Candidate genes underlying QTL for flowering time and their interactions in a wide spring barley (*Hordeum vulgare* L.) cross

Ana M. Casas [a],*, Carlota R. Gazulla [a], Arantxa Monteagudo [a], Carlos P. Cantalapiedra [a,1], Marian Moralejo [b], M. Pilar Gracia [a], Francisco J. Ciudad [c], William T.B. Thomas [d], José L. Molina-Cano [e], Scott Boden [f,2], Bruno Contreras-Moreira [a,g,3], Ernesto Igartua [a]

[a] *Department of Genetics and Plant Production, Aula Dei Experimental Station (EEAD-CSIC), Avda Montañana 1005, E-50059 Zaragoza, Spain*
[b] *University of Lleida - AGROTECNIO Center, Avda. Rovira Roure 191, 25198 Lleida, Spain*
[c] *Instituto de Tecnología Agraria, Junta de Castilla y León, ITACyL, P.O. Box 172, 47071 Valladolid, Spain*
[d] *The James Hutton Institute, Invergowrie, Dundee DD2 5DA, UK*
[e] *Institut de Recerca i Tecnologia Agroalimentàries (UdL-IRTA), Avda Rovira Roure 191, 25198 Lleida, Spain*
[f] *John Innes Centre, Norwich Research Park, Norwich NR4 7UH, UK*
[g] *Fundación ARAID, calle María de Luna 11, 50018 Zaragoza, Spain*

ARTICLE INFO

ABSTRACT

Response to vernalization and photoperiod are the main determinants controlling the time to flowering in temperate cereals. While the individual genes that determine a plant's response to these environmental signals are well characterized, the combinatorial effect on flowering time of allelic variants for multiple genes remains unresolved. This study investigated the genetic control of flowering-time in a biparental population of spring barley, derived from a wide cross between a late-flowering European and an early-flowering North-American cultivar. While the major flowering time genes are not segregating in the Beka × Logan cross, large variation in flowering was observed. We identified five QTL, with both parents found to contribute early alleles. The catalog of QTL discovered aligns with several candidate genes affecting flowering time in barley. The combination of particular alleles at *HvCEN*, *HvELF3* and *HvFT1* in Logan are responsible for the earliness of this cultivar. Interestingly, earliness for flowering could be further enhanced, with Beka found to contribute three early alleles, including a QTL co-locating with a *HvFD-like* gene, suggesting that there are diverse aspects of the flowering-time pathway that have been manipulated in these two cultivars. Epistatic interactions between flowering-time QTL or candidate genes were observed in field data and confirmed under controlled conditions. The results of this study link photoperiod-dependent flowering-time genes with earliness per se genes into a single model, thus providing a unique framework that can be used by geneticists and breeders to optimize flowering time in barley.

1. Introduction

Appropriate timing of flowering is an essential component of agronomic adaptation of cereal crops. It is an intricate genetic process, which involves the interplay of a remarkable number of genes. This is further complicated by the presence of several alleles with distinct phenotypic effects at some loci, with polymorphisms resulting from base substitution, InDels and copy number variation [1–3]. Work done in Arabidopsis and in cereals suggests a richness of interactions among genes [4], whose effect is only detectable with appropriate genetic screens. Therefore, much work is still required to unravel completely the genetic control of flowering time.

Flowering of cereals must occur during a narrow time window, specific to each region, coincident with the optimum environmental conditions for maximizing grain-filling efficiency. Allelic diversity at genes regulating the responses to photoperiod and

* Corresponding author.
  E-mail address: acasas@eead.csic.es (A.M. Casas).
[1] Present address: Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid (UPM) and Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid 28223, Spain.
[2] Present address: School of Agriculture, Food and Wine, Waite Research Institute, University of Adelaide, Glen Osmond, SA 5064, Australia.
[3] Present address: The European Bioinformatics Institute (EMBL-EBI), Wellcome Trust Genome Campus, Hinxton, UK

vernalization, or the early maturity (*eam*) loci, favored the adaptation of temperate cereals to a wide range of environments [5]. For winter cereals, there are new discoveries even for the already well-known vernalization and long-day photoperiod pathways [6–8]. Spring cultivars of crops like barley and wheat lack the vernalization mechanism and photoperiod responsiveness acting in most winter cultivars. Therefore, the genes determining flowering time in spring cultivars, beyond vernalization and long day response, are a breeding target of particular interest. Fine control of flowering time will be particularly necessary for breeders, to fine tune flowering of future cultivars to new environmental conditions by new combinations of day-length and temperature that will occur because of climate change.

The classical study by Laurie [9] revealed the approximate genomic position of a good number of genes affecting plant development and flowering time in barley. The functions and phenotypic effects of the main vernalization and photoperiod genes (by their current names, *VrnH1*, *VrnH2*, *VrnH3*, *PpdH1*, *PpdH2*) were described in that article. Many studies have confirmed their main findings [10–16]. Others have expanded the catalog of alleles at these loci [17–19], and some new QTL and genes for flowering time determination have been revealed.

HvCEN is an orthologous gene of *Antirrhinum CENTRORADIALIS*, and contributes to the determination of growth habit, as its alleles are split among spring and winter barleys [20]. *HvELF3* was identified as the barley orthologue of the *Arabidopsis thaliana* circadian clock regulator *EARLY FLOWERING3* [21,22]. Mutations in this gene were selected empirically to expand cultivation of spring cultivars into regions at Northern latitudes with short cultivation seasons. Some of those mutations produce defective proteins that disturb the sensing of the circadian clock mechanism, which results in a constitutive long-day growth and early-flowering phenotype, including increased expression of *FLOWERING LOCUS T* (*HvFT1*) and production of gibberellic acid [21,23]. *HvPHYC* reportedly disrupts circadian expression of clock genes, and interacts with *PpdH1* to accelerate flowering under non-inductive short days [6]. Breeders in Japan [24] and in Australia [8] used a mutation in *HvPHYC* conferring earliness.

Since the discovery of *HvFT1* as the candidate gene for *VrnH3* [12,25], natural variation for this gene, with polymorphisms in the promoter and first intron [12,26], as well as copy number variation [18,19], was revealed. As summarized by Song [27], induction of *FLOWERING LOCUS T* is the output of the photoperiod pathway. Flowering time is correlated with *FT1* expression in barley and wheat [12,15,28,29]. In temperate cereals, photoperiod responses are caused by interactions between circadian-clock regulated components, such as CONSTANS, and light signaling. In barley, *HvCO1* seems to be one of these components, as it displays an oscillating diurnal expression profile that peaks after the end of the light period [13,21,30].

