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ORIGINAL PAPER

HvFT1 (*VrnH3*) drives latitudinal adaptation in Spanish barleys

Ana M. Casas · Abderrahmane Djemel · Francisco J. Ciudad · Samia Yahiaoui · Luis J. Ponce · Bruno Contreras-Moreira · M. Pilar Gracia · José M. Lasa · Ernesto Igartua

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Abstract In barley, three genes are responsible for the vernalization requirement: *VrnH1*, *VrnH2* and *VrnH3*. The winter growth habit of barley requires the presence of a recessive *VrnH1* allele, together with an active *VrnH2* allele. The candidate for *VrnH3* (*HvFT1*) has been recently identified, with evidences pointing at a central role in the integration of the vernalization and photoperiod pathways. Functional polymorphisms have been proposed, but experimental evidence of their role on agronomic performance and adaptation is needed. We examined allelic variation at the promoter and intron 1 of the *HvFT1* gene in a landrace collection of barley, finding a high diversity level, with its geographic distribution correlated with latitude. Focusing on genotypes with winter alleles in *VrnH1* and *VrnH2*, an association analysis of the four main *HvFT1* haplotypes found in the landrace collection detected differences in time to flowering. Landraces with the intron 1 *TC* allele, prevalent in the south, flowered 6–7 days earlier than those with the *AG* allele, under natural conditions. These results were validated in an independent F_2 population. In both data sets, the effect found was similar, but in opposite direction to that described in literature. The polymorphism reported at intron 1 contributes to variation in flowering time under field conditions. We have found that polymorphisms at the promoter also contribute to the effect of the gene on flowering time under field and controlled conditions. The variety of *HvFT1* alleles described constitutes an allelic series that may have been a factor in agro-ecological adaptation of barley.

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A. M. Casas (✉) · A. Djemel · S. Yahiaoui · L. J. Ponce · B. Contreras-Moreira · M. P. Gracia · J. M. Lasa · E. Igartua
Department of Genetics and Plant Production,
Aula Dei Experimental Station, CSIC, P.O. Box 13034,
50080 Zaragoza, Spain
e-mail: acasas@eead.csic.es

F. J. Ciudad
ITA, Instituto de Tecnología Agraria, Junta de Castilla y León,
P.O. Box 172, 47071 Valladolid, Spain

B. Contreras-Moreira
Fundación ARAID, Paseo María Agustín 36, Zaragoza, Spain

Present Address:
A. Djemel
Misión Biológica de Galicia, CSIC,
P.O. Box 28, 36080 Pontevedra, Spain

Present Address:
S. Yahiaoui
Institut National de la Recherche Agronomique d'Algérie,
02 rue frères Ouaddek, Hassen Badi El-Harrach, Alger, Algeria

Introduction

Flowering time is an important factor in the adaptation of barley varieties to environmental conditions and maximizing yield potential (Boyd et al. 2003; Cockram et al. 2007a; Cuesta-Marcos et al. 2009), by synchronizing the plant cycle to the prevailing environmental conditions. Flowering time is a complex trait that shows an almost continuous variation in cereals. The investigation of the genetic control of flowering time in barley has benefited from the comparative use of floral pathways in *Arabidopsis thaliana* (Cockram et al. 2007a) and rice, via the identification of candidate genes through orthology.

The variation in flowering time is mainly due to variations in genes regulated by day length (photoperiod) or long exposures to low temperature (vernalization) (Laurie et al. 1995; Trevaskis et al. 2003; Dubcovsky et al. 2005). In barley, three genes are responsible for the vernalization requirement: *VrnH1* (isolated by map-based cloning in diploid wheat, Yan et al. 2003), *VrnH2* (identified by positional cloning, Yan et al. 2004) and *VrnH3* (identified by homology to a known gene from *Arabidopsis thaliana*, Yan et al. 2006). *VrnH1* is induced by vernalization and promotes the transition from vegetative to reproductive development. *VrnH2* is a floral repressor that delays flowering until the plants are vernalized. The *VrnH3* gene seems to be orthologous to the *A. thaliana* floral pathway integrator *FT* (*FLOWERING LOCUS T*) gene (Yan et al. 2006; Faure et al. 2007; Turck et al. 2008; Kikuchi et al. 2009). In *A. thaliana*, *FT* expression increases in the leaves when plants are exposed to inductive day length. In barley, expression of orthologous *HvFT1* (synonymous to *VrnH3*) is induced by long day conditions and promotes flowering (Hemming et al. 2008).

The winter growth habit of barley requires the presence of a recessive *VrnH1* allele, together with an active *VrnH2* allele (Cockram et al. 2007b; Hemming et al. 2009). Vernalization induces *VrnH1* under both short and long days, which then represses *VrnH2*. Distelfeld et al. (2009) reported that the interactions among the three vernalization genes generate a feedback regulatory loop that once started, leads to an irreversible induction of flowering. The function of *HvFT1* has started to be unraveled only recently. There is now mounting evidence supporting the role of the FT protein in *Arabidopsis* (and corresponding proteins in other species) as an important part of the florigen (Corbesier et al. 2007; Tamaki et al. 2007). Kikuchi et al. (2009) presented strong evidence suggesting that *HvFT1* plays a central role in promoting flowering, integrating the photoperiod and vernalization pathways. *HvFT1* expression seems to be regulated by the major photoperiod response genes: *PpdH1* under LD conditions and *PpdH2* under SD conditions. There are evidences on the adaptive role played by *VrnH1*, *VrnH2* and *PpdH1* during the expansion of the crop, facilitating its adaptation to new agroecological niches (Cockram et al. 2007a; Jones et al. 2008). Does *VrnH3-HvFT1* also have an adaptive role? We know that the phenotypic effect of *HvFT1* on flowering time can be very large (Yan et al. 2006), and therefore may be an important factor for the final determination of barley flowering time. Other open questions on this gene are: to what environmental cue does *VrnH3* respond, temperature or photoperiod? What effect does it have on flowering time under natural conditions?

To address these questions, we analyzed the polymorphism and the phenotypic effect of this gene on a collection of Spanish barley landraces and its variation at the sequence level, and validated its effect on a segregating population.

