage 53 according to the international Biologische Bundesanstalt, Bundessortenamt et CHemische Industrie (BBCH) scale (Meier *et al.*, 1994; Alburquerque *et al.*, 2008). The Dynamic model (Fishman *et al.*, 1987) was then used to calculate chilling accumulation as the chill portion (CP) from 1 October until dormancy release, corresponding to the chilling requirements. Higher CP values mean that more chilling is required to break dormancy. Among the different models used to calculate chilling accumulation (Campoy *et al.*, 2011a), the Dynamic model was chosen on the basis of better performance in different climatic conditions (Luedeling & Brown, 2011).

Heat requirements were evaluated over 3 yr (2010–2012) in the R × G progeny. The model proposed by Richardson *et al.* (1974) was used to calculate heat accumulation as growing degree hours (GDH) between dormancy release and the flowering date.

Flowering dates were evaluated over 6 yr for R × L (2006 and 2008–2012) and 5 yr for R × G (2008–2012), when 70% of floral buds reached the full bloom stage in the field. For each

genotype, the whole tree of one clone was observed every 1 or 2 d during the flowering period. A single person evaluated the flowering date, which was recorded as the number of days from 1 January of that year (day of year). Four years of phenotyping data for the R × L family have already been used for QTL detection for flowering using a Simple Sequence Repeat (SSR) marker-based map (Dirlewanger *et al.*, 2012).

Statistical analysis of phenotypic data

Mean, minimum and maximum values of traits were calculated for the different years of evaluation. Measure of between-year stability was considered as a broad-sense heritability (broad-sense heritability, H_{BS}) and was estimated by using measures of each year as a random replication according to methods proposed by Dirlewanger *et al.* (2012). Estimates were obtained from the analyses of variance based on the random linear model:

$$Y_{ij} = \mu + y_i + g_j + e_{ij},$$

where Y_{ij} is the phenotypic value of the jth progeny in the ith year; μ is the mean value of the trait; y_i is the random effect of the ith year on the trait; g_j is the random genotypic effect of progeny j; and e_{ij} is the year × genotype interaction, that is, the residual of the model. Observations on a progeny are repeated measurements.

H_{BS} was estimated using the following equations:

$$H_{BS} = \frac{s_g^2}{s_g^2 + \frac{s^2}{n}}$$

where $s_g^2 = (MS_g - MS_{residual})\ n^{-1}$ is the genetic variance, MS_g is the estimated mean square of genotypes, $MS_{residual}$ is the estimated mean square of residual error, s^2 is a measure of variability attributable to genotype × year interaction, and n is the number of years in the experiment.

The statistical analyses were performed using the RCommander package of the software R 2.15.0 (R_Development_Core_Team, 2012).

Genotyping and map construction

The two progenies and their parents were genotyped using single nucleotide polymorphism (SNP) markers from the RosBREED cherry 6K SNP array v1 (Peace *et al.*, 2012). Mapping analyses were performed using JoinMap® 4.0 (van Ooijen, 2006) according to the mapping protocol previously described by Klagges *et al.* (2013).

R × L progeny was used previously for genetic map construction using SSRs (Dirlewanger *et al.*, 2004) and SNPs (Cabrera *et al.*, 2012; Klagges *et al.*, 2013), but for the QTL analysis, the initial map of 687 markers developed by Klagges *et al.* (2013) was simplified. Markers situated closer to each other than 2 cM were deleted to ease computing analysis. The R × G progeny was used for the first time in this study to construct a new sweet cherry genetic linkage map using the same array and protocol.

Haldane's mapping function was used to convert recombination frequency into map distance as in MultiQTL. Although the peach physical map based on peach physical genome v1.0 provided a reference for comparisons of linkage map output, no fixed order for the SNP markers was forced. The graphical presentation of linkage maps was performed using MapChart software version 2.2 (Voorrips, 2002).

Quantitative trait locus analysis

Multiple interval mapping was performed using MultiQTL V2.6 software (MultiQTL Ltd, Haifa, Israel; http://www.multiqtl.com). According to the principles of multiple interval mapping, a QTL with the greatest effect is first found and is taken as a cofactor to control the genetic background and then a search for another QTL is carried out in a different position. This procedure, repeated until no further QTL is found, takes into account the effects of QTLs present on other chromosomes.

Two types of analysis were performed. Each year was analysed independently for each trait in order to examine the stability of the QTLs. An analysis combining all years was performed using the multiple environment option available, increasing the accuracy of the QTL detection.

To confirm the presence of a QTL as statistically significant, the type I error at the chromosome level (α_{chr}) was calculated using the following relationship: $\alpha_{chr} = 1-(1-\ [1-\ (1-\alpha_g)^{1/M}])^m$, where M is the total number of markers used for the QTL detection on each map and m is the number of markers in the linkage group (LG) (Saintagne *et al.*, 2004). A genome-wide type I error (α_g) of 5% was chosen. The proportion of the phenotypic variation explained by the QTL (EV) was calculated as: $EV = ¼(d^2/\alpha_{ph}^2)$, where d^2 is the estimated substitution effect of the QTL ($d = X(A) - X(B)$, A and B being the two homozygote genotypes at the marker loci) and α_{ph} is the phenotypic variance of the trait. The graphical presentation of linkage maps and QTL was obtained using MapChart software version 2.2.

In silico candidate gene research

Chromosomal regions for *in silico* candidate gene analysis were initially selected based on the location of the QTLs associated with chilling requirements and flowering date. Predicted peach protein sequences derived from scaffolds underlying the major QTL on LG4 were downloaded from the Genome Database for Rosaceae (http://www.rosaceae.org/node/355) and blasted against the National Center for Biotechnology Information (NCBI) nr database using blastp in the program Blast2GO (Conesa *et al.*, 2005) with a *E*-value cut-off of 0.001. Blast2GO was then used for the gene ontology annotation.