The role of other proteins interacting with FT, once it is translocated to the shoot apical meristem (SAM), is a hotspot for research. It is well established that the outcome of the pathway, which integrates environmental and endogenous signals inductive to flowering, is an increased expression of *FT* [31,32]. Research on the endogenous signals that interact with FT to determine the fate of SAM cells has progressed remarkably in Arabidopsis [31,33], tomato [34] and, to a lesser extent, in cereals [35–37]. As a general rule, the fate of the cells in the SAM is determined by the balance of signals between the promoting FT-FD complex and repressor complexes in which FT is substituted by TFL1 or CEN in Arabidopsis [38,39] and rice [40], or SP in tomato [34]. The elucidation of the interplay of signals that act in the SAM in each crop species is of particular relevance, as they can affect the size of the reproductive sink and, ultimately, yield.

This study presents results of a biparental population of spring barley, a wide cross between an early North-American and a late European cultivar. The catalog of QTL discovered matches well with several candidate genes affecting flowering time in barley, described over the last years. The information on QTL and their interactions will be combined with gene expression analysis and gene sequencing to provide a more complete picture and new hypotheses about the determination of flowering time in spring barley.

2. Materials and methods

2.1. Plant material

The Beka × Logan doubled haploid (DH) population was initially reported by Moralejo [41]. It is a cross between European and North-American spring barley lines. Beka (Bethge XIII × Kneifel), bred in France, was widely grown in Spain during the last century. Logan is a very early cultivar, developed in North Dakota from the cross (ND7085 × ND4994) × ND7556. The population consists of 102 DH lines.

2.2. Field trials

Seven field trials were carried out at three sites, Lleida (northeast Spain) in 1999, 2001, 2002, 2005, and 2006, Valladolid (central Spain) in 1999, and Dundee (eastern Scotland) in 2002. The trials of year 2002 followed an alpha-lattice design, with two replicates. All other trials were unreplicated. All trials at Lleida were autumn-sown (Nov-Dec), whereas trials at Valladolid and Dundee were sown in spring (March-April). Sowing dates and phenotypic values are summarized in Table S1. Days from sowing to anthesis (50% of tillers with 2 cm of awns visible) were recorded at each trial.

2.3. Genotyping

Beka and Logan are spring genotypes with similar genetic constitution, i.e. *VrnH1-1*, *vrnH2*, *ppdH1*, and *PpdH2*. The population was already genotyped with 120 markers, microsatellites and AFLPs [41], but the map presented a gap of over 60 cM around the region of *VrnH3*. Polymorphisms in *VrnH3* (*HvFT1*) between the parents, both CNV and sequence differences in the promoter, were previously identified [19]. The population was genotyped for *HvFT1* (promoter InDels) and three microsatellite markers (EBmac0603, Bmag0914, and GBM1116), to saturate the region and provide better resolution on chromosome 7H. Genomic DNA previously isolated by Moralejo [41] was utilized. Primers used for genotyping these and other genes (*HvELF3*, *HvCEN*, *HvGA2ox3*, *HvFD-like* and *HvCO1*), are summarized in Table S2. Joinmap 4 [42] was employed for map construction. The genetic map, printed with MapChart [43], is provided as Fig. S1.

2.4. Data analyses

Multi-environment QTL analysis was performed using Genstat 17 [44]. Simple interval mapping was employed to identify an initial set of cofactors for use in iterative rounds of composite interval mapping. The final set of cofactors was used in a multi-environment mixed model to test whether each represented a QTL main effect or a QTL × Environment, and to estimate allelic effects at each environment. The Li and Ji method (default in Genstat 17) was used to estimate a 5% genome-wide significance threshold for the $-\log_{10}(P)$ values. Minimum cofactor distance was set to 30 cM, and the minimum distance to declare

independent QTL was set to 20 cM. Interactions between QTL were analyzed using the REML option in Genstat 17, in a model that included the markers next to the peaks of the six QTL, and up to three-way marker-by-marker interactions. Environments and markers by environments were set as random factors, and markers representing the QTL and their interactions as fixed factors. The genotypic variance not explained by these QTL was pooled in the residual, together with true experimental error. Broad-sense heritability of flowering time on an entry-mean basis was calculated in Genstat, using the VHERITABILITY command within the REML procedure [44]. To evaluate differences in gene expression, analyses of variance was performed considering genotype as fixed factor. Multiple comparisons were obtained by Fisher's protected Least Significant Differences (LSD) with the R package 'agricolae' [45]. To test whether variation in gene expression was associated with flowering time in the greenhouse experiment, Pearson correlations were calculated using the R package 'corrplot' [46] and the function Cor.test to obtain the significance of correlations. These last analyses were carried out in R [47].

2.5. Sequencing major genes

Beka and Logan *HvELF3*, *HvCEN*, *HvGI*, *HvFT2*, *HvGA2ox3*, *HvTFL1*, *HvFD*-like, *HvFT1* and *HvCO1* were sequenced, based on PCR-amplified overlapping fragments. Different sets of primers were designed to amplify each gene (Table S2). Then, BLASTN sequence comparisons [48] were carried out against the latest barley Morex V2.0 reference [49], Morex RefSeqv1.0 High-Confidence genes [50], and Morex, Barke and Bowman whole genome barley sequences [51] at the IPK (http://webblast.ipk-gatersleben.de/barley/, accessed January 30, 2020) BLAST server. The online tool SIFT [52] (http://sift.bii.a-star.edu.sg accessed January 30, 2020) was used to predict the likely impact of amino acid substitutions on protein function. Scores below 0.05 are predicted to affect protein function. GenBank accession numbers for the nucleotide sequences described in this manuscript are KT199230–KT199239, and MN887592–MN887599.

2.6. Gene expression analysis

The parents and selected DH lines of the population were employed. To focus on variation in the three main QTL found, i.e. QTL1, 2 and 5, eight DH lines representing all allelic combinations at *HvELF3*, *HvCEN*, and *HvFT1*, were considered. Barley plants used for expression analyses were grown in pots, in a greenhouse, at (20 ± 4) °C with supplementary lighting (16 h light/8 h dark). Seeds were sown on Oct 15. Aiming to capture differences in gene expression during development, leaf tissue (last expanded or flag leaf) was harvested 23 (around jointing) and 38 (at a later reproductive stage) days after sowing, 2 h before dark. At each sampling time, four samples per genotype, coming from different plants and pots, were analyzed.

RNA was purified with the NucleoSpin RNA Plant kit (Macherey-Nagel). Primers for qRT-PCR are shown in Table S2. Amplifications were carried out using Power SYBR Green Supermix (Thermo Fisher Scientific, Carlsbad, CA, USA) on an ABI7500 real time PCR system (Applied Biosystems, Carlsbad, CA, USA). Gene expression at each time point was normalized to the expression of Actin, considering efficiencies.

2.7. Searching for transcription factor motifs within HvFT1 promoters

A DNA motif of Hordeum vulgare VRN1, three motifs of Arabidopsis thaliana and Solanum tuberosum CONSTANS and one motif of A. thaliana FHY3 transcription factors were compiled from published experimental data [53–57], and annotated in

footprintDB [58], with the following links: floresta.eead.csic.es/footprintdb/index.php?db = EEADannot&motif = VRN1, floresta.eead.csic.es/footprintdb/index.php?db = EEADannot&motif = CONS1, floresta.eead.csic.es/footprintdb/index.php?db = EEADannot&motif = CONS2, floresta.eead.csic.es/footprintdb/index.php?db = EEADannot&motif = StCONSTANSlike1, http://jaspar.genereg.net/matrix/MA0557.1, (accessed January 30, 2020).