Materials and methods

Plant material

The Spanish Barley Core Collection (SBCC, http://www.eead.csic.es/barley) is a set of 159 inbred lines derived from landraces, plus 16-old cultivars. The landraces constitute a representation of barley cultivated in Spain prior to the introduction of modern cultivars, and have complete passport data (Igartua et al. 1998; Yahiaoui et al. 2008).

An F_2 population, 'Esterel' × 'SBCC016' developed at the EEAD/CSIC in the framework of the Spanish National Barley Breeding Program was also used for this study. The parents were 'Esterel' ('7,761' × 'Plaisant'), a French winter cultivar with a strong vernalization requirement, and the Spanish line 'SBCC016' (from Luna, Zaragoza, Spain). This line exhibited a winter growth pattern and apparently weaker vernalization requirement than typical winter cultivars such as 'Esterel'. Out of the five major flowering time genes related to vernalization and photoperiod responses, the population segregates for *VrnH1*, *PpdH2* and *VrnH3*. It is dominant and monomorphic for *VrnH2* and *PpdH1*.

Genotyping

It was carried out on leaf samples harvested from individual plants. After homogenization (Mixer Mill model MM301, Retsch), DNA was extracted according to the protocol described in the NucleoSpin® Plant II Kit (Macherery-Nagel). DNA amplification was carried out for markers representing major flowering time candidate genes: *HvBM5A* (*VrnH1*),*HvFT3* (*PpdH2*) and *HvFT1* (*VrnH3*). From this point on, we will use the names of the candidate genes for the sake of simplicity, except where stated otherwise.

Allelic variation in the promoter and first intron of *HvFT1* was examined in the SBCC. The first intron of *HvFT1* was amplified with primers HvFT1.1F (5′-acgtacgtccc ttttcgatg-3′) and HvFT1.2R (5′-atctgtcaccaacctgcaca-3′) which gave a 506-bp fragment. To differentiate between the two polymorphic sites in this intron, digestion of the amplified DNA was carried out with *Tsp509* I (A/T) or *Bcl* I (G/C). Two indels in the proximal promoter of *HvFT1* were characterized (Fig. 1) using the DNA of 'Calicuchima-sib' or 'Morex', respectively: indel1 (at 2,526 in Fig. 1) was amplified with primers FT1ind.1F (5′-atttatgccgccaatcgac-3′) and FT1ind.2R (5′-ggaatgtctgccaattagctc-3′) that generated a fragment of 139 or 135 bp; indel 2 (position 2,908 in Fig. 1) was amplified with primers FT1ind.3F (5′-actagagcggagagc agcag-3′) and FT1ind.4R (5′-actgaggaggtggtgaatgg-3′) that

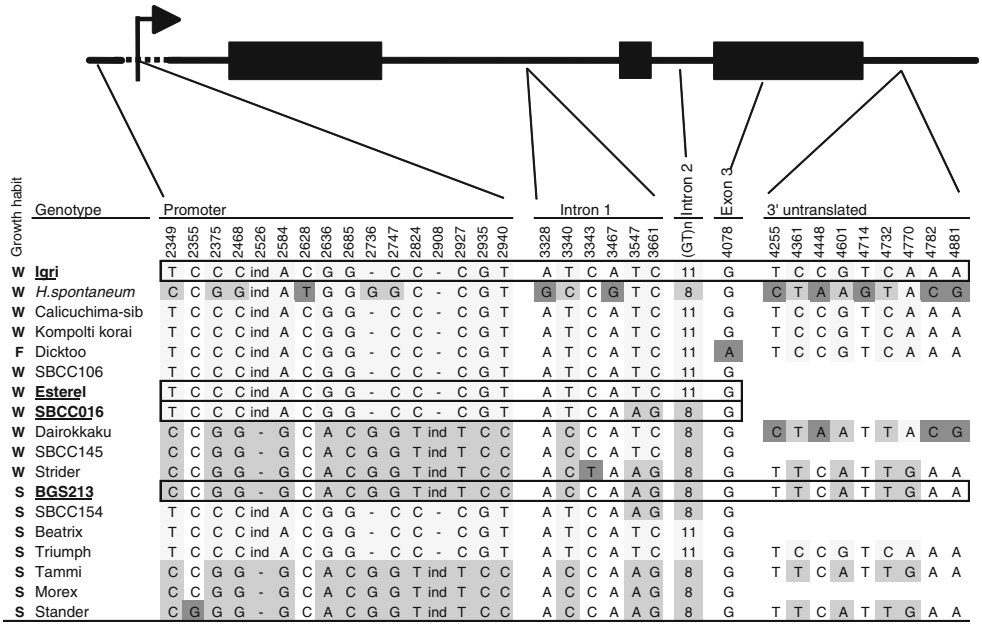

Fig. 1 Structure of the gene *VrnH3* (*HvFT1*) showing the promoter, three exons and two introns. The sequences of four entries from the Spanish Barley Core Collection (SBCC), 2 cultivars ('Esterel' and 'Beatrix') and 12 sequences downloaded from GenBank, are aligned to show the variability found in several regions of the gene. Polymorphisms are labeled with respect to the coordinates of the 'Calicuchima-sib' (EU007825) sequence deposited in GenBank. A color code identifies the similarity of the sequence with typical winter cultivar 'Igri' (*light gray*) or spring line 'BGS213' (*dark gray*). The GenBank accession numbers are: 'Igri' (DQ898517), *H. spontaneum* 'PBI004-7-0-015' (DQ898516), 'Calicuchima-sib' (EU007825), 'Kompolti korai' (EU007828), 'Dicktoo' (EU007827), 'Dairokkaku' (EU007826), 'Strider' (EU007830), 'BGS213' (DQ898515) 'Triumph' (DQ898520), 'Tammi' (EU007831), 'Morex' (DQ100327 and EU331775) and 'Stander' (DQ898519 and EU331781)

generated a product of 142 or 146 bp. We also examined another polymorphism in the distal part of the promoter (SNP927 at position −2,150 from the transcription start site). Primers SNP927.F (5′-aggatcgctaagacgttgga-3′) and SNP927.R (5′-aggccacgacctcaagtatg-3′) produced a 279-bp fragment. A polymorphism was detected after digestion with *Aci* I (recognition sequence CCGC).