Results

Phenotypic analysis, correlations and heritability

All three flowering traits showed high interannual variability (Supporting Information Table S1). In the cool winter 2009/

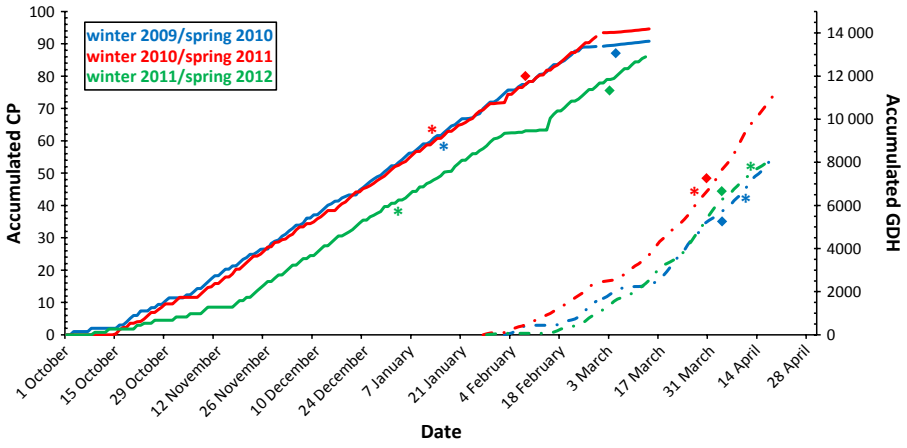

Fig. 1 Chill portion (CP) (continuous line) and growing degree hours (GDH) (dashed line) accumulation in the field for the years of study (blue, 2009/2010; red, 2010/2011; green, 2011/2012). The values for fulfillment of chilling and heat requirements for each year are indicated for the sweet cherry (*Prunus avium*) cultivars 'Regina' (squares) and 'Garnet' (stars).

spring 2010, we observed the highest chilling requirements and the latest flowering date (75.4 CP and 11 April for R × G and 12 April for R × L, respectively). In spring 2011, we observed that flowering dates were earlier (31 March for R × G and 4 April for R × L). In winter 2011/spring 2012, the lowest mean value of chilling requirements (58.7 CP) was observed. Chill and heat accumulations on the experimental field were markedly different between years (Fig. 1). Chill accumulation was lower and delayed in 2012 compared with 2010 and 2011. No chill accumulation was achieved in the first 2 wk of February 2012 because of an abnormal frost period (current dormancy models do not consider the chill effect of temperatures below 0°C). Also, variation was observed for heat accumulation according to the year of evaluation. In 2010, heat accumulation was lower and more irregular than in 2011 and 2012 (Fig. 1).

These different patterns of chilling and heat accumulation were associated with the distribution of each trait according to the year of evaluation (Fig. 2). For example, in 2011 flowering date showed a low distribution amplitude (8 and 9 d for R × G and R × L, respectively) and earlier mean value as a consequence of the high chill and high heat accumulated compared with 2010 and 2012 (Fig. 2c,d). In contrast, in 2010 the mean flowering date was later than in 2011 or 2012 because of the lower heat accumulation recorded.

No trait followed a normal distribution according to the statistical Shapiro–Wilk test, except for heat requirements in R × G in 2011 (Fig. 2b). Distributions were markedly skewed in R × G for chilling and heat requirements in 2012, the year of lower and more delayed chill accumulation (Fig. 2a,b). Transgressive segregants, having a phenotype beyond those of their parents, were identified for the three traits.

As expected, flowering date and chilling requirements were positively correlated with one another from year to year, and heat requirements were generally, but not always, negatively correlated with chilling (Table 1). Surprisingly, no significant correlation was found for heat requirements for either 2010 or 2011 with 2012, the year with delayed chill accumulation. Similarly, heat requirements were positively correlated to flowering date in all years but the year with delayed chill accumulation.

H_{BS} was 84% for chilling requirements and 43% for heat requirements in R × G. Regarding flowering date, H_{BS} was 92% and 91% in R × L and R × G, respectively.

Linkage maps

For R × L, the parental maps used for QTL detection consist of 136 and 127 SNP markers over eight LGs covering 712.4 and 710.4 cM for 'Regina' and 'Lapins', respectively (Table S2).

For R × G progeny, 723 SNP markers were included in the initial map. Markers were organized into eight LGs. These maps used for QTL detection consisted of 142 and 137 markers covering 657.6 and 823.6 cM for 'Regina' and 'Garnet', respectively (Table S2).

QTL analysis

Chilling requirements For the R × G progeny, QTLs were detected on five LGs, LG1 for 'Garnet' and LG1, 2, 4, 6 and 7 for 'Regina' (Fig. S1, Table 2a). A major QTL was detected for all years on LG4 of 'Regina' with a very small confidence interval and an explained variation (EV) of 22%, 18.1% and 16.2% in 2010, 2011 and 2012, respectively (Fig. 3). The QTL on LG1 of 'Garnet' was detected in 2010 and 2012 analyses with an EV of 13.4% and 14.8%, respectively. QTLs on 'Regina' LG1 and LG2 were detected only in 2010. Using multi-year analysis, these QTLs were detected as well as other QTLs with lower EV on LG6 and LG7 of 'Regina'.

Heat requirements For the progeny R × G, QTLs were detected on seven LGs, LG1, 3, 6 and 8 for 'Garnet' and LG1, 4 and 7 for 'Regina' (Fig. S1, Table 2b). QTLs detected were different for each year. QTLs with the highest EV were detected in 2011: on LG4 of 'Regina' (EV = 33.3%) and on LG6 of 'Garnet' (EV = 21.1%). Using multi-year analysis, these QTLs, except that located on LG7 of 'Regina', were detected. Surprisingly, this QTL on LG7 was the only one detected in 2012, the year of delayed chill accumulation. QTLs with the highest effect were detected on LG4 of 'Regina' and LG6 of 'Garnet' with an EV of 10.9%. Another QTL with a lower EV was detected on LG8 of 'Garnet', only by the multi-year analysis.