These motifs, together with a CCAA[A|T] box [54,57] and a NF-CO motif [59], were scanned along the Beka and Logan upstream sequences reported in this work with software matrix-scan 'quick', an upstream-noorf order 2 Markov *Hordeum vulgare* background and a minimum weight of 3.7 [60].

3. Results

The range of variation for flowering time varied among individual environments from 11 to 19 days, and was 13.5 days for the mean of the seven environments (Table S3). Broad-sense heritability on an entry-mean basis, calculated across all locations was 0.956. This high value was not unexpected for this trait, particularly in a population without known variation in photoperiod and vernalization response genes, which usually cause genotype by environment interaction.

3.1. Preliminary QTL analysis

In the previous study of this population [41], a single QTL for flowering time was identified on chromosome 2H. Addition of new field phenotyping data, and the inclusion of several markers in the region of *HvFT1*, including markers within the gene, remarkably increased the quality of the analysis. A first QTL analysis identified six QTL (Fig. S2).

3.2. Main candidate genes in the QTL regions and QTL reanalysis

We then searched for putative candidate genes in the QTL regions, based on other studies [61], and the availability of a reference genome [50]. The findings for each QTL (Fig. 1) and polymorphisms identified (Fig. 2) are the following:

For QTL1 (1H.2), the peak marker Ctig4047 (or scssr04163) mapped on 1HL, was close to the position of the *Mat-a*, or *HvELF3* locus [21,22], suggesting that *HvELF3* (HORVU1Hr1G094980) could underlie this QTL, in which Logan carries the early allele. There were many polymorphisms between the parental sequences (Table S4), including three predicted amino acid changes: Ala315Gly, Trp669Gly, and Leu698Pro, with SIFT scores of 0.59, 1.0, and 0.11, all tolerated. According to the nucleotide sequences, both genotypes had a functional protein. *HvELF3* was further genotyped in the population, and fully co-segregated with Ctig4047.

For QTL2 (2H.1), the peak at marker EBmac0623 on 2H was the only QTL previously associated with flowering time [41]. It was proposed to be an effect of *Eam6*, or *eps2S*, later identified as *HvCEN* [20], the barley homolog of *Anthirrinum CENTRORADIALIS* (HORVU2Hr1G072750). Logan carries the early allele. Beka and Logan only differ in the non-coding region (two SNPs in intron 2 and another polymorphism in the 3′UTR), and represent haplotypes III and I, respectively, as coded by Comadran [20]. In the predicted protein sequence, both haplotypes carry Ala135. *HvCEN* was genotyped in the population and fully co-segregated with EBmac0623.

In QTL3 (3H), the AFLP marker E41M47_1 on 3H was the closest to the peak. Several flowering time genes had been reported in that region of 3H, including the circadian clock gene *GIGANTEA* (*HvGI*) [62], a member of the *FLOWERING LOCUS T* family (*HvFT2*) [16,25], and genes of the gibberellic acid pathway (*HvGA20ox2* and *HvGA2ox3*) [23]. No polymorphisms between the parents of
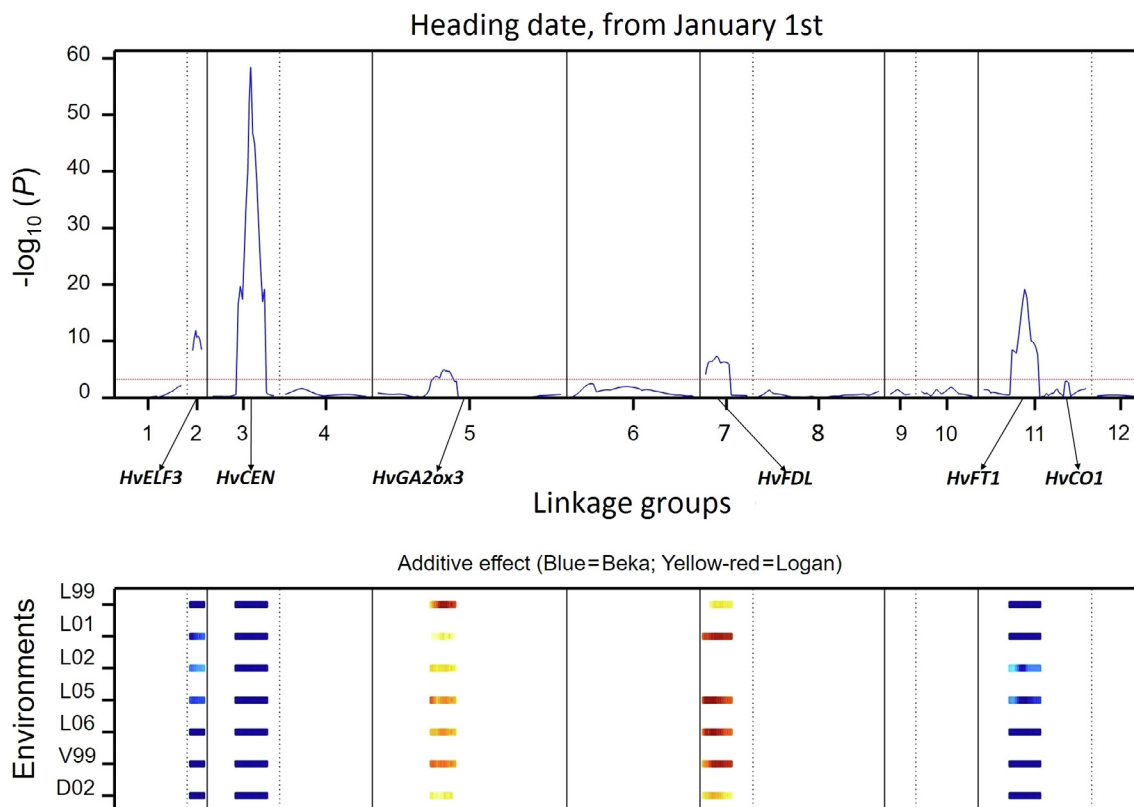
Fig. 1. Multi-environment QTL analysis for days to flowering from January 1st for seven field experiments carried out with the Beka × Logan population. QTL analysis after mapping *HvELF3*, *HvCEN*, *HvGA2ox3*, *HvFD-like6*, *HvFT1*, and *HvCO1*. Dashed lines mark linkage groups within chromosomes. The peaks above the threshold (solid line parallel to *X*-axis) indicate presence of QTL significantly affecting the trait. In the lower part of the figure, field trials are coded with L (Lleida), V (Valladolid) or D (Dundee) and two digits for the year; the coloured dashes indicate the significance of the QTL and its direction, in each environment. Bars, blue means that the early allele came from Logan, yellow–brown from Beka, with intensity proportional to the size of the effect.
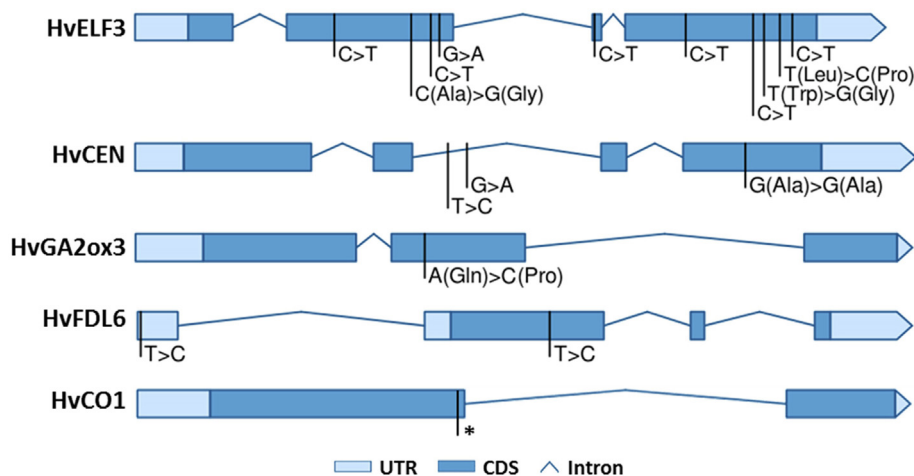