The F_2 population was genotyped for the first intron of *HvFT1* as described. Differences in the size of the first intron of *VrnH1* were detected with primers HvBM5A.88F (5′-gaatggccgctactgcttag-3′) and HvBM5A.85R (5′-tctcataggttctagacaaagcatag-3′), amplifying through the critical region for vernalization. The difference in size was based on the presence of a barley-specific MITE that was absent in 'Esterel'. *PpdH2* was tested using *HvFT3*-specific primers, HvFT3.1F (5′-atccattggttgtgtggctca-3′) and HvFT3.2R (5′-atctgtcaccaacctgcaca-3′), which amplify a fragment of 430 bp, through exons 1 and 2 of the 'SBCC016' gene. Amplified products were run on 2% agarose gels and visualized by ethidium bromide staining. Six additional SSR markers that map around *HvFT1* on 7HS were assayed in the 'Esterel' × 'SBCC016' population.

The SBCC was genotyped with DArT markers (Wenzl et al. 2004) at Triticarte (http://www.triticarte.com.au/). Only 750 markers with known position in a barley consensus map (Wenzl et al. 2006) were used to analyze the correlation between these bi-allelic markers and latitude of the recollection site of the landraces.

Sequencing

The nature of the polymorphism at *HvFT1* was investigated by sequencing this gene in 'Esterel', 'SBCC016' and four other genotypes. Primers (Table S1) were designed to amplify overlapping fragments, based on the 'Calicuchima-sib' sequence deposited in GenBank (EU007825). Sequencing was carried out in six genotypes: 'Esterel' (winter, 6-row), 'SBCC016' (winter, 6-row), 'SBCC106' (winter, 6-row), 'Beatrix' (spring 2-row), SBCC145 (winter 6-row) and 'SBCC154' (facultative, 2-row). All of them were parents of mapping populations currently under study. Amplicons from two independent PCR reactions were sequenced from both ends with forward and reverse primers. Sequences were assembled, aligned and searched for polymorphisms using the software package ClustalW2 (Larkin et al. 2007). We also performed a BLAST search (Altschul et al. 1997) against the GenBank nucleotide sequence library, to search for further similar public sequences.

Experimental setup

The SBCC was evaluated under field conditions in ten agronomic trials sown in November or December across Northern Spain during 3 years (2002–2004). These trials were sown following alpha designs, with three replications, in plots of six rows, 1.5 m wide by 7 m long. Flowering date and other agronomic traits recorded in these trials are described in Yahiaoui et al. (submitted). Two additional trials were late sown in April. Flowering date was estimated as the date in which 50% of the stems presented at least 2 cm of protruding awns, expressed in days from 1 January. The SBCC was also evaluated under controlled conditions, in four treatments combining the presence or absence of vernalization (V or NV), ensued by either long or short days (SP or LP). A first batch of seeds was sown in pots and transferred to a vernalization chamber. Vernalization was provided for 56 days, under 10-h light and day/night temperatures of 11°C/5°C. Two weeks before the end of the vernalization period, another batch of seed was sown in pots, directly within the long- and short-photoperiod glasshouses. By the end of the vernalization period, both vernalized and unvernalized plants reached approximately the same developmental stage. At that time, vernalized plants were moved to the long- and short-photoperiod greenhouses, where the day lengths had been set at 17 h (long) and approximately 10 h (short, natural day length during winter time). Four plants per line were tested at each of the four resulting treatments. The treatments were coded as VLP (vernalization ensued by long days), VSP (vernalization ensued by short, natural photoperiod), NVLP (no vernalization, long days) and NVSP (no vernalization, short photoperiod). The variable measured was the total number of leaves produced on the main stem. Sensitivities to vernalization (in long and short days, VER-LP and VER-SP) and photoperiod (in vernalized and unvernalized plants, PHOT-V and PHOT-NV) were calculated as differences between the treatments.

The F_2 population 'Esterel' × 'SBCC016' was sown in winter (4 February 2008) under natural conditions. We expected that the rather late sowing date would induce the occurrence of possible differences in the vernalization requirement due to the polymorphism at the vernalization loci and a lack of sufficient number of cold days to induce full vernalization for strict winter types. Three hundred F_2 seeds (plus parents) were germinated and grown for 1 week under greenhouse conditions (21°C, 16-h light/8-h dark photoperiod) and transferred at the same growing stage (one leaf) to two microplots, which consisted of three rows of 12-m long and 1-m wide. Ten plants of each of the two parents were planted in two different zones of the seed beds, to provide a measure of experimental error. The experiment was carried out at the Aula Dei Experimental Station in Zaragoza in long seed beds commonly used for crossing blocks. The plants were watered every week with drip irrigation. Weeds were removed by hand, and general purpose insecticide was sprayed as needed to prevent insect damage. The traits recorded were: (1) initiation of stem elongation, determined for each plant by grasping and pressing the base of the primary tiller with two fingers to feel the bulge caused by the first node (stage 31 Zadoks); and (2) flowering time or heading, recorded as the moment when 2 cm of the awns protruded from the flag leaf on the primary tiller (i.e., when it reached stage 49 in the Zadoks scale) (Zadoks et al. 1974). The initiation of stem elongation and heading of the primary tiller were recorded daily for a period of 2 months, from the end of March until the end of May.

Data analysis

The SBCC phenotypic data were subjected to an association analysis with the polymorphisms found in *HvFT1*. This germplasm collection presents a marked population structure (Yahiaoui et al. 2008). To account for this structure, we could have used the Q matrix calculated in that study. Instead, we preferred to use *VrnH1* as a proxy for population structure, because it is closely linked with it (unpublished) and also there is direct interaction between *VrnH3* and *VrnH1* in the metabolic pathway that leads to the promotion of flowering in response to environmental cues (Distelfeld et al. 2009). The analyses were performed using the REML routine provided in Genstat 12 (Payne et al. 2009), including *VrnH1* plus the polymorphisms at *HvFT1* as fixed factors. The averages provided for the allelic or haplotypic classes according to *HvFT1* polymorphisms are best linear unbiased estimators, corrected for unbalances in other terms of the model (*VrnH1*).