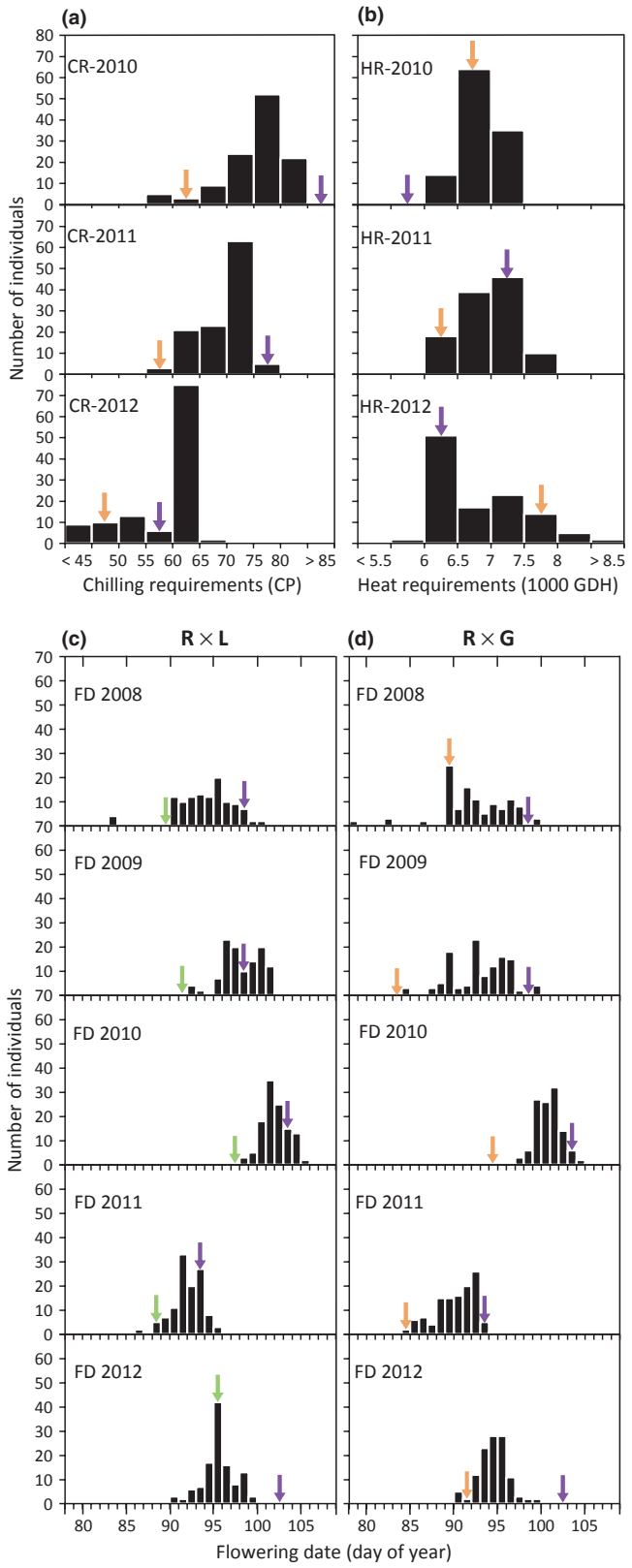

Fig. 2 Distribution of phenological traits in sweet cherry (*Prunus avium*): chilling requirements (CR) in chill portion (CP) (a) and heat requirements (HR) in growing degree hours (GDH) (b) in 'Regina' × 'Garnet' progeny (R × G) and flowering date (FD) in 'day of the year' in 'Regina' × 'Lapins' (R × L) (c) and R × G (d) progenies. Data for each parent are indicated by arrows ('Regina', purple; 'Garnet', orange; 'Lapins', green).

Flowering date One major QTL was detected in the two progenies and for all years on LG4 of 'Regina' with a small confidence interval and an EV that reached 31.8% and 45.8% for R × L and R × G, respectively (Fig. 3, Table 2c,d). For the R × L progeny, QTLs were also detected on LG1 of 'Lapins' in 2011 and 2012 and on LG5 of 'Regina' in 2009 and 2010. Two other minor QTLs were only detected with multi-year analysis, on LG2 of 'Lapins' and LG8 of 'Regina'. More QTLs were detected within the R × G than within the R × L progeny; in fact, QTLs were detected on all linkage groups. QTLs for 'Regina' on LG6 and LG7 were both detected for 3 yr, although the maximum EV was 10.5%. In contrast, QTLs on LG1 and LG6 of 'Garnet' were detected only for 1 yr but with high EV, 17.1% in 2008 and 14.4% in 2011, respectively. Using multi-year analysis, all QTLs detected in single-year analyses were found except the QTL on LG3 of 'Regina'. Also, five new QTLs were detected (on LG2 of 'Regina' and LG2, 3, 5 and 8 of 'Garnet') but all had small EV.

Haplotype analyses Haplotypes were constructed with SNP markers segregating in the major QTL on LG4; nine and three markers were used for R × G and R × L, respectively (Table S3a). As parental maps were built for QTL analysis using markers only segregating in one parent, two alleles were obtained in each diploid progeny. The mean values of chilling requirements, heat requirements and flowering date for the two haplotypes were calculated for each year of evaluation (Table S3b). Significant differences between the two haplotypes were found for each year for chilling requirements and flowering date. As for heat requirements, a significant difference was only obtained for 2011. For chilling requirements, the differences between the haplotypes ranged from 3.77 to 5.15 CP, for flowering date, values ranged from 1.7 to 3.8 d of year and for heat requirements the difference between the haplotypes was 504.8 GDH (2011). The greatest difference was observed in 2012 for chilling requirements and in 2009 for flowering date for the two progenies. In all cases the 'Regina' haplotype increased the value for each trait.

In silico candidate gene research

In silico candidate gene analysis was performed within small confidence intervals found for the QTLs on LG4. The region covered by the confidence intervals was reduced thanks to the high number of phenotyping years and to the high heritability of the traits. Other QTLs were dismissed for *in silico* candidate gene analysis because of the large size of the confidence interval. These intervals on LG4, expressed in bp to represent the physical position in peach, overlapped to a considerable extent: 6580 288–9412 384 bp for flowering date in R × L, 6789 121–8045 747 bp for flowering date in R × G, and 7075 445–8196 058 bp for chilling requirements in R × G (Fig. 3). In order to include QTLs for both flowering date and chilling requirements, 2830 000 bp from 6580 288 bp of scaffold 4 were screened. In this region, 429 genes were predicted in the peach ('Lovell') genome (Table S4), of which one had no Blast hit in databases and 47 had Blast hits with unknown proteins. Data

Table 1 Spearman correlation coefficients of chill (CR) and heat (HR) requirements and flowering date (FD) in progenies of sweet cherry (*Prunus avium*) during different years of observation

R × L	FD-2006	FD-2008	FD-2009	FD-2010	FD-2011					
FD-2008	0.59*									
FD-2009	0.63*	0.78*								
FD-2010	0.59*	0.75*	0.88*							
FD-2011	0.70*	0.67*	0.73*	0.75*						
FD-2012	0.66*	0.66*	0.74*	0.69*	0.70*					
R × G	CR-2010	CR-2011	CR-2012	HR-2010	HR-2011	HR-2012	FD-2008	FD-2009	FD-2010	FD-2011
CR-2011	0.63*									
CR-2012	0.70*	0.66*								
HR-2010	−0.45*									
HR-2011		0.25		0.35*						
HR-2012			−0.34*	0.27	0.09					
FD-2009							0.82*			
FD-2010	0.51*			0.46*			0.77*	0.88*		
FD-2011		0.52*			0.92*		0.74*	0.84*	0.77*	
FD-2012			0.62*			0.06	0.74*	0.79*	0.78*	0.80*