Fig. 2. Gene models for *HvELF3*, *HvCEN*, *HvGA2ox3*, *HvFD-like6*, and *HvCO1*. Identified polymorphisms are shown as vertical black bars on their corresponding positions within each gene. Each polymorphism shows both the Beka (left) and the Logan (right) alleles, as well as the respective encoded aminoacids for non-synonymous SNPs. "Ala" is also shown for *HvCEN* SNP on exon 4, highlighting that there is no protein sequence change. The variant shown, as an asterisk, for *HvCO1* indicates the position where the ORF is interrupted in Logan.

the population were found in *HvGI* (HORVU3Hr1G021140 and HORVU3Hr1G021150), nor in a partial sequence of *HvFT2* (HORVU3Hr1G027590). The only differences were in *HvGA2ox3* (HORVU3Hr1G072810). However, when this gene was further genotyped in the population and positioned in the genetic map, it fell at 77.6 cM, 16.0 cM distal from the QTL. Therefore,

involvement of *HvGA2ox3* was discarded, and no potential candidate gene was identified for QTL3.

At QTL4 (5H.1), the peak corresponds to marker HvM30, on 5HS. *HvTFL1* [16] and a barley homolog of *TaFDL6* [35,36] are in the same region of 5HS. From here on, we refer to this last gene as *HvFD-like (HvFDL)*. There was no polymorphism for *HvTFL1*

(HORVU5Hr1G042230) between the parents of the population. *HvFDL6* (HORVU5Hr1G045180), a gene annotated by Schoonheim [63] as ABA responsive element binding factor 3, has a transcript with 4 exons, but only 3 are protein coding. We identified several polymorphisms, including a SNP in the first (non-coding) exon and a synonymous variant in exon 2, (T/C, 170Thr). This last polymorphism was used to genotype *HvFD-like* in the population, which fully co-segregated with HvM30, the QTL4 peak marker. Beka carries the early allele.

The QTL5 (7H.1) coincides with *HvFT1* (HORVU7Hr1G024610), in the middle of a peak which was completely excluded from the results previously published [41], due to lack of polymorphic markers for a stretch of over 60 cM. Besides differences in the number of copies (2 in Beka and 1 in Logan) [19], the parents also differed in the nucleotide sequence of the gene, particularly in the promoter region. Within ~ 1500 bp upstream to exon 1, there were 26 SNPs and 4 indels differentiating Beka and Logan. Beka promoter haplotype coincides with the "promoter-late" haplotype described by NItcher et al. [18], whereas Logan haplotype is the same as the "promoter-early" described in the same work. The sequences of the parents in the coding region, although partial, are more similar, with only one SNP in intron 1 and one microsatellite in intron 2 (Table S4). Both genotypes carry the same 'TC' haplotype in intron 1, commonly used as diagnostic for functional differences at this gene. Given these evidences, if *HvFT1* is the functional gene behind QTL5, it is likely related to polymorphism in its regulation. A prediction of *cis*-regulatory elements of transcription factors VRN1 and CONSTANS along the Beka and Logan upstream *HvFT1* sequences found a proximal VRN1 site (CCGTAATAAG) only in Logan (position − 397, weight 4.6) and Morex (position − 391, weight 4.6), but not in Beka (Fig. S3). As VRN1 has a regulatory effect on *HvFT1*, these differences may account for the phenotypic effect of this QTL.

QTL6 (7H.1) peak was close to the AFLP marker, E35M49_14 at 67 cM. By using common markers across barley genetic maps [64], we could locate this QTL close to the flowering time gene *CONSTANS*, *HvCO1* [65]. *HvCO1* (HORVU7Hr1G043030 or HOR-VU7Hr1G043040 in the current reference genome), on chromosome 7H, was also polymorphic between the parents of the population and was included in the analysis. Beka carries a complete, functional gene, but Logan lacks the CCT domain (Fig. S4).

A new QTL analysis was then performed, including marker information from all possible candidate genes. In this second analysis, only five QTL were detected, coded as QTL1–5, as indicated in Fig. 1 and Table 1. The peak of the QTL analysis was coincident with the position of four of the genes included, i.e. *HvELF3*, *HvCEN*, *HvFD-like*, and *HvFT1*. On the other hand, the region of *HvCO1* was no further associated as a main factor with flowering time in the final QTL analysis. Its test statistic fell just below the threshold (Fig. 1).

3.3. QTL interactions

An analysis of variance including the closest markers to the QTL peaks, plus *HvCO1*, was carried out. It must be noted that the

combination of all six regions would produce 64 haplotypes. Out of these, only 43 were present in the population. The absence of some haplotypes reduces precision in the calculation of interactions. However, the REML analysis corrects for unbalanced frequencies, and the consistency of effects across seven field trials adds soundness to the results. The limited number of lines prevented the calculation of interactions higher than order 3. There were three significant double interactions and five significant terms of three-way interaction. All QTL or genes were involved in interactions. QTL1 (*HvELF3*) and QTL2 (*HvCEN*) were present in five interactions each, QTL3 (E41M47_1), QTL5 (*HvFT1*) and *HvCO1* were present in three interactions and QTL4 (*HvFDL*) in two (Table 2, S5). The most frequent interaction occurred between *HvCEN* and *HvELF3*, which carried over two triple interactions *HvCEN* × *HvELF3* × *HvFT1* and *HvCEN* × *HvELF3* × *HvFDL*. The double interaction itself was of quantitative nature (Table S5). The contribution of this double interaction to the two triple ones probably indicates that the real interactions are these last ones, or others of higher rank that could not be detected. *HvFT1* had almost no effect when *HvELF3* and *HvCEN* were Logan/Beka haplotype, and had a rather large effect in the other three cases for this triple interaction, particularly when all three alleles came from Logan, producing marked earliness. In the *HvELF3* × *HvCEN* × *HvFDL* case, the effect of *HvFDL* was overridden by the presence of Beka alleles at the other two QTL, resulting in late flowering. Another triple interaction also involves *HvCEN*, with *HvFDL* and *HvFT1*, but it was barely significant. The last two triple interactions involved QTL3 (E41M47_1) by *HvCO1*, with either *HvELF3* or *HvCEN*. Therefore, although *HvCO1* did not reach significance as main effect QTL, it was significant as an interactive QTL. The interactions detected in the field were also seen in the small number of lines tested in the greenhouse experiment.

