ANALYZING SEQUENCE POLYMORPHISMS AND *HvFT1* EXPRESSION IN A BIPARENTAL BARLEY POPULATION

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SUMMARY:

Time of flowering in barley has a large impact on yield, and many researches have focus on finding polymorphisms in genes underlying the adaptation of different cultivars to changing environmental conditions. Previous studies have identified three vernalization and two photoperiod genes, which have a major role in the determination of flowering. Other genes (*HvFT1*, *HvCEN, HvELF3* or *HvLUX)* also contribute to fine tune the time to heading within spring or winter barley cultivars.

In this study we use a doubled haploid population, derived from the cross of two spring two-row barley cultivars, Beka and Logan. Previous QTL analyses had identified three major QTL controlling flowering time in this population. The aim of this study was to analyze the expression of three genes, *HvELF3*, *HvCEN* and *HvFT1*, candidates for the major QTLs segregating in this population. Gene expression was analyzed in eight doubled haploid lines with contrasting alleles for these genes. Plants with the Logan allele in the three genes flowered the earliest. Expression of HvFT1 was dependent on the genetic background. Higher HvFT1 expression, and earlier flowering, was detected in plants with the Logan allele in the three genes. Most of the results were coherent with the previous literature.

RESUMEN:

La fecha de floración en la cebada tiene gran impacto en la producción de las cosechas y por tanto, existe un interés por conocer los polimorfismos de los genes que están detrás de la adaptación de diferentes cultivares a ambientes con condiciones ambientales muy diversas. Estudios previos han identificado tres genes de vernalización y dos de fotoperiodo, que juegan un papel importante en la determinación de la fecha de floración. Otros genes, (*HvFT1*, *HvCEN, HvELF3* o *HvLUX)* también contribuyen a este fenómeno, tanto en variedades de primavera, como en las de verano.

En este estudio hemos usado una población biparental, que deriva del cruce entre dos variedades de primavera, Beka y Logan. A partir de análisis de QTLs se han identificado tres QTL que controlan la fecha de floración en esta población. El objetivo de este estudio era analizar la expresión en los tres genes, *HvELF*, *HvCEN* y *HvFT1*; principales candidatos de los QTLs identificados. Se analizó la expresión en las ocho líneas de la población, las cuales, tenían diferente combinación de alelos cada una. Por un lado se vio como las plantas con alelos de Logan florecían antes. Además, las plantas que tenían alelos de Logan para los tres genes, presentaban mayor expresión de *HvFT1* que el resto. La mayoría de los resultados que se obtuvieron fueron coherentes con los observados en otros estudios similares.

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1. INTRODUCTION:

Barley (*Hordeum* vulgare L.) is a major cereal grain known from ancient times. It was one of the first grains in the Fertile Crescent, in Western Asia and near the Nile River. 10,000 years ago the crop was domesticated from its wild relative *H.vulgare ssp. spontaneum.* (1)

Nowadays, barley ranks fourth in importance among cereals after wheat, maize and rice. The main producing countries worldwide are Russia, Ukraine and France. Spain has an important production on a worldwide scale. In 2012, the area harvested was 2.676.200 ha and the production was 5,976,900 tons. (2)

The reason for its importance is due to its wide ecological adaptation, including extreme latitude and altitude. This is the result of a long domestication process started centuries ago. The crosses between efficient cultivars led to new varieties that could be adapted to new regions with diverse climatic conditions.

At the beginning, the improvement of barley cultivars was made by observing the phenotype and selecting the best seeds. Nowadays, the use of molecular tools allows to associate physical traits with the genotype, and facilitates the obtaining of varieties with desirable characteristics for specific regions and assures bumper crops under very different conditions.

In this project we studied the relation between some recently described QTLs and the date of flowering in a barley population. By quantitative PCR we were able to follow the expression of some genes which are part of the flowering process.

2. BACKGROUND

Barley is diploid species with 14 chromosomes. It has been considered as an important model organism for genetic studies. Its diploid genome has facilitated the study of other crops such as wheat or rye.

2.1. Domestication of barley:

During domestication of barley cultivars, farmers selected seeds with favorable traits, and over time, they converted wild species in bountiful crops. This transition started around 10 000 years before present and has led to an accumulation of characteristics that facilitate agricultural production. The most important traits acquired are non-brittle rachis, an increase in the size and number of seeds, changes in plant architecture, improved seed fertility, loss of seed shattering, and adaptation of flowering time to different areas. (1)

Barley can be classified in different groups:

- Two-rowed and six rowed barley: Depending on the development of lateral kernels. Two-row barley has lower protein content, so it is usually used for malting, while six-row barley, with a higher protein content is best suited for animal feed.
- Hulled and “naked” seeds: Based on the seed covers. Naked barley has an outer hull easier to remove and generally falls of during harvesting.
- Spring and winter barley: Depending on the growth habit. Winter barley is planted in autumn because it requires a cold treatment before being competent to flower. Spring barley is planted in different times of the year, depending on the region.

All these diverse characteristics appeared due to the unconscious selection of mutations during the domestication process.