Analysis of variance for the phenotypic traits of the 'Esterel' × 'SBCC016' population was performed using the general linear model (GLM) procedure of the SAS System for Windows (SAS Institute Inc., Cary, NC, USA) with segregating markers and their interactions as fixed variables. The error term corresponded to the plant variation within the parents. When the interactions were not significant, they were pooled with the residual term.

In the population 'Esterel' × 'SBCC016', a genetic map of chromosome 7HS was produced using JoinMap 4.0 (Kyazma B.V.). QTL analysis for flowering time was carried out with the appropriate procedure provided in the software GenStat Release 12.1 (VSN International Ltd).

Apart from SBCC entry codes, all accession numbers reported in this study are from GenBank. The accessions for the *HvFT1* nucleotide sequences first described in the manuscript are: 'Esterel'—HM133574; 'Beatrix'—HM133575; 'SBCC106'—HM133576; 'SBCC016'—HM133577; 'SBCC 154'—HM133578 and 'SBCC145'—HM133579.

Results

Variation in *HvFT1*

The landrace-derived inbred lines of SBCC presented similar frequencies of the two *HvFT1* haplotypes reported by Yan et al. (2006) at the first intron (*AG* and *TC*), and one individual with a recombinant haplotype *TG* (Table 1). To find out whether these polymorphisms corresponded to the ones previously described, we sequenced a region of approximately 3,900 bp, in eight amplicons covering the promoter and the entire intronic and exonic regions, except between three and ten amino acid residues at the 3′ end of the third exon. Sequencing this gene in four SBCC lines and two cultivars revealed the same two SNPs in the first intron, one SSR in the second intron, as described by Yan et al. (2006), and a variety of polymorphisms in the promoter region (Figs. 1, S1). No sequence from these SBCC lines was similar to 'BGS213' (*AG* haplotype, Yan et al. 2006) nor to other spring genotypes ('Morex', 'Tammi' and 'Stander'). We found a new allele in lines 'SBCC016' and 'SBCC154', as compared to the alleles described by Yan et al. (2006). Both genotypes carry the *AG* haplotype in the first intron, similar to the spring genotype 'BGS213' (DQ898515), but their promoter is identical to that of winter genotypes such as 'Igri' (DQ898517), 'Calicuchima-sib' (EU007825) or 'Kompolti korai' (EU007828). Also, the sequences of 'SBCC145' and 'Dairokkaku' (EU007826) are very similar to each other and differ only in one SNP. These genotypes represent a mixture of the two types of prevalent promoters and also present several new SNP. The other lines sequenced by us, 'Esterel', 'Beatrix' and 'SBCC106', all present sequences similar to other spring or winter cultivars.

Table 1 Number of lines in the SBCC classified according to *HvFT1* haplotypes defined by polymorphisms at the promoter (SNP927, indel 1, indel 2) and at the first intron

HvFT1				
SNP927	Indel 1	Indel 2	Intron 1	No. lines
Winter lines				
C	139	142	AG	36
C	139	146	AG	8
C	139	142	TC	76
C	135	146	AG	13
C	135	146	TG	1
C	135	146	TC	6
Spring or facultative lines				
C	139	142	AG	8
C	135	146	AG	9
C	139	142	TC	1
C	135	146	TC	1

Overall, there are four main alleles (Fig. 1), constituted by combinations of two main types of sequences at the proximal region of the promoter and at the first intron, exemplified by 'Morex', 'Dairokkaku', 'Kompolti korai' and 'SBCC016'. 'Strider' (EU007830) presents a fifth allele, with two polymorphisms in the promoter and one in the first intron (Figs. 1, S1). We believe that this is a functionally different allele, as 'Strider' is a strict winter cultivar (von Zitzewitz et al. 2005). It features characteristic winter alleles at *VrnH1* and *VrnH2*, same as 'BGS213' (Yan et al. 2006). The spring habit of this last genotype has been attributed to its *VrnH3* allele. 'Strider' presents a very similar *VrnH3* sequence to 'BGS213' (and other spring cultivars), at least in the introns and proximal regions of the promoter. As 'Strider' and 'BGS213' exhibit different growth habits, they should present some functional polymorphism elsewhere in *VrnH3*. We have found several polymorphisms between 'Strider' and four spring genotypes at positions 927, 1,966 and 3,343 (Figs. 1, S1), which are therefore candidates for functional polymorphisms.

Yan et al. (2006) already pointed out that the promoter of *HvFT1* may play a functional role in the regulation of this gene. The sequence analysis revealed several polymorphisms that could be investigated further in order to shed more light on the functional role of the promoter. We characterized the SBCC for several polymorphisms, namely the two indels in the proximal region, and SNP927. The results of these analyses are presented in Tables 1 and S2, together with the polymorphism at the other two main vernalization genes *VrnH1* and *VrnH2*. A large majority of the lines, 140, can be classified as winter types, according to the haplotypes at *VrnH1* and *VrnH2*, and also to phenotypic and expression analyses (Casao et al. 2011a, b). Overall, we found six *HvFT1* haplotypes among the winter lines and four among the rest (Table 1).

HvFT1 latitudinal distribution

The *HvFT1* polymorphisms observed in the SBCC presented different relationships with latitude. The correlation coefficient of the intron 1 haplotypes with latitude was high, 0.55. The correlation coefficients of SNP927 and the two indels with latitude were moderate to low (0.23, 0.07 and 0.03, respectively). These coefficients were calculated just for the 156 accessions from the Iberian Peninsula (i.e., disregarding accessions from the Canary Islands). The significance of the coefficient for intron 1 haplotypes is apparent when compared with the correlations obtained for 750 DArT markers (Fig. 2), neutral in principle, representing 460 unique positions in the genome according to the genetic map of Wenzl et al. (2006). The average correlation of DArT markers with latitude was 0.13 (in absolute values, as represented in Fig. 2), with a standard deviation of 0.15.