R × G, 'Regina' × 'Garnet' progeny; R × L, 'Regina' × 'Lapins' progeny. Traits are given as xx-yyyy: xx, trait acronym; yyyy, year of evaluation. Significant correlation: *, $P < 0.001$.

mining based on the predicted function of proteins and their potential involvement in flowering and dormancy resulted in the selection of nine candidate genes (Table 3, Fig. S2). Among these, candidate genes with gene ontologies related to different processes were found: negative regulation of flower development, *NUCLEAR PORE ANCHOR (NUA), PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1 (PIE1), EMBRYONIC FLOWER2* (*EMF2*) and *ACTIN-RELATED PROTEIN 4-LIKE* (*ARP4*); photoperiodism, *ARP4*; gibberellin metabolism, *ENT-KAURENE SYNTHASE* (*KS*), *GA20 OXIDASE* (*GA20ox*) and *GIBBERELLIN 2-OXIDASE* (*GA2ox*); and vernalization, *EMF2* (Table 3). In the same interval, 13 target genes of miRNAs (ppa024905m, ppa022903m, ppa011349m, ppa018824m, ppa001477m, ppa008801m, ppa000550m, ppa008304m, ppa019339m, ppa000901m, ppa016261m, ppa008637m, and ppa003898m) already reported in peach by Barakat *et al.* (2012) were found.

Discussion

Despite considerable efforts to understand the effects of the environment on growth and flowering, very little is known about the molecular and genetic mechanisms that control flower development in sweet cherry (Yu *et al.*, 2012). The present study clearly shows the polygenic control of chilling requirements and flowering date in sweet cherry.

Phenotypic analysis, correlation and heritability

The time-consuming process of evaluating chilling requirements is a major drawback for phenotyping this important breeding trait. Previous studies within the Rosaceae family have consisted of 2-yr analysis in almond (Sanchez-Perez *et al.*, 2012), apricot (Olukolu *et al.*, 2009) and peach (Fan *et al.*, 2010). This 3-yr QTL study of chilling requirements performed in a Rosaceae species under markedly different winter and spring conditions allowed us to identify interesting features of the interaction of chilling requirements and heat requirements in a segregating population. The skewness from normality and differences in segregation could be related to the environmental effect. For example, the bimodal distribution of flowering date in R × L during 2010 (Fig. 2c) can be related to the irregular and delayed heat accumulation period (Fig. 1), whereas the skewed distribution of chilling requirements during 2012 (Fig. 2a) may be associated with the delayed chill accumulation and the frost period at the beginning of February. The delayed and reduced chill accumulation recorded in 2012 can be associated with the reduced fruit set and productivity found for many high-chill varieties (data not shown), a negative effect of insufficient chilling fulfillment described for temperate fruit trees (Erez, 2000).

The mean values and distributions of chilling requirements, heat requirements and flowering date are related to the different patterns of chill and heat accumulation recorded between years. For each year, the mean value of chilling requirements is related to the total chill accumulation in the field. Across years, the higher the chill accumulated in the field conditions the higher the chilling requirements calculated. This is in agreement with previous findings in sweet cherry (Alburquerque *et al.*, 2008), apricot (Campoy *et al.*, 2012), almond (Egea *et al.*, 2003) and peach (Pawasut *et al.*, 2004; Fan *et al.*, 2010). In addition, significantly different chilling requirements have been found in clonal plant material cultivated in different climatic conditions in peach (Balandier *et al.*, 1993) and apricot (Campoy *et al.*, 2012). As for heat requirements, the lower the chilling requirements of a cultivar the higher the heat requirements, as previously found in peach (Pawasut *et al.*, 2004) and apricot (Campoy *et al.*, 2012), except for 2011 when no significant correlation was found. The later flowering date values recorded in 2010 for both families are related to the lower heat accumulation recorded in this year (Table S1). In 2011, chilling accumulated was similar to that recorded in 2010, but heat accumulation was considerably higher, which led to an advance in flowering date for both populations (Figs 1, 2c,d).

Table 2 Quantitative trait loci (QTLs) detected in sweet cherry (*Prunus avium*) using year-by-year and multi-year analysis for: (a) chill requirements and (b) heat requirements for 'Regina' × 'Garnet' progeny (R × G) and (c, d) flowering date for (c) 'Regina' × 'Lapins' progeny (R × L) and (d) R × G. QTLs detected every year are shaded in gray

(a)

R × G	LG	L(cM)	CI (95%)	LOD	EV	d
Years						
2010	R1	54.5	[14.6;94.4]	4.0	9.8	−4.0
	R2	49.5	[26.0;73.0]	3.7	9.6	−4.0
	R4	22.3	[17.5;27.0]	8.3	22.0	6.0
	G1	27.1	[0.0;103.5]	3.5	13.4	−3.6
2011	R4	20.6	[15.7;25.5]	6.6	18.1	4.0
2012	R4	22.3	[3.5;41.2]	5.1	16.2	5.3
	G1	55.6	[0.0;126.6]	3.6	14.8	−4.4
Multi-years	LG	L(cM)	CI (95%)	LOD	EV_{mean}	d_{mean}
	R1	35.4	[0.0;74.6]	8.5	7.4	−3.1
	G1	39.4	[0.0;104.8]	7.9	9.7	−3.1
	R2	52.9	[27.3;76.7]	7.6	6.5	−2.8
	R4	21.0	[18.9;23.1]	20.8	17.5	5.0
	R6	80.4	[66.1;81.7]	7.6	6.0	−2.7
	R7	50.8	[30.9;57.6]	7.3	5.8	2.7

(b)

R × G	LG	L(cM)	CI (95%)	LOD	EV	d
Years						
2010	R1	67.0	[47.9;86.0]	4.9	15.6	273
	G1	37.2	[0.0;126.4]	3.5	9.8	221
	G3	95.0	[68.1;100.3]	4.2	13.4	253
2011	R4	22.5	[11.6;33.4]	9.7	33.3	524
	G6	21.6	[9.3;33.9]	6.0	21.1	−418
2012	R7	12.9	[0.9;25.0]	3.6	13.7	−460
Multi-years	LG	L(cM)	CI (95%)	LOD	EV_{mean}	d_{mean}
	R1	61.9	[28.3;95.5]	6.0	6.4	137
	R4	19.0	[11.0;27.1]	10.7	10.9	208
	G1	75.7	[0.0;151.2]	5.9	5.3	175
	G3	98.9	[89.1;100.3]	8.3	7.9	220
	G6	24.5	[16.9;32.0]	11.0	10.9	−308
	G8	55.0	[17.1;72.1]	7.0	6.3	219