2.2. Flowering of barley:

Flowering time, as explained in the introduction, depends on the vernalization requirement of a cultivar, and its photoperiod sensitivity. As seen before, barley can be classified in two types: spring and winter barley. In winter varieties, prolonged exposure to cold accelerate flowering. It occurs during winter, when temperatures do not exceed 10ºC. This phenomenon is called vernalization. These cereals are planted in autumn, whereas spring varieties are planted in different times of the year and do not have vernalization requirement. (3)

The spring growth habit appeared after domestication of winter varieties. Independent mutations led to phenotypes that did not require vernalization, this modification converted flowering time in a highly variable phenotype (4).

Remains of the first barley crops were found in the Fertile Crescent, and over time, varieties without the need for an extended period of cold were extended to regions with severe winters, leading to a great variety of cultivars that are

suitable for different geographic regions and climatic conditions. Cultivars from regions with mild winters, have an intermediate vernalization requirement, alleles from these cultivars might offer advantages in areas like the Mediterranean region, were winter is not so cold (5).

2.3. Vernalization genes:

The genes underlying the flowering process are:

- *Vrn-H1* gene encodes a MAD-box transcription factor which regulates the transition of the vegetative shoot apical meristem to the reproductive phase. It is induced by vernalization, and it depends on the length of the cold treatment, the longer is the exposure, the greater is the effect. (6, 7)
- *Vrn-H2* is a floral repressor. It encodes a protein with zinc-finger motif, which mediates DNA binding. The main role of *Vrn-2* is to block flowering in long days, by repressing *Vrn-3*. (6). It is under the control of circadian-clock. (7)
- *Vrn-H3* seems to be an orthologous of the *Arabidopsis thaliana FLOWERING LOCUS T* gene (FT). (8, 9) *Vrn-3,* or *HvFT1*, is a promoter of flowering that encodes a phosphatidylethanolamine-binding protein (PEBP) (8). It is induced by long days and interacts with the *PHOTOPERIOD1* gene (*Ppd-H1*). *Ppd-H1* is a pseudo-response regulator gene expressed with a diurnal pattern; it moderates day-length induction of *HvFT1* by controlling *CONSTANS* (*CO*) activity. (3)
  Different alleles of the *HvFT1* gene have polymorphisms in the promoter and first intron. In the promoter, the haplotypes differ in nine linked SNPs and two indels (8, 10). Two SNPs in the first intron, TC or AG, are associated with differences in flowering time (8, 10). These regions of the gene are presumably important for the repression of *HvFT1*, and might contain a binding site for the Vrn-2 protein. (3)
  In spring cultivars, *HvFT1* can carry more than one copy of the gene (11, 12). Increased copy number has been associated with early flowering (11).

Spring barley has evolved from the ancestral winter growth habit. In addition to allelic variation in *HvFT1*, deletions at *Vrn-2* and, different alleles at *Vrn-1*, resulting from deletions of a cis-regulatory motif in the first intron of this gene confer spring behavior. (13).

2.4. Interactions between *Vrn-H* genes:

Flowering is explained by a model that integrates vernalization and photoperiod pathways. In general, barley planted in late summer or fall does not flower before winter because both vernalization and day length response pathways are inactive. *Vrn-1* expression is low and *Vrn-2* activity represses the possible

induction of *HvFT1*. *Vrn-1* is slightly up-regulated during winter, due to low temperatures, and then represses *Vrn-2*. Low levels of *Vrn-2* transcripts, add up to the increase of the day-length, cause high levels of CO protein and favor the activation of *HvFT1*. This promotes consecutive stages of reproductive development. Flowering occurs in spring or early summer (Figure 1). (3)