3.4. Validation in selected DH lines

To evaluate the effect of the different gene/QTL combinations on gene expression, eight selected DH lines, with different combinations of *HvELF3*, *HvCEN*, and *HvFT1* were analyzed (Table 3; Fig. 3). The chosen DH lines have the Beka allele at QTL4 in the region of *HvFD-like* on 5H, Logan at QTL3 peak on 3H, and Beka at *HvCO1* on 7H (all but one line, with the Logan allele). Some expression differences were evident, particularly at the second sampling date. Expression of *HvFD-like* was similar between the parents of the population (Fig. 3). Higher *HvFT1* expression, and earlier flowering, was detected in the lines carrying the Logan allele at both *HvELF3* and *HvCEN* (lines 2771 and 2820). In contrast, parent Logan, which shared the same two alleles, and had a lower *HvFT1* expression, although flowering occurred quite early. *HvELF3* expression was low and quite similar among all lines. Regarding *HvCEN*, the level of expression was variable, but not significantly different amongst plants. Yet, *HvCEN* expression was more stable in plants with the Logan allele in *HvELF3*. The only line with a Logan allele at *HvCO1* had the latest heading date and presented very low expression levels of *HvCEN* and *HvFT1*. Minor differences in *HvFT1* expression were detected at an earlier sampling date (Fig. S5). The correlation coefficients of gene expression levels with

Table 1
QTL for flowering time detected by composite interval mapping in the Beka × Logan DH population in seven field trials.

QTL number	Linkage group	Position (cM)	Closest marker	Confidence interval	−log$_{10}$ (*P*)	Additive effect							QTL × E	Candidate gene(s)
						L99	L01	L02	L05	L06	V99	D02		
1	1H.2	2.9	Ctig4047	0.0–8.1	14.0	−1.5	−0.9	−1.4	−0.7	−1.2	−1.2	−1.7	<0.001	*HvELF3*
2	2H.1	34.3	EBmac623	31.8–36.2	49.1	−3.2	−1.8	−2.7	−1.5	−2.0	−2.1	−2.5	<0.001	*HvCEN*
3	3H	59.6	E41M47_1	54.3–79.7	4.7	0.5	0.5	0.5	0.5	0.5	0.5	0.5	ns	*HvGI/HvFT2*
4	5H.1	10.7	HvM30	1.4–23.3	11.4	0.8	0.8	0.8	0.8	0.8	0.8	0.8	ns	*HvFDL*
5	7H.1	37.1	HvFT1	35.2–41.0	17.8	−1.7	−1.4	−1.4	−0.7	−1.3	−1.1	−1.7	<0.001	*HvFT1*

Table 2
Probabilities of significance from an analysis of variance of flowering date recorded at seven field trials, in the Beka × Logan DH population.

Term	P-value [a]	Term	P-value [a]
HvELF3	<0.001	HvELF3.HvCEN.HvFDL	<0.001
HvCEN	<0.001	HvELF3.HvCEN.HvFT1	0.015
HvFD-like	<0.001	HvELF3.HvCEN.E41M47_1	0.070
HvFT1	<0.001	HvELF3.HvCEN.HvCO1	0.322
E41M47_1	0.002	HvELF3. HvFDL.HvFT1	0.318
HvCO1	<0.001	HvELF3.HvFT1.E41M47_1	0.188
HvELF3.HvCEN	<0.001	HvELF3.HvFT1.HvCO1	0.099
HvELF3.HvFDL	0.889	HvELF3.HvFDL.E41M47_1	0.613
HvELF3.HvFT1	0.815	HvELF3.HvFDL.HvCO1	0.057
HvELF3.E41M47_1	0.033	HvELF3.E41M47_1.HvCO1	0.004
HvELF3.HvCO1	0.355	HvCEN.HvFDL.HvFT1	0.048
HvCEN.HvFDL	0.362	HvCEN.HvFDL.E41M47_1	0.287
HvCEN.HvFT1	0.938	HvCEN.HvFDL.HvCO1	0.575
HvCEN.E41M47_1	0.211	HvCEN.HvFT1.E41M47_1	0.167
HvCEN.HvCO1	0.257	HvCEN.HvFT1.HvCO1	0.256
HvFDL.HvFT1	0.181	HvCEN.E41M47_1.HvCO1	0.002
HvFDL.E41M47_1	0.097	HvFDL.HvFT1.HvCO1	–
HvFDL.HvCO1	0.267	HvFDL.HvFT1.E41M47_1	0.865
HvFT1.E41M47_1	0.280	HvFDL.E41M47_1.HvCO1	0.468
HvFT1.HvCO1	<0.001	HvFT1.E41M47_1.HvCO1	0.587
E41M47_1.HvCO1	0.496		

The sources of variance are the trials plus six markers close to QTL peaks representing the five QTL found (1–5): *HvELF3*, *HvCEN*, E41M47_1, *HvFD-like*, and *HvFT1*, plus *HvCO1*. The analysis of variance was done on genotypic means per environment, taking as error the residual genotypic and genotype by environment variance.
 [a] Probability of the *F*-test. *P*-values below 0.05 are underlined.

date of awn tipping gave only one significant result ($r = -0.66$), with *HvFT1*.

4. Discussion

The combination of QTL analysis of comprehensive field data with sequence information and expression analysis of genes in selected lines provides new evidence to expand the catalog of genes, gene interactions, polymorphisms, and allelic effects that modulate flowering time in spring barley. The increasing quantity and quality of barley genomics resources allowed us to find putative candidate genes behind four of the five QTL detected. The potential candidates were suggested by position, but their involvement is further supported by sequence polymorphism, phenotypic effect matching functional expectations, and the patterns of interactions observed. The detection of interactions between QTL could indicate the involvement of the underlying genes in the same pathway. A higher number of interactions (like for QTL1/*HvELF3* and QTL2/*HvCEN*) indicates either an upstream or an integrator position, whereas a lower number of interactions (QTL4/*HvFDL*)

indicates a downstream position. For most cases, there are previous evidences, either from barley or from other species, supporting the interactions detected.