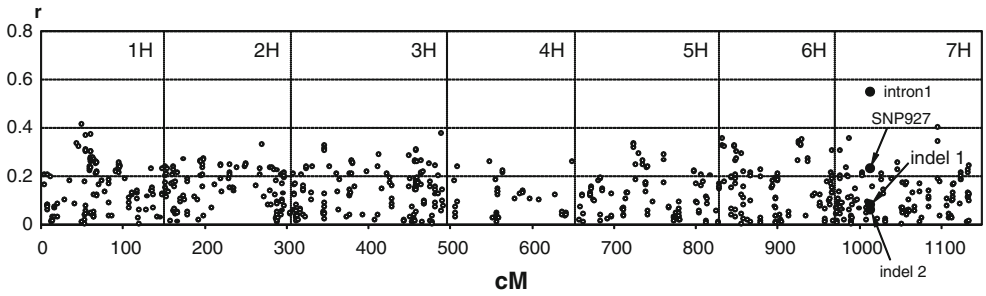

Fig. 2 Genome scan presenting the correlation coefficients of 750 DArT markers (*small empty circles*) mapped to the seven barley chromosomes with latitude (*decimal*). The correlation coefficients for *HvFT1* polymorphisms are indicated by *larger filled circles*

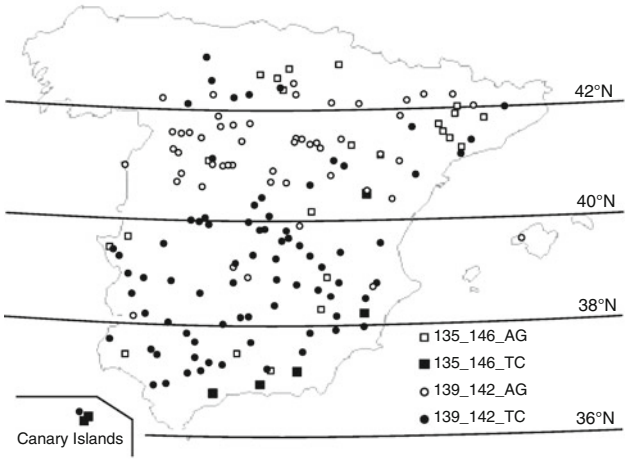

Fig. 3 Distribution of *HvFT1* haplotypes found in the Spanish Barley Core Collection lines over the Iberian Peninsula. *Lines* are placed according to latitude and longitude of their collection sites

The highest coefficient shown by DArT markers was 0.41. Given the distribution of correlation coefficients for neutral markers, a correlation of ±0.55 would occur by chance with a probability of 0.0006. Thus, it seems that the relationship of *HvFT1* distribution with latitude is not accidental. Individuals carrying the *TC* haplotype prevailed in the southern half of the country, whereas *AG* haplotypes were much more common in the northern half (Fig. 3). The distributions of other *HvFT1* polymorphisms were not related with latitude. The small class (7 individuals) carrying the haplotype *135–146-TC* was apparently restricted to southern latitudes, including two lines from the Canary Islands. Actually, its correlation with latitude was significant (0.23), but too low to stand out of the coefficients for the DArTs.

Effect of *HvFT1* in the SBCC

The agronomic evaluation of the SBCC provided a good estimation of flowering time of Spanish landraces under standard and late sowing conditions.

Out of the 159 landrace-derived lines, only 19 can be considered spring or facultative types. The majority are winter types, presenting the two characteristic *VrnH1* alleles found in Spanish barleys, 93 similar to 'SBCC106', and 47 like 'SBCC058' (Casao et al. 2011b). We carried out an association analysis of the polymorphisms at *HvFT1* with the phenotypic traits, only for the 140 winter lines, to avoid the confounding effects of the small number of spring and facultative lines (Tables 2, S2). The association was performed using mixed models, including *VrnH1* polymorphism as an additional factor. Each polymorphism conveys somewhat different information, as there was not complete linkage between any of them (Table 1). The analyses were performed for each polymorphism separately (not shown), and then we ran a combined analysis for the two most relevant polymorphisms found (Table 2). The analyses of variance for intron 1 revealed significant effects for heading time at both sowings. On the contrary, the indels by themselves did not explain heading time variation, but they did explain part of some traits measured under controlled conditions. Therefore, it seems that both intron and promoter contributed to phenotypic effects. Indel 1 was significant at more variables, and with larger effects than indel 2 (5 vs. 3), suggesting that the functional polymorphism was closer to indel 1. Therefore, indel 2 was dropped for further analyses.

The analyses of variance in Table 2 include indel 1 and intron 1 of *HvFT1*, and their interaction, and *VrnH1* as fixed factors. *HvFT1* induced significant earlier heading, mostly explained by intron 1 (*TC* allele 6–8 days earlier), but also for indel 1 (*135* allele 2–3 days earlier) in autumn sowings. Indel 1 effect was significant for all treatments and sensitivities involving long days. The *135* allele was more responsive to long days than the *139* allele, with little influence from the intron. Under non-inductive conditions (NVSP), the effect of the intron polymorphism was prevalent, though a small interaction was also detected. Under controlled conditions, the class *135-AG* was consistently the latest under short days. But it also showed the strongest response to long days, featuring significantly larger

Table 2 Phenotypic results of field and greenhouse experiments for 140 lines from the Spanish Barley Core Collection with different *HvFT1* alleles

Trait	*HvFT1*				Winter lines				Spring or facultative (n = 19)
	VrnH1	id1	id1	int.	*135-AG* (n = 14)	*135-TC* (n = 6)	*139-AG* (n = 44)	*139-TC* (n = 76)	
Days from January 1st									
Heading date (fall sowing)	ns	**	**	ns	120.6b	112.7d	122.5a	115.9c	121.8
Heading date (April sowing)	**	ns	ns	ns	163.9	158.1	160.7	158.9	156.1
Number of leaves									
VLP	ns	*	*	*	6.91a	6.07b	6.96a	6.82a	6.26
VSP	*	ns	ns	**	13.43a	11.36b	12.33b	12.23b	12.16
NVLP	**	**	ns	ns	11.05b	10.56b	11.94a	11.74a	7.79
NVSP	ns	ns	**	*	14.51a	12.49bc	13.41b	12.88c	13.28
VER-LP	**	**	ns	ns	2.54b	2.65b	3.69a	3.51a	0.31
VER-SP	*	ns	ns	ns	1.46	1.35	1.19	1.05	0.95
PHOT-V	**	*	ns	ns	6.52a	5.30b	5.37b	5.41b	5.90
PHOT-NV	**	**	ns	ns	3.46a	1.93b	1.47b	1.14b	5.49

The significance values for the factors included in the analyses are shown together with the averages of *HvFT1* allelic classes (days from 1 January for field trials; number of leaves for the controlled conditions experiment). *VrnH1* was included as a factor to account for population structure (see text). The comparison of allelic means was done using the intra-allelic sums of squares as the error term, for 140 winter lines. Spring and facultative lines were not included in the analyses of variance, and their results are shown for the sake of comparison

Means followed by the same letter within each row are not significantly different for $P < 0.05$

id1 indel 1, *it1* intron 1, *Int.* indel 1 × intron 1 interaction

*, **, ns, significant for $P < 0.05$, $P < 0.01$ and non-significant, respectively

sensitivities to photoperiod (PHOT-V and PHOT-NV) than any other class.