(c)

R × L	LG	L(cM)	CI (95%)	LOD	EV	d
Years						
2006	R4	32.6	[10.3;54.9]	4.8	18.7	2.2
2008	R4	29.1	[19.9;38.3]	6.4	19.0	3.2
2009	R4	28.3	[20.6;36.0]	11.5	31.8	3.1
	R5	9.2	[0.0;41.33]	2.8	6.6	1.2
2010	R4	27.8	[18.8;36.8]	9.6	27.4	1.7
	R5	13.8	[0.0;57.5]	3.2	8.3	0.5
2011	R4	29.7	[16.7;42.8]	5.6	19.6	1.6
	L1	131.7	[66.6;152.2]	3.3	13.1	1.3
2012	R4	29.9	[21.5;38.3]	8.5	25.4	2.0
	L1	122.0	[50.4;152.2]	3.2	12.9	1.4
Multi-years	LG	L(cM)	CI (95%)	LOD	EV_{mean}	d_{mean}
	L1	146.9	[144.2;149.6]	17.1	8.0	1.4
	L2	65.5	[45.4;70.3]	8.0	3.3	0.8
	R4	29.6	[27.9;31.2]	47.0	21.2	2.2
	R5	5.2	[0.0;36.51]	11.3	4.4	0.7
	R8	45.4	[30.9;55.0]	9.3	4.0	0.8

(d)

R × G	LG	L(cM)	CI (95%)	LOD	EV	d
Years						
2008	R3	33.4	[6.2;60.6]	2.7	5.5	−1.9
	R4	22.0	[16.6;27.3]	12.1	30.5	4.6
	R6	75.7	[52.9;81.7]	4.2	9.1	−2.5
	R7	6.3	[0.0;22.5]	3.4	6.9	−2.0
	G1	109.6	[62.4;151.2]	4.3	17.1	3.1
2009	R1	19.0	[0.0;52.2]	3.7	5.9	−1.5
	R4	22.8	[18.5;27.2]	24.1	45.8	4.4
	R6	71.7	[61.8;81.6]	7.7	10.5	−2.1
	R7	55.9	[45.3;57.6]	6.6	8.5	1.8
2010	R4	21.5	[15.7;27.2]	15.3	42.1	2.0
	R7	34.9	[0.0;57.6]	2.7	5.9	0.1
2011	R4	20.0	[13.1;26.9]	13.7	36.7	2.8
	R6	66.6	[23.4;81.7]	3.1	6.8	−1.2
	G6	24.3	[6.6;42.1]	4.8	14.4	−1.8
2012	R4	21.6	[18.2;25.1]	12.5	36.2	2.3
Multi-years	LG	L(cM)	CI (95%)	LOD	EV_{mean}	d_{mean}
	R1	19.5	[0.0;50.6]	9.9	4.0	−0.9
	G1	111.2	[96.2;126.3]	14.2	7.5	1.4
	R2	29.5	[16.9;42.2]	7.4	2.9	−0.8
	G2	14.2	[1.0;27.4]	10.5	5.4	−1.2
	G3	88.0	[31.3;100.3]	7.4	4.1	0.8
	R4	20.4	[18.4;22.5]	76.1	36.3	3.1
	G5	11.5	[0.0;28.7]	6.8	3.5	−0.9
	R6	69.1	[49.1;81.7]	11.8	4.1	−1.0
	G6	23.8	[19.2;28.4]	20.5	12.1	−1.9
	R7	51.0	[19.7;57.6]	13.5	4.7	0.8
	G8	53.8	[10.8;72.1]	6.9	3.4	0.9

LG, linkage group; L, distance from the beginning of the chromosome to the point of maximum LOD in the interval; CI, confidence interval; LOD, logarithm of the odds ratio; EV, variation explained by the QTL in percentage of the total variation explained; EV_{mean}, mean of EV in the multiple environment approach; d, difference X(A) − X(B) according to the year of evaluation, where A and B are the two homozygotes at the marker loci; d_{mean}, mean of d in the multiple environment approach; (+/−), the sign varies according to the year of evaluation.