This study interrogates two spring genotypes, which do not differ in vernalization or photoperiod response, but show different flowering behavior. The range of average flowering in the population exceeded 13 days, which is rather large, and provides ample genetic variation for breeders, outside the classical *Vrn* and *Ppd* genes. Beka was selected in France, from Central European ancestors, a region with humid and cool summers, and is late flowering. Logan was selected in North Dakota, a northern region with cold winters and short summers and is early flowering, in accordance with the region's short cropping season.

The QTL detected in this study fit well with the findings of an association study focused on the duration of different developmental phases of spring barley [61]. These authors found QTL for at least one phase in the same regions detected in our study, but only in the subset of accessions sensitive to long days (carrying the *PpdH1* allele). In the other subset (*ppdH1*), which encompasses most spring barleys (including the parents of our population), the only QTL in common were QTL2 (*HvCEN*) and *HvCO1*. Our parents belong to this last group (recessive *ppdH1*) and, nonetheless, revealed a richness of QTL possibly due to the large genetic distance between them. Another study with a NAM population involving *Hordeum spontaneum* parents, also found flowering time QTL associated to *HvELF3*, *HvFT1*, *HvCEN*, and *HvCO1* [66]. The last two genes acted epistatically, consistent with the interactions detected in our study (with additional intervention of QTL3 in a three-way interaction). The *ppdH1* background of our population allowed the identification of other components of the flowering pathway that are downstream of *PpdH1* and would otherwise be dominated by its strong effect.

We could put our sequences in the context of barley allelic diversity (Table S6) by comparing them to different barley genotypes [50,51]. Logan has the same sequence as cultivar Bowman in *HvELF3*, *HvCEN*, *HvFD-like* and *HvFT1* and differs from those of cultivars Barke or Morex. This is not surprising, as Logan (ND11231-11) has Bowman (ND4994) in its pedigree (http://genbank.vurv.cz/barley/pedigree/pedigree.asp, accessed January 30, 2020). This last genotype is relevant in barley genetics, as Druka [67] described a set of introgression lines in Bowman for different mutant phenotypes, which are used for gene discovery. Our finding that Bowman, being like Logan, most likely carries "early" alleles for at least three important flowering time genes should be kept in mind when analyzing the phenotypic results of those mutants. The Bowman background could have an influence on the phenotype of other mutant flowering time genes, intensifying or smoothing the effect of a particular mutation. For instance, some Bowman introgression lines were used to identify *eam8*/*mat-a*, syn. *HvELF3* [21,22], *eam10*, syn. *HvLUX1* [68] or *eam5*, syn. *HvPHYC* [6].

Table 3
Haplotypes for flowering time genes, and markers closest to QTL, for selected DH lines of the Beka × Logan population.

HvELF3 (Ctig4047)	HvCEN (EBmac623)	HvFDL (HvM30)	HvFT1	HvCO1	Genotype	Awn emergence
B	B	B	B	B	Beka	53
L	L	L	L	L	Logan	48
B	B	B	B	B	2814	54
B	B	B	L	L	2785	59
L	B	B	B	B	2775	47
L	B	B	L	B	2779	49
B	L	B	B	B	2796	51
B	L	B	L	B	2799	51
L	L	B	B	B	2771	45
L	L	B	L	B	2820	43

B, Beka; L, Logan; Awn emergence, days from sowing to 50% of plants with emerged awns, for plants grown in a greenhouse under 16 h light/8h dark.
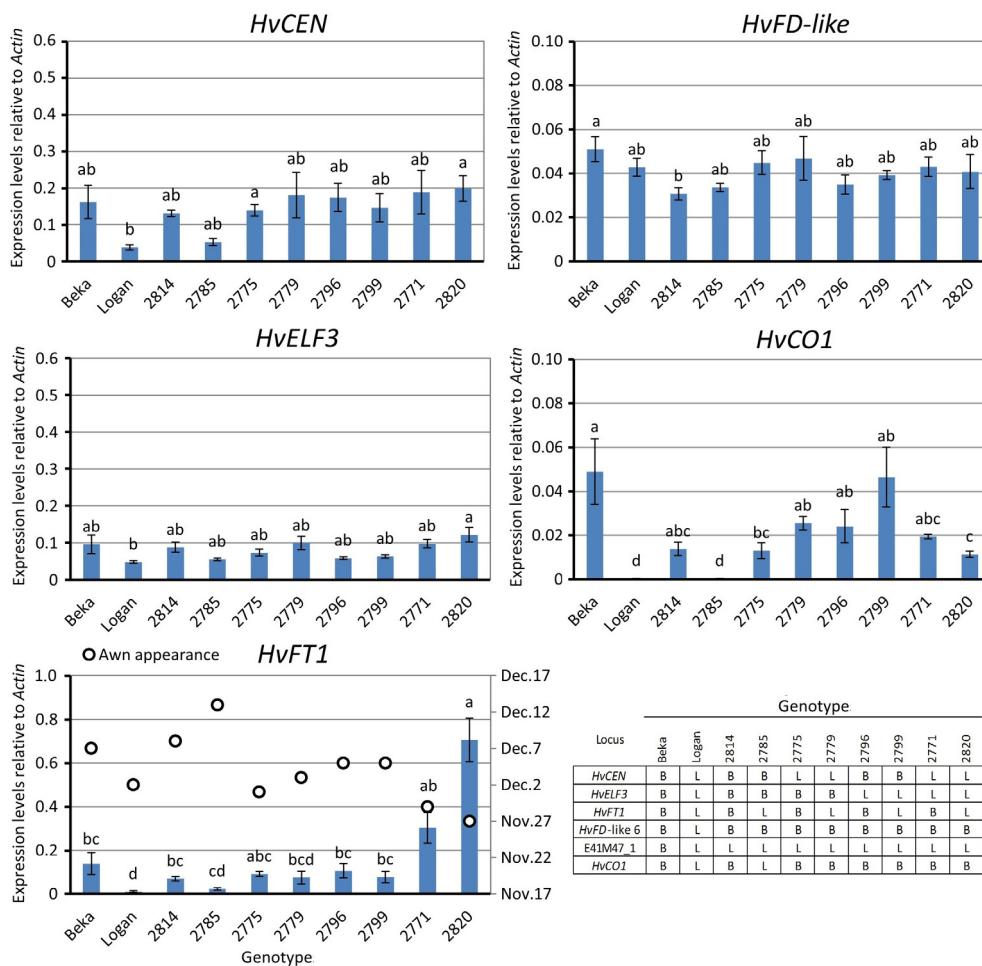
Locus	Beka	Logan	2814	2785	2775	2779	2796	2799	2771	2820
HvCEN	B	L	B	B	L	L	B	B	L	L
HvELF3	B	L	B	B	B	B	L	L	L	L
HvFT1	B	L	B	L	B	L	B	L	B	L
HvFD-like 6	B	L	B	B	B	B	B	B	B	B
E41M47_1	B	L	L	L	L	L	L	L	L	L
HvCO1	B	L	B	L	B	B	B	B	B	B

Fig. 3. Gene expression analysis by qRT-PCR, 38 days after sowing. Relative expression of *HvCEN*, *HvELF3*, *HvFT1*, *HvFD-like* and *HvCO1*, normalized to *Actin*, in the parents and selected DH lines of the Beka × Logan population. Days until awn emergence are shown for comparison. Bars represent means ± SEM. Bars with the same letter are not significantly different at $P < 0.05$ according to ANOVA that included genotypes. Genetic composition of the lines is also described.