The class *135-TC* was earliest at all greenhouse treatments and field sowings (significantly at the autumn sowings and in the VLP treatment). This class seems to have a different promoter than *135-AG*. The fragment of the promoter between positions 715 and 1,416 was sequenced in all six winter *135-TC,* and all were identical to 'SBCC145' (Fig. S1). Therefore, the *135-TC* class may represent a distinct allele with many polymorphisms in the promoter compared to the other classes.

The allelic variation detected through association may have been influenced by some other genes. Therefore, in the next experiment we further tested the effect of *HvFT1* in an independent set of materials with random assortment of independent genes, such as a biparental population.

Validation of *HvFT1* effect in an F_2 population

The population 'Esterel' × 'SBCC016' was used for this purpose. The number of plants finally scored for phenotypic traits and molecular markers was 242 F_2 plants and 33 parent plants. Only healthy plants that reached flowering were kept for further analysis. The phenotypic distributions of days to stem elongation and days to flowering showed transgressive segregation, as the variation span for the population clearly exceeded the range of the parents (Table 3). The distribution of *HvFT1* over the population showed a deficit of homozygotes for the 'SBCC016' allele (59:141:42, 'Esterel': heterozygous:'SBCC016', chi-square = 9.0, $P = 0.01$).

Table 3 Means and ranges of the distribution of agronomic traits in the 'Esterel' × 'SBCC016' F_2 population and parents

	Days to stem elongation[a]	Days to flowering[a]
'Esterel'	116.1 a	139.1 a
'SBCC016'	112.4 b	135.1 b
F_2 population	113.6	137.7
Range (min, max)	14 (105, 119)	28 (124, 152)

[a] Values followed by different letters within traits are significantly different ($P < 0.05$)

We carried out a QTL analysis restricted to the chromosome arm 7HS, where this gene was located, to ensure that there was a flowering time QTL in this region. We found a marked peak for flowering date at *HvFT1* (Fig. 4), with an effect of 3.5 days (7 days between homozygous classes, 'SBCC016' contributing the late allele). Therefore, the effect of this gene on flowering time was confirmed, and their diagnostic markers were used in further analyses.

The three major genes that were segregating in the population—*HvFT1*, *HvBM5A* (candidate for *VrnH1*) and *HvFT3* (candidate for *PpdH2*)—were included in a joint

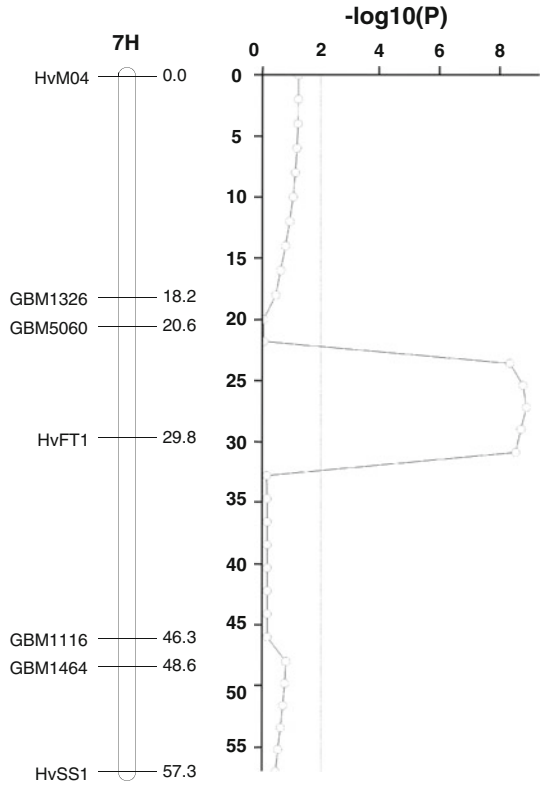

Fig. 4 Partial map of chromosome 7HS for the F_2 population 'Esterel' × 'SBCC016', and QTL analysis for flowering time

analysis of the phenotypic traits. In this analysis, *HvFT1* still presented a large effect on flowering time and a smaller but significant effect on time to stem elongation (Table 4). The heterozygous class presented averages closer to the 'late' allele for these two traits. Therefore, significant additive and dominance effects were found for this gene (Table 5). No significant interactions were detected between *HvFT1*, *HvFT3* and *HvBM5A* (data not shown). 'Esterel' itself was 4.0 days later than 'SBCC016', even though the combined effect of the three genes examined (*HvFT1*, *HvFT3* and *VrnH1*) indicated that 'Esterel' should be around 3 days earlier than 'SBCC016'. Obviously, there must be other heading date QTL in this population of rather large effect that must account for the lateness of 'Esterel'.

Table 5 Means of allelic classes and genetic effects for *HvFT1* in the 'Esterel' × 'SBCC016' F_2 population

Traits	*HvFT1* alleles[a]			Genetic effects	
	TC/TC	TC/AG	AG/AG	a[b]	d[c]
Stem elongation	112.6a	114.0b	114.0b	0.7**	0.7**
Flowering time	133.2a	138.5b	140.5c	3.7**	1.7**

[a] Values followed by different letters within traits and genes significantly different ($P < 0.05$)

[b] Additive effect (homozygote AA − homozygote aa)/2, significantly different from 0, $*P < 0.05$, $**P < 0.01$

[c] Dominance effect (heterozygote − ((homozygote AA + homozygote aa)/2)), significantly different from 0, $*P < 0.05$, $**P < 0.01$

Discussion

Experimental proofs on the effect of the *FT* gene family on the determination of flowering time at several species are mounting: these have been reported for wheat (Yan et al. 2006; Bonnin et al. 2008), rice (Takahashi et al. 2009) and Arabidopsis (Schwartz et al. 2009). In barley, some studies found an effect of this gene under controlled conditions (Yan et al. 2006; Kikuchi et al. 2009) and also under natural field conditions (Stracke et al. 2009; Wang et al. 2010).