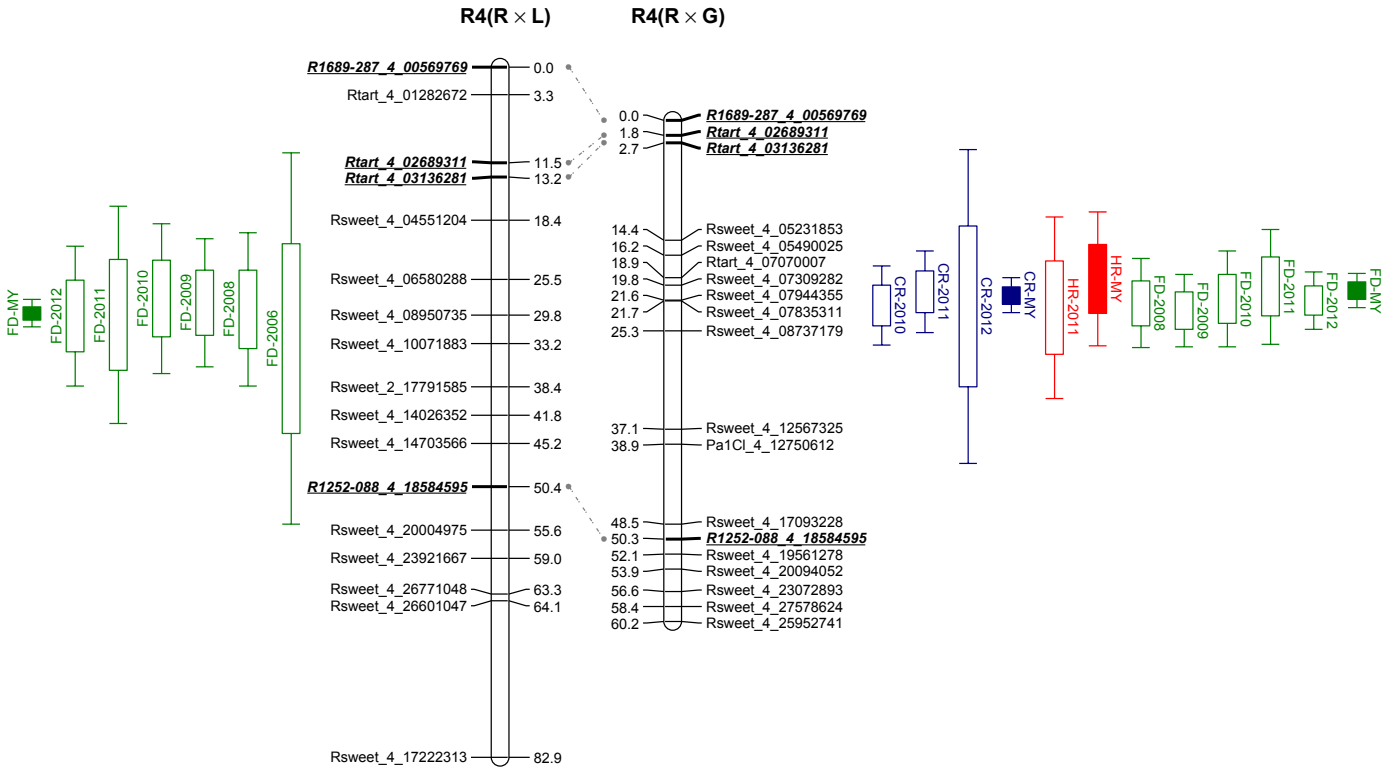

Fig. 3 Quantitative trait loci (QTLs) located in linkage group 4 (LG4) of the sweet cherry (*Prunus avium*) cultivar 'Regina' (R4) detected using 'Regina' × 'Lapins' (R × L; left) and 'Regina' × 'Garnet' (R × G; right) progenies. Results for each year are indicated by open bars and are given as xx-yyyy (xx, trait acronym; yyyy, year of evaluation). Those obtained using multi-year analyses in MULTIQTL are indicated by closed bars and given as xx-MY. Bars are blue for chilling requirements (CR), red for heat requirements (HR) and green for flowering date (FD). Solid bars denote the most probable position of the QTL obtained from the highest LOD score position given by the MIM and the bootstrap (1000 permutations) ± SD. Confidence intervals (α<5%) are represented as lines. The distance between markers is represented in cM. Markers present in both families are in bold, italic and underlined.

Table 3 Candidate genes located in the quantitative trait loci (QTLs) on linkage group 4 in sweet cherry (*Prunus avium*)

Gene	Description	Peach gene ID	Transcript start	Sequence length (aa)	Match ID (accession)	Similarity (%)	E-value	Gene ontology – biological process
NUA	Nuclear-pore anchor-like	ppa000061m	6823 622	2039	XP_004293787.1	81	0E0	Negative regulation of flower development; protein import into nucleus
KS	ent-kaurene synthase	ppa001902m	7070 520	745	AEN74904.1	91	0E0	Gibberellin biosynthetic process
KS	ent-kaurene synthase	ppa018714m	7078 032	383	AEN74904.1	77	0E0	Gibberellin biosynthetic process
KS	ent-kaurene synthase	ppa019543m	7087 518	337	AEN74904.1	75	3.19E-111	Gibberellin biosynthetic process
PIE1	Photoperiod-independent early flowering 1	ppa000063m	7488 534	2030	XP_002882790.1	74	0E0	Negative regulation of flower development; chromatin remodelling
GA20ox	Gibberellin 20-oxidase	ppa020361m	7983 415	316	XP_002517548.1	77	1.32E-146	Gibberellin process; oxidation-reduction process
GA2ox	Ggibberellin 2-oxidase	ppa008414m	8581 870	334	ACT99992.1	92	0E0	Gibberellin catabolic process; oxidation-reduction process
EMF2	Embryonic flower 2	ppa002685m	8728 803	646	XP_004300676.1	88	0E0	Vernalization response; negative regulation of flower development; histone methylation
ARP4	Actin-related protein 4-like	ppa006685m	8820 787	401	XP_002275561.2	94	0E0	Long-day photoperiodism, flowering

H_{BS} has been used as an index of reliability of phenotypic selection for genetic characteristics, and the accuracy of QTL analysis depends considerably on the level of H_{BS} (Beavis, 1998). We have shown that, in our mapping populations, H_{BS} was sufficiently high (> 0.8) to enable genetic analysis of chilling requirements and flowering date, indicating that the values for the two traits

were consistent over years. H_{BS} values for flowering date in both families were within the range of those found in other Rosaceae species: 0.84 in apple (*Malus domestica*) (Celton *et al.*, 2011); 0.92 in rose (*Rosa* ssp.) (Kawamura *et al.*, 2011); 0.89 and 0.85 in peach; 0.67 in apricot and 0.88 in sweet cherry (Dirlewanger *et al.*, 2012); and 0.91 in sour cherry (Wang *et al.*, 2000). The H_{BS} of chilling requirements (0.83) was similar to that found in peach (0.79) (Fan *et al.*, 2010). To our knowledge, this is the first reported H_{BS} value for sweet cherry, showing the high genetic control of this trait in this species. In contrast, the H_{BS} value for heat requirements was much lower (0.43) compared with the values for flowering date and chilling requirements. This is in agreement with the value of 0.54 found in a previous study in peach (Fan *et al.*, 2010). The lower H_{BS} for heat requirements indicates a stronger genotype × year interaction for this trait. This is in agreement with the reduction of heat requirements after the application of chilling beyond the fulfillment of the cultivar's chilling requirements, noted by several authors in different species (Couvillon & Erez, 1985; Ghelardini *et al.*, 2010; Okie & Blackburn, 2011). Correlations between years for flowering date were similar in R × L and R × G, but higher than those found for chilling requirements in R × G. This may be explained by the higher amplitude observed between the earliest and latest flowering genotypes compared with the amplitude between the high and low chill or heat genotypes (Fig. 1a,b). However, in the case of heat requirements, two out of three correlations were not significant. Nonsignificant correlations of heat requirements coincided with 2012, the year of abnormal delayed chill accumulation. This is consistent with the influence of the residual effect of dormancy release (insufficient chilling accumulation) on heat requirements found in *Prunus* (Couvillon & Erez, 1985; Okie & Blackburn, 2011). The correlation between chilling requirements and flowering date showed significant and constant values for the different years of evaluation, as previously found in almond (0.8–0.91; Sanchez-Perez *et al.*, 2012) and peach (0.70–0.67; Fan *et al.*, 2010). In contrast, correlations of these traits with heat requirements showed high variability between years, suggesting a strong influence of the environmental conditions. These results are in agreement with the effect of inadequate fulfilment of chilling requirements over heat requirements in peach (Okie & Blackburn, 2011). Similar results were found in elm (*Ulmus* spp.) (Ghelardini *et al.*, 2010).