Finding allelic variation for *HvELF3*, *HvCEN* and *HvFT1*, possibly related to a phenotypic effect, is one of the key results of this study. It provides breeders with additional tools to modify flowering time in spring barley. The ELF3 protein sequences predicted for Beka and Logan present three amino acid differences, which could be associated with differences in flowering time. *HvELF3* sequences of these two genotypes fall into two different clades of the phylogenetic tree constructed by Xia [69], after resequencing the natural variants of this gene in 134 barley accessions. Beka's allele is identical to Igri's (HQ850272) and groups together with late flowering genotypes (average 74.6 days), whereas Logan's is similar to Bowman's, located in a cluster of early flowering genotypes (average 68.5 days). This observation matches our finding of, earlier flowering (1.5–3 days difference) associated with the *HvELF3* Logan allele. This effect is smaller than reported for different *elf3* mutant alleles [21,22,69], but confirms the presence of ample genetic and phenotypic variation for flowering time associated to this gene. Mutant *elf3* (*mat-a.8*) plants display disrupted expression of circadian clock genes, resulting in elevated expression of the floral activator *HvFT1*, and accelerated reproductive development [21–23]. Boden [23] showed increased production of gibberellin (GA) in the *elf3* (*mat-a8*) mutant plants and suggested that ELF3 suppresses flowering under non-inductive photoperiods by blocking GA production and *FT1* expression.

Epistatic interactions between flowering time QTL or candidate genes have been reported in different studies, pointing to functional relationships and the presence of genetic networks [19,66,70]. *HvCEN*, likely a paralog of *HvFT1*, is one of those candidate genes interacting with other flowering time genes, and is present in a large number of interactions in our study. Comadran [20] suggested that *HvCEN* was a repressor of flowering, with a heading time delaying effect. In this sense, Bi [71] have recently shown that *Hvcen* mutants flower early and show a reduction in spikelet number per spike, tiller number, and grain yield. Comadran [20] hypothesized that amino acid 135 caused the functional differences in this gene. Haplotype II (carrying Pro135) is present in most winter barleys, whereas haplotype III (Ala135) is found in spring barleys. The alleles of Beka and Logan correspond to haplotypes III and I, respectively, both carrying Ala135. Nevertheless, the Logan allele at this QTL is associated with earlier flowering in our study. Therefore, either we are looking at the effect of a linked gene, or there are other functional polymorphisms in *HvCEN*, or in its regulatory sequences. We are not the first to detect a phenotypic effect when confronting haplotypes III and I. Borràs-Gelonch [72] found a large effect heading date QTL in the Steptoe × Morex population, in the vicinity of *HvCEN*, with Morex (haplotype I, as Logan) as the early allele. These two results from independent studies indicate that either there is another functional

polymorphism at *HvCEN,* different from the one proposed by Comadran [20], or there is a second gene tightly linked to *HvCEN* affecting flowering time. In support of the first possibility, we identified another SNP polymorphism (A/G) downstream *HvCEN*, in position chr2H:523379223, where Logan carries the same allele as the reference genotype Morex (G). Polymorphism at the 3′ region could be a cause for regulatory control of *HvCEN*. This was demonstrated in *Arabidopsis thaliana*, in which *TFL1*, paralog of *HvCEN,* is expressed in all shoot meristems but not in floral meristems [73]. These authors showed that *TFL1* expression was based on distinct *cis*-regulatory regions; the most important being located 3′ of the coding sequence. Recent findings by Bi [71] further support expression differences of *HvCEN* haplotypes. They found differential tissue expression of *HvCEN* haplotype I (Bowman) and haplotype III (Bonus). Taken together, these results indicate that the possibility of a polymorphism with functional consequences in the 3′ region of *HvCEN*, causing different phenotypes for haplotypes I and III, deserves further exploration.

The interaction between QTL1 and QTL2, possibly associated with *HvCEN* and *HvELF3*, supports the recent results of Bi [71]. They analyzed *hvelf3 hvcen* double mutants, with high *HvFT1* expression under SD, suggesting that, under conditions where *HvFT1* is expressed, either under LDs or in the *hvelf3* mutant background, *HvCEN* genetically interacted with *HvFT1* to modulate floral development. These authors could not rule out that other genes might be influencing the observed phenotype, as many light-dependent transcripts were miss-regulated in the *hvelf3* mutant [21].

The position of QTL2 (possibly *HvCEN*) at the center of the pathways affecting flowering time in spring barleys, in interaction with other genes and not as earliness per se QTL, is a new finding of this study. We want to remark that the alleles of the two parents belong in the spring germplasm pool. This is of particular interest for plant breeders, as it should be possible to work with them in crosses with minimum linkage drag. The combination of particular alleles at *HvCEN*, *HvELF3*, and *HvFT1* in Logan are responsible for the earliness of this cultivar, but there is room for increased earliness considering the early alleles contributed by Beka. This was actually evidenced in transgressive segregation seen in the greenhouse experiment, with only 8 lines and the parents. There were 2 lines later than the late parent (Beka), and 3 lines earlier than the early parent (Logan). On the other hand, the lateness conferred by *HvCEN*, *HvELF3* and *HvFT1* alleles from Beka is almost unaltered by the haplotypes at the other minor QTL (3 and 4). If the candidate for QTL2 is not *HvCEN*, then the result is equally interesting, as it would point to a different gene with great potential to modulate an important agronomic and adaptive trait.

Beka and Logan show two of the polymorphisms described for *HvFT1*. As mentioned earlier, they differ in the sequence of the promoter and the number of copies of the gene, late vs. early, 2 vs. 1, respectively [19]. In a previous study with the Beka × Mogador population, we reported that, within the dominant *VrnH1* spring class, plants with the Beka allele (2 copies of *HvFT1*) flowered earlier [19]. Both Beka and Logan carry the dominant *VrnH1* spring allele and, in the current study, the Logan allele was associated with earlier flowering, as reported for other genotypes with a similar *HvFT1* promoter haplotype [18,26]. This is yet another indication of the richness of alleles and allelic effects at this locus.