It is not clear what kind of stimuli these genes are responsive to, or if these are the same for every species. But, there is a wide consensus on the central role of *FT* genes in the pathway toward flowering, as integrators of the vernalization and photoperiod routes (Turck et al. 2008), and therefore may be influenced by both length of day and temperature. Yan et al. (2006), Hemming et al. (2008) and Kikuchi et al. (2009) found differences in expression of *HvFT1* alleles in response to length of day, whereas Yan et al. (2006) and Schwartz et al. (2009) also reported differential expression of barley, wheat and Arabidopsis *FT* genes in response to temperature. These last authors also reported a remarkable influence of thermocycle on *FT* expression in Arabidopsis.

Further evidence has been produced by several groups actively working on these genes worldwide. Currently, it can be safely assumed that *FT* genes respond to

Table 4 Analyses of variance for flowering time and time to stem elongation of the F_2 population 'Esterel' × 'SBCC016'

Sources of variation	Days to stem elongation		Days to flowering	
	DF	MS	DF	MS
HvBM5A	2	8.6	2	43.6
HvFT3	1	1.1	1	290.9**
HvFT1	2	24.5**	2	583.7**
Residual (genotypic)	1	434.9**	1	1,455.1**
Error (parents)	32	3.5	32	14.6

DF degrees of freedom, *MS* mean squares

$*P < 0.05$, $**P < 0.01$

environmental cues, and that they definitely affect flowering time. Taking all these into account, we can hypothesize that they are good candidates for playing a relevant role in crop adaptation.

Several studies in barley have detected flowering time QTL in bin 4 of chromosome 7H, where *HvFT1* is located (Hayes et al. 1993; Laurie et al. 1995; Cuesta-Marcos et al. 2008; Borràs-Gelonch et al. 2010). However, these results do not necessarily imply an effect of *HvFT1* on flowering time. In only one case ('Steptoe' × 'Morex') there was a coincidence of polymorphism in *HvFT1* sequence (Kikuchi et al. 2009) and effect on flowering time (Hayes et al. 1993). In another case, although a QTL was detected in that region (Laurie et al. 1995, population 'Igri' × 'Triumph'), no polymorphism was found in the sequences of the parents (Faure et al. 2007). Furthermore, no QTLs for flowering time were detected in that region for the 'Dicktoo' × 'Morex' (Pan et al. 1994) and the 'Sloop' × 'Halcyon' (Read et al. 2003) populations, even though they present the polymorphism allegedly related with function at the first intron of *HvFT1* (Hemming et al. 2008; Karsai et al. 2008).

The polymorphisms that determine functional differences in *HvFT1* are yet to be unequivocally identified. Up to now, the hypothesis put forward by Yan et al. (2006) for functional polymorphisms in *HvFT1* suggested that mutations in the first intron differentiated plants with dominant and recessive *VRN3* alleles, though they did not discard a possible role of the promoter. They summarized the polymorphisms found as two haplotypes at the first intron (*TC*, late winter, and *AG*, early, spring), and several more at the promoter.

The two alleles tested by Yan et al. (2006) at the cross 'BGS213'/*H. spontaneum* produced a difference of 50–60 days in time to flowering under long days in plants not exposed to vernalization ('BGS213' allele earlier). These alleles were the most distinct among the genotypes sequenced by these authors (Fig. 1). The differences between *HvFT1* alleles found both in the SBCC and in the population 'Esterel' × 'SBCC016' were much smaller, around 6–7 days and with opposite sign (*TC* early, *AG* late). In fact, our experiments and Yan's probably focused on different alleles. The studies by Yan et al. (2006), Stracke et al. (2009), Wang et al. (2010) and ours taken together suggest the existence of an allelic series at *HvFT1*, with a wide range of effects on flowering time.

The four SBCC lines sequenced represented three different *HvFT1* haplotypes. The rest of the SBCC lines (155) were characterized by two indels in the promoter and the two diagnostic SNPs at the first intron. Interestingly, spring lines sequenced in this and previous studies present a diverse array of possible alleles at *HvFT1* (Fig. 1) but, possibly, their effect is overridden by the spring alleles at *VrnH1* and *VrnH2*.

Our findings strongly support the existence of several regions of *HvFT1* regulation, in the promoter and in intron 1.The haplotypes described in the winter lines of the SBCC represent a combination of promoter and intron 1 polymorphisms with distinct phenotypic effects. There were three haplotypes of intron 1, although one (*TG*) was in minority and, phenotypically, close to the *AG* lines. There were three haplotypes at the promoter, attending to the marker analysis (Table 1), though similar classes *C-135–146-TC* and *C-135–146-AG* actually carried two different promoters ('Dairokkaku' and 'Strider' in Fig. 1, for instance). Also, we suspected that haplotypes *C-139–142-AG* and *C-139–146-AG* actually shared the same promoter, as they did not differ phenotypically (not shown). They may represent different ancestral intragenic recombination breakpoints between the two regulatory regions. We did not find the typical promoter of spring genotypes (line 'BGS213', cultivars 'Tammi', 'Morex', 'Stander' in Fig. 1). This was deduced from the fact that all SBCC lines had the same *Aci* I restriction profile in SNP927 as winter cultivar 'Strider', and different from spring cultivar 'Morex'.