Transgressive segregation, the appearance of individuals in segregating populations beyond the parental phenotypes, was evident among the R × G population for chilling requirements and heat requirements and among R × L and R × G for flowering date. Both positive (later dormancy release of flowering than in 'Regina', the late-flowering/high-chill cultivar) and negative (earlier dormancy release of flowering than in either 'Lapins' or 'Garnet', early-flowering/low-chill cultivars) transgressive segregants were found, and could be useful for future breeding for climatic adaption in sweet cherry.

Linkage maps

Total coverage for the four parental maps was similar to those previously published for sweet cherry (Klagges *et al.* (2013). Parental maps were compared with the complete maps (Klagges *et al.*, 2013) and no rearrangement was found (data not shown). In R × L, distortions in the marker distributions at the top and bottom of LG6 were found concentrated only on 'Lapins'. These distortions could be associated with the presence of male sterility and the gametophytic S locus, located at the top and bottom of LG6, respectively (Dirlewanger *et al.*, 2004; Tao & Iezzoni, 2010). However, in R × G, distortions in marker distributions were found only at the bottom of Garnet's LG6, probably associated with the gametophytic S locus.

Genetic determinism

As population sizes were limited, only QTLs with large effects were statistically significant and stable; therefore, the number of QTLs detected in this study should be considered a minimum estimate. Six QTLs for chilling requirements and six QTLs for heat requirements were mapped in R × G, whereas six and 11 QTLs for flowering date were mapped in R × L and R × G, respectively. QTLs with the highest EV were located in the syntenic region in both progenies for flowering date. This similarity of results in two genetic backgrounds (different male parents) gives robustness to the QTL detection performed. QTL clusterings were clear and consistent with the strong correlations previously shown between chilling requirements and flowering date (Table 1). This was especially clear for the major QTL detected on LG4 of 'Regina' in both R × L and R × G populations (Table 2, Fig. 3). This QTL colocalizes with a major QTL found in almond (Sanchez-Perez *et al.*, 2012), apricot (Dirlewanger *et al.*, 2012), peach (Fan *et al.*, 2010; Dirlewanger *et al.*, 2012) and sweet cherry (Dirlewanger *et al.*, 2012). However, whether this pattern is attributable to pleiotropic effects of a single gene or to tightly linked genes is still to be determined. In spite of this, interesting colocalizations with candidate genes were found on LG4. However, other major QTLs for chilling and flowering phenology have been found in *Prunus* species. One major QTL for chilling requirements was identified on LG1 in peach colocalizing with a QTL for flowering date, and overlapping the evergrowing (EVG) region (Fan *et al.*, 2010). Another QTL mapped on LG7 for chilling requirements colocalized also with a QTL for flowering date. These colocalizations suggested that a unique temperature sensor regulated both traits. Several candidate genes for dormancy release control have been reported in peach, such as *DORMANCY ASSOCIATED MADS-box* (*DAM*) genes (Leida *et al.*, 2012). Similarly, major QTLs for flowering phenology have been found in LG1 and LG5 in apricot (Campoy *et al.*, 2011b; Socquet-Juglard *et al.*, 2013). Within the sweet cherry progenies studied, QTLs for chilling requirements, heat requirements and flowering date were also detected on LGs 1, 5 and 7, but all of them had minor to moderate effects and were not stable across years. Nevertheless, a colocalization for chilling requirements and flowering date was found on LG1 and LG7 of 'Regina', by using multi-year analyses (Fig. S1). Whether the differences in QTL detection found between these closely related species are attributable to differences in genetic determinism or just differences in allelic donation of progeny parents is still to be

determined. The use of different genitors, and in particular of low-chill varieties, for establishing new mapping progenies as well as association mapping analyses will help to resolve this uncertainty.

The identification of the QTL on LG4 in progenies from different, albeit related, genetic backgrounds analysed over several years gives robustness to our results. In addition, the significant differences observed for the two haplotypes for each year for chilling requirements and flowering date indicate that by using the selected SNP markers it is possible for breeders to develop an approach to build ideotypes better adapted for the predicted environmental conditions resulting from climatic change. A difference of nearly 4 d for flowering date was observed between the two haplotypes, while the amplitude within the complete population was 15 d. Moreover, according to the high synteny among *Prunus* (Dirlewanger *et al.*, 2004) and the colocalization of other QTLs for flowering date within *Prunus* (Dirlewanger *et al.*, 2012), we could expect a similar mechanism of control in other *Prunus* species.

The low H_{BS} of heat requirements and the colocalization with QTL regions for chilling requirements suggest not only low genetic control underlying this character but also that it is affected by the level of chilling requirement fulfillment. In addition, this raises the need for a more accurate phenotyping method to unambiguously characterize dormancy release. Currently, forcing methods are the most frequently used option, but they remain insufficient for high-throughput phenotyping of complete dormancy release and for standardizing data from different species.

Candidate gene analysis

A scan over the region comprising the confidence intervals for the QTLs involved in chilling and flowering phenology on LG4 allowed us to identify candidate genes for these traits (Table 3). The gene ontologies of these genes were associated either with the gibberellin biosynthesis pathway or with negative regulation of flower development. In contrast, no obvious function related to the control of dormancy was found for the 13 target genes of miRNAs located in the same region. The putative roles and interactions of the candidate genes are discussed in the following sections.

Candidates genes involved in the gibberellin biosynthesis pathway: *KS*, *GA20ox* and *GA2ox*

KS converts *ent*-copalyl diphosphate (CDP) to ent-kaurene, an early intermediate in gibberellin biosynthesis (Yamaguchi, 2008). There is evidence that ent-kaurene biosynthesis is controlled by environmental conditions such as photoperiod (Zeevaart & Gage, 1993) and temperature (Moore & Moore, 1991) as well as by plant development (Silverstone *et al.*, 1997).

GA20ox is a key enzyme for gibberellin biosynthesis (Yamaguchi, 2008) and promotes flowering in *Arabidopsis thaliana* (Rieu *et al.*, 2008).

GA2ox is an enzyme related to gibberellin inactivation (Yamaguchi, 2008).