Logan lacks the second exon of *HvCO1*, producing a truncated, non-functional protein. The level of expression in the only lines (2785 and Logan itself) with a Logan allele at *HvCO1* was barely detectable, significantly lower than the rest (Fig. 3). These two genotypes also had low levels of *HvFT1* expression. These results point to an interaction between these two genes, also revealed as a low but significant interaction between them for field flowering date. This agrees with other reports illustrating that *HvFT1* expres-

sion is enhanced by *HvCO1* [13,30]. However, these two lines, 2785 and Logan, flowered with 11 days of difference in the greenhouse, indicating that at the time of sampling (10–22 days before flowering), *HvFT1* expression was not fully correlated with flowering date. Either the expression of this gene diverged later for those two genotypes, or there are other ways of promoting inflorescence development in Logan. In any case, considering the 10 genotypes tested in the greenhouse experiment, *HvFT1* expression was the only one significantly correlated with flowering date, supporting its role in promoting inflorescence development. The explanation of this difference may lie in the different haplotypes of the two lines at other QTL. Line 2785 was also late in the field trials (116.6 days on average), but not as late as line 2814 (118.1 days), which was relatively earlier under controlled conditions, probably due to environmental differences. The greenhouse experiment was carried out under a longer photoperiod (16 h) than experienced in most field trials. The greenhouse environment likely enhanced the factor that delayed flowering of line 2785 more than that of line 2814 under controlled conditions, which is consistent with the effect of a gene responsive to long days or involved in the circadian clock. The fact that *HvCO1* only appeared as an epistatic QTL, and not as a main one, indicates its interactive nature. There was a retarding effect of the Logan allele at this locus when the Logan allele was also present at QTL3, but not when the Beka allele was present (Table S5).

In *A. thaliana*, Tiwari [57] described that the flowering time regulator CONSTANS binds to the promoter of *FT*, via a unique *cis*-element. In barley, Deng [54] showed that the VERNALIZATION 1 protein binds to the promoter of *HvFT1*. These results prompted us to search for the binding motifs identified in those studies in the Beka, Logan and Morex *HvFT1* promoters (Fig. S3). A few differences were apparent between the sequences of our two parents, which coincided with the polymorphism described previously [18] as promoter-early and promoter-late. While preliminary, the most relevant seems to be a proximal VRN1 site found only in Logan. Although Beka and Logan carry the same *VrnH1-1* allele, we cannot discard an enhanced activation of *FT1* by VRN1 in Logan due to its extra VRN1-landing site, as reported [54].

The action of the FT protein in the SAM is rich in interactions. *FT* transcription is induced in the leaves and its protein travels through the phloem to the SAM, where it plays a central role in triggering flowering [74,75]. In *A. thaliana*, FT forms a heterodimeric complex with the bZIP transcription factor FD, up-regulating the expression of the meristem identity gene *AP1* at the shoot apex [76,77]. This protein–protein interaction was also observed in rice, although in this monocot the complex includes as well a 14-3-3 protein [78]. Similar interactions have been reported in wheat, where the orthologous FT protein interacts with an FD-like protein and has the ability to bind in vitro the promoter of *VrnH1*, the wheat homolog of *AP1* [35]. Amongst the different *TaFDL* genes discovered in that study, two *TaFDL* proteins, *Ta*FDL2 and *Ta*FDL6, showed very strong interactions with *Ta*FT [35]. Later, the same authors demonstrated that FT, other FT-like proteins and different FD-like proteins could interact with multiple wheat and barley 14-3-3 proteins [36]. They also proved that a flowering activation complex including FT1, TaFDL2, and Ta14-3-3C could bind to the VRN1 promoter in vitro. We have identified a barley homolog of *TaFDL6*, possibly associated with the flowering time QTL on 5H. Given the scenario of interactions found in other monocots, one can speculate that the product of the *HvFD-like* gene may play a role in the regulation of flowering in barley. However, no major differences in *HvFDL* expression were detected between the parents or DH lines of the population (Fig. 3).

We have attempted to piece together our findings within a single framework. The early flowering cultivar, Logan, contains early

alleles for the major upstream regulators of the flowering pathway that are expressed in leaves, including *FT1*, the central integrator of flowering, and *ELF3*, which modulates the expression of *FT1* and production of GA [21,23]. We hypothesize that the early *ELF3* allele alleviates the requirement for a responsive *CO1* allele to transcriptionally activate *FT1* [21], and that the early *CEN* allele improves the potential for the increased expression of FT1 protein to promote flowering [20,38]. In spring barley, FT1 and GA act in a dominant manner to increase expression of down-stream components of the flowering pathway, including meristem identity genes in the developing apex [23]. In Logan, this dominant effect occurs in a genetic background that includes late alleles for QTL3 and QTL4 (possibly *FDL*), which we predict attenuates the florigenic effect of *FT1* in the developing apex. We propose that this combination of alleles achieves an early flowering phenotype while minimizing any yield penalty that may be caused by enhanced flowering signals reducing the number of spikelets that form on the inflorescence. Conversely, the late flowering cultivar, Beka, contains alleles of *FT1*, *ELF3* and *CEN* that delay the transition to flowering, especially when grown under shorter photoperiods. We propose that the early allele of *CO1* facilitates robust transcriptional activation of *FT1* as the photoperiod extends into longer day-lengths. Thus, the early alleles of QTL3 and *FDL* would mediate potent induction of meristem identity genes, as FT1 is transported from the leaves to the apex. Taken together, this model proposes that flowering time has been optimized in spring barley by balancing the strength of leaf-derived flowering signals with the potential for a developing apex to respond to these signals, which is supported by the observation that earliness for flowering could be further enhanced by combining early alleles for all QTL identified in this study. From a breeder's point of view, Logan contributes alleles that confer earliness in the first part of flowering pathways, and Beka contributes earliness at the late stages, obviously with less overall effect, due to the accumulative nature of development. Validation of this model requires further experimentation; however, the use of QTL analysis to predict gene and protein interactions provides a valid set of hypotheses to investigate in future studies. It is remarkable that, out the 5 QTL described in this paper, three correspond most probably to genes that encode proteins known to form complexes in several species, revealing an unexpected application of QTL analysis as predictor of protein interactions.

These hypotheses should be validated with further experimentation, but offer the advantage of integrating in a single model genes from the photoperiod pathway and genes known as earliness per se. These genes, so far, were loosely connected. Having them in a unique framework, supported by the results of this study, allows for better control of barley flowering time by geneticists and breeders, and enhanced possibilities for prediction of outcomes that will be needed to construct mechanistic models of barley development.

CRediT authorship contribution statement

Ana M. Casas, Ernesto Igartua, and José L. Molina-Cano: conceived this work. **José L. Molina-Cano, María P. Gracia, Francisco J. Ciudad, and William T.B. Thomas:** carried out the field experiments and data collection. **Ana M. Casas, Carlota Ruiz-Galluza, Arantxa Monteagudo, and Marian Moralejo:** performed the laboratory work. **Carlos P. Cantalapiedra and Bruno Contreras-Moreira:** curated the data and performed the bioinformatic analysis. **Ana M. Casas, Ernesto Igartua, and Arantxa Monteagudo:** run the QTL and statistical analyses. **Ana M. Casas, Scott Boden, Bruno Contreras-Moreira, and Ernesto Igartua:** drafted, reviewed and edited the document. All authors read and approved the manuscript.

Declaration of competing interest

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cj.2020.07.008.

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