Therefore, the data suggest the presence of just three different functional types of *HvFT1* promoters in the SBCC, exemplified by cultivars 'Dairokkaku', 'Calicuchima-sib' and 'Strider'. The association analysis of the phenotypic data provides enough resolution to separate, to some extent, the effects of promoter and intron 1. In the treatments without vernalization, the statistical significance of the effects due to the two regions was almost opposite. It seems that lines with allele *135* at indel 1 were more responsive to long days. The class *135-AG* was always significantly the latest under a short photoperiod. Class *135-TC* seems also more responsive to long days, but the results are not conclusive, given the small class size and the possible presence of a different promoter, as mentioned earlier. The two most abundant classes in the SBCC, *139-AG* and *139-TC*, had very similar responses under controlled conditions (only different at NVSP), but a large difference in flowering time in autumn field trials. This result was confirmed by a similar effect (7 days) between the two homozygous classes in a field sowing of the population 'Esterel' × 'SBCC016', representing exactly the same polymorphism. It seems that the experiments under controlled conditions, though useful to discriminate physiological responses, do not predict field flowering accurately. The possible effect of the first intron was much more evident under natural conditions, whereas the effect of the promoter was less marked (though significant) in the field. Wang et al. (2010) also found a new *HvFT1* haplotype conferred by a new SNP detected in the first intron at the *H. spontaneum* accession 'ISR42-8'. This allele conferred lateness in spring-sown field trials com-

pared to the allele contributed by a spring cultivar, but just by 1.9 days. This figure is closer to the effects detected in our experiments of 6–7 days, though for apparently different alleles.

The latitudinal distribution of *HvFT1* seems non-random, suggesting a role for its diversity in response to daylength. Under field conditions, the *TC* allele was earliest. This is the allele prevalent in southern Spain, where crop cycles are shorter and heading time occurs even in March. Besides, it is the region where temperatures rise in a more steep way during spring, and thus more prone to terminal water stress. The presence of the *TC* allele in southern barleys, which seems to hasten flowering under short photoperiods, would provide an additional mechanism of defense to induce flowering before temperatures rise too much and terminal stress damages the crop. The promoter carried by lines in the class *135-TC* seems to induce even more earliness, which would be consistent with their presence in the most southern latitudes, corresponding to the mildest winters (the only exception, a line collected above 40°N was actually a spring genotype).

The lines sequenced were representative of the three main populations out of the four identified in Spanish barleys (Yahiaoui et al. 2008). The 'Esterel' allele is present in the SBCC ('SBCC106') and the phenotypic effect of the *HvFT1* alleles for 'Esterel' × 'SBCC016' agree quite well with the differences found in the SBCC. Therefore, though we cannot claim to have captured all genetic and phenotypic diversity at *HvFT1* in the SBCC, the different sets of data (ecogeographic, association in the SBCC and linkage mapping in 'Esterel' × 'SBCC016') are coherent and present a credible picture of allelic diversity at *HvFT1* in the SBCC, with effect on flowering time and with a clear latitudinal distribution.

HvFT1 had a large effect on flowering time and less on time to stem elongation. This meant that most of this gene's effect on plant development was evident after the jointing stage, and it might be responding to different environmental cues than *VrnH1* (Turner et al. 2005; Cockram et al. 2007a; Hemming et al. 2008). The particular conditions of the winter sowings in this experiment, a realistic late sowing date for the region, featuring increasing day length and variable temperatures, may have resulted in a combination of environmental conditions causing a significant effect of *HvFT1* on flowering date. This effect may be of importance in explaining the adaptation role of this gene. The *TC* allele of *HvFT1* would confer earliness, at least in late sowings, which may be convenient for barley plants growing in midspring in Mediterranean climates to escape from rapidly rising temperatures and the risk of drought and heat stress.

The complex regulation of *HvFT1* suggested in this study actually agrees quite well with the latest findings in cereal and model species. Bonnin et al. (2008) also reported putative functional polymorphisms in non-coding intronic regions of orthologous genes *FTA* and *FTD* in wheat, although they did not examine the promoter regions. Interestingly, *FTA*, *FTD* and *HvFT1* have the same overall structure, and the regulatory control of these genes may be conserved among Triticeae. Intronic regions in vernalization response genes are important for repression before vernalization and might contain binding sites for repressor proteins (Trevaskis et al. 2007). There is also evidence for a role of polymorphisms at the promoter region in the regulation of the *FT* gene family in *A. thaliana* (Schwartz et al. 2009). So, the existing evidences point at the presence of regulatory polymorphisms in *FT* genes at the non-coding, intronic and promoter regions, at least.

A mechanism for the regulation of *FT* in *A. thaliana* has been recently put forward (Tiwari et al. 2010), involving two tandem binding sites for the CONSTANS protein in the proximal part of the promoter (denominated CORE sites), and at least four sites (known as 'CCAAT' boxes) for the union of NUCLEAR FACTOR Y/HEME ACTIVATOR PROTEIN/CONSTANS heterodimeric complexes, in a more distal region. Therefore, CONSTANS can act both as a DNA-binding transcription factor and as a co-activator. In this model, the level of expression of *FT* was found to be proportional to the number of occupied binding sites, as if the flowering signal accumulated along the promoter region. We screened the available barley sequences for these motifs to check whether this regulatory mechanism was conserved. While we could not find matches of CONSTANS proximal binding sites, up to six 'CCAAT' motifs were found in the barley promoter, in a 2,100 bp window, as shown in Figure S1. The four proximal 'CCAAT' boxes (3–6) were conserved in all genotypes analyzed, but interestingly two of the polymorphisms identified in this work mapped very close to boxes 1 and 2. In particular, SNP 1660 corresponds to the first nucleotide in box 2, and the polymorphism in position 962 is immediately after box 1. Both of them are in complete linkage disequilibrium with indel 1 for the genotypes shown in Fig. 1.

HvFT1 diversity revealed in this and previous studies may help to explain the classical descriptions on *VrnH3* distribution (or *Sh3*, by its old denomination). Reports of the presence of the dominant (spring) allele of this gene limited its range to low or high latitudes (Takahashi and Yasuda 1971) or high altitudes (von Bothmer et al. 2003). Actually, these studies seemed to focus on extremely early spring genotypes, characterized by the 'Tammi' *HvFT1* allele, also described in Yan et al. (2006). It seems sensible that very early cultivars occur at regions featuring very short growing seasons. But the diversity of this gene seems to extend beyond the classical spring/winter two-allele model. The present results widen the scope for the role of the diversity of *HvFT1* on barley adaptation, especially

among winter types. The diversity of polymorphisms and effects found at this gene may provide breeders with additional genetic variability to fine-tune plant development to local environmental conditions.

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