Bioactive gibberellins act as key mediators between the perception of environmental signals and the resulting growth responses. Gibberellins have important roles in promoting transcription of the *FLOWERING TIME LOCUS* (*FT*), *TWIN SISTER OF FT* (*TSF*) and *SQUAMOSA PROMOTER BINDING PROTEIN-LIKE* (*SPL*) genes during floral induction in response to long days in *A. thaliana* (Porri *et al.*, 2012). Increases in gibberellin concentrations were reported to be necessary for endodormancy release (Lavee, 1973). Recent evidence indicates that gibberellin biosynthetic genes are induced by long-term chilling exposure in dormant buds, being associated with the acquisition of growth ability (Rinne *et al.*, 2011). In peach, endogenous GA concentrations are fine-tuned during dormancy/activity transitions in flower buds (Reinoso *et al.*, 2002). Transcriptome analysis of Japanese pear (*Pyrus pyrifolia* Nakai) flower buds at endodormancy and endodormancy release stages revealed that transcripts of GA20ox were less abundant in ecodormancy, whereas the genes encoding GA2ox showed up-regulated expression in ecodormancy (Bai *et al.*, 2013). In almond, the distinct expression pattern of both *PdGA20OX* and *PdGA2OX* suggested that, before anthesis, there is a genetic reprogramming of the gibberellin metabolic pathway (Barros *et al.*, 2012). In a similar fashion, regulation of the expression of *KS* and/or *GA20ox* and/or *GA2ox* detected in the QTL region may play a role in endodormancy release in sweet cherry.

Candidate genes involved in negative regulation of flower development: *NUA*, *PIE1*, *ARP4* and *EMF2*

NUA is the *A. thaliana* homologue of TRANSLOCATED PROMOTER REGION (TPR). Mutations of *NUA* have been associated with early flowering under short days and long days and altered expression of several flowering regulators such as *FLC*, *FT* and *SUPPRESSOR OF OVEREXPRESSION OF CONSTANS1* (*SOC1*) (Xu *et al.*, 2007).

PIE1 is the orthologue in *A. thaliana* of *Saccharomyces cerevisiae Swr1* (a member of SWItching 2/Sucrose Non Fermenting 2 familly), the catalytic subunit of the ATP-dependent chromatin remodelling complex SWR1. *PIE1* is required for high-level expression of *FLC*, possibly via H2A.Z deposition onto target chromatin including FLC (Deal *et al.*, 2007). Other homologues of SWR1 complex components in *A. thaliana* include ARP4 and ARP6. Mutations in any of these homologues cause an early-flowering phenotype through the reduction of *FLC* expression (Choi *et al.*, 2007). Thus, *FLC* activation, and the resulting creation of a vernalization requirement in *A. thaliana*, require deposition of H2A.Z via PIE1, ARP4, and ARP6 (for a review, see Kim *et al.*, 2009).

The *EMF* genes are required to maintain vegetative development in *A. thaliana* (Moon *et al.*, 2003). The *EMF* genes have been shown to regulate flowering time because modulation of EMF activities generates transgenic plants that flower at different times (Aubert *et al.*, 2001; Yoshida *et al.*, 2001). *EMF2* encodes a Polycomb group protein. The *A. thaliana* Polycomb repressive complex 2 (PRC2) subunits CURLY LEAF, EMF2 and FERTILIZATION INDEPENDENT ENDOSPERM repress the expression of *FLC* (Jiang *et al.*, 2008). In addition, PRC2 plays a prominent role in establishing an epigenetic cellular memory for

cold-regulated expression states of AGAMOUS-LIKE 19 and FLC during the vernalization response (Alexandre & Hennig, 2008). Therefore, EMF2 may be implicated not only in the control of flowering date but also in the control of chilling requirements. The possible role of chromatin modifications in dormancy and flowering regulation has been previously suggested (for a review, see Horvath, 2009).

The proteins encoded by these candidate genes regulate, directly or indirectly, the expression of the floral pathway integrator FLC, which represses the expression of *FT*. As we did not find an *FLC* orthologue in either the peach genomic sequence or in the 'Regina' sweet cherry transcribed sequences (L. Le Dantec, J. Quero-García & E. Dirlewanger, unpublished data), another protein may be an FLC substitute in these perennial trees. DAM factors are considered the main candidates for the control of bud dormancy and meristem growth cessation in peach and other perennial plants, through gene expression regulation (Mazzitelli *et al.*, 2007; Horvath *et al.*, 2008, 2010; Yamane *et al.*, 2008; Li *et al.*, 2009; Sasaki *et al.*, 2011; Leida *et al.*, 2012; Verde *et al.*, 2013). Moreover, *DAM5* and *DAM6* expression patterns in peach are consistent with a role as quantitative repressors of bud break (Jimenez *et al.*, 2010; Yamane *et al.*, 2011). Additionally, analysis of chromatin modifications reinforced the role of epigenetic mechanisms in *DAM6* regulation and bud dormancy release (Leida *et al.*, 2012) Thus, *DAM6* could be a potential *FLC* substitute. Repression of *DAM6* may be necessary for dormancy release after fulfilling the chilling requirement in peach and sweet cherry. In peach, specific chromatin histone modifications in the *DAM6* gene occurred during bud dormancy progression and release and were associated with repression of *DAM6* (Leida *et al.*, 2012). In sweet cherry, a colocalization for chilling requirements and flowering date was found on LG1 overlapping the evergrowing region where the *DAM6* gene has been found in peach (Bielenberg *et al.*, 2008). Taken together, these data suggest possible regulation of the expression of *DAM6* leading to endodormancy release by one or a combination of the candidate genes detected on the GL4 QTL, through: (1) histone modification (via PRC2 containing EMF2) and/or (2) the deposition of histone variant H2A.Z (via the cherry SWR1 complex homologue containing PIE1 and ARP4) and/or (3) an unknown mechanism involving NUA.

Further analysis including transcriptomic, association genetics and epigenetics approaches could provide new understanding of the complex process involved in dormancy release and flowering time control.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Linkage maps and QTL analysis in two sweet cherry (*Prunus avium*) progenies 'Regina' × 'Lapins' and 'Regina' × 'Garnet' for chilling requirements (CR), heat requirements (HR) and flowering date (FD).

Fig. S2 QTL and candidate genes in sweet cherry (*Prunus avium*).

Table S1 Mean values, SD and value range for each trait for each sweet cherry (*Prunus avium*) progeny during different years of evaluation

Table S2 Markers used in the parental maps for QTL detection in sweet cherry (*Prunus avium*)

Table S3 Haplotype analysis using SNP markers spanning the QTL on linkage group 4 in sweet cherry (*Prunus avium*) progenies 'Regina' × 'Garnet' (R × G) and 'Regina' × 'Lapins' (R × L)

Table S4 Genes located in the QTL on linkage group 4 in sweet cherry (*Prunus avium*) with the corresponding Blast2GO analysis

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