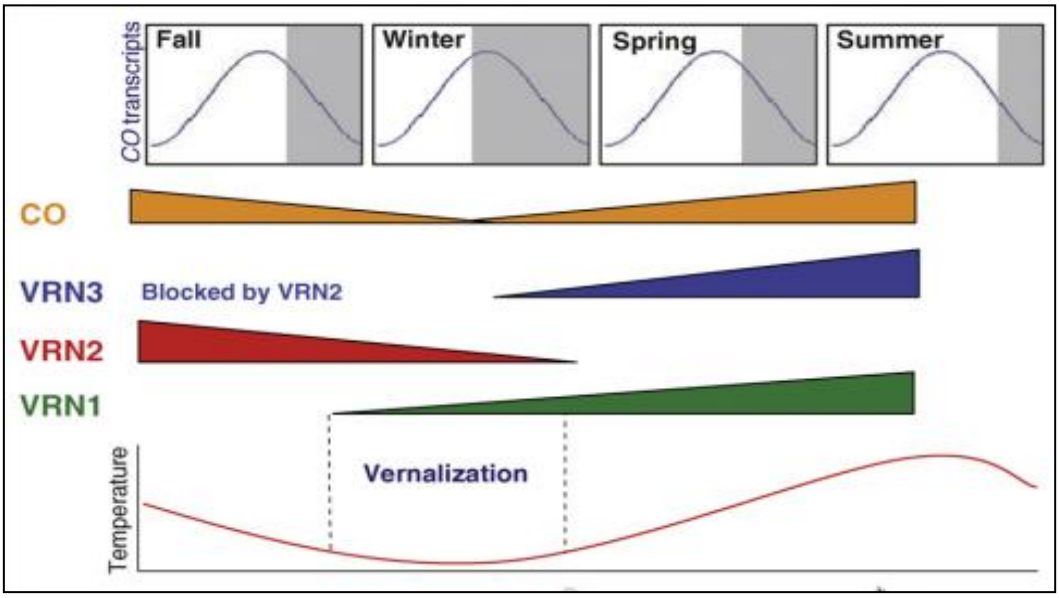

Figure 1. *Expression levels of the Vrn-H genes during time. The red line represents the seasonal variation in temperature. Rectangles on top indicate the duration of light (white) and dark (grey) in each season.* Taken from Distelfeld et al. (2009)

2.5. Photoperiod response:

The photoperiod pathway is also involved in the flowering process. The circadian clock provides diurnal and seasonal control of flowering. Two genes have been identified and characterized:

- *Ppd-H1*, mentioned above, up-regulates FT1 expression, which is mediated by the activity of CO. Both genes are regulated by transcriptional outputs from the circadian clock (Figure 2). The recessive allele of this gene, *ppd-H1*, delays flowering under long days and shows reduce expression of FT1 (14).
- *Ppd-H2*, encodes a distinct PEBP, *HvFT3*, and promotes flowering under short day conditions (5).

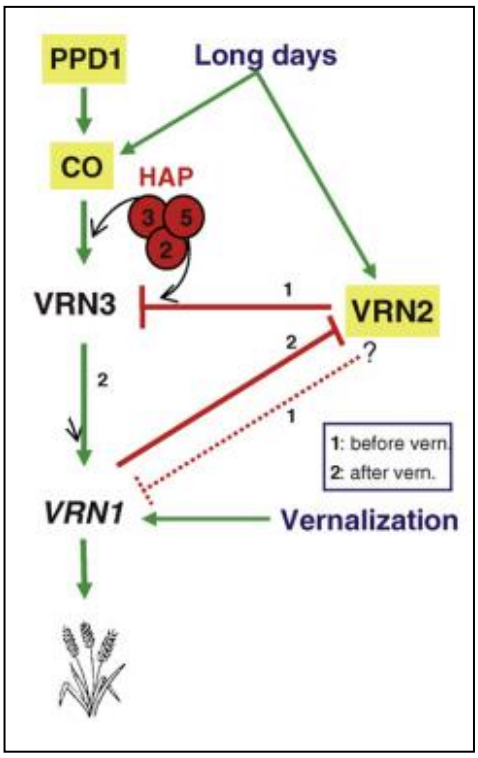

Figure 2. *Model of flowering that integrates vernalization requirement (Vrn-H1, Vrn-H2) and photoperiod response (Ppd-H1, CO). Vernalization induces Vrn-H1 under both long or short days, which repress Vrn-H2.*
Vrn-H3 responds to photoperiod signals by interacting with trimeric HAP2/3/5 complexes. Taken from Distelfeld et al. (2009)

Other components of the circadian clock have been related with the photoperiod response and its effect in flowering. The gene *EAM8* was identified as a barley orthologous of the *A. thaliana* circadian clock regulator *EARLY FLOWERING 3* (15, 16). There is strong evidence that mutations on this gene cause early flowering phenotype in plants, by increasing the expression of *FT1*, but this process is independent of allelic variation at *Ppd-H1*.

2.6. Importance of early flowering:

The development of DNA markers facilitates the characterization of quantitative trait loci (QTL), regions within genome that contain genes related with a particular quantitative trait.

DNA markers have an enormous potential in plant breeding, and have been used to determine regions underlying time to flowering.

Other studies identified the location of the genetically assigned *Earliness per se* locus *Eps2* (or *Eam6*) on chromosome 2H, one of the major determinants of flowering time under Mediterranean conditions (17, 19). The region predicted contains more than 300 genes, and by screening all genes within the interval, candidates for EPS2 were identified. One of these is *HvCEN*, a homolog of *Antirrhinum CENTRORADIALIS* (18). Two main alleles related with a winter and spring growth habit, have been identified. The difference between the alleles consists in a non-synonymous substitution. The haplotype Pro135 is found in cultivars with winter growth habit while haplotype Ala135 is linked with the spring growth habit. This gene is considered as a contributor to environmental adaptation (18).

3. PREVIOUS RESULTS:

This study is based on a spring x spring barley population, Beka x Logan, previously analyzed for flowering time under Mediterranean climate (19). QTL analyses for date to heading using data from three field trials identified only one major QTL on chromosome 2H, in the region of *Eam6* or *Eps2*. Lately, we identified polymorphism for *HvFT1* between the parents of the population. This gene and three other markers in the same region were analyzed in the whole population, allowing a better definition of chromosome 7H (Figure 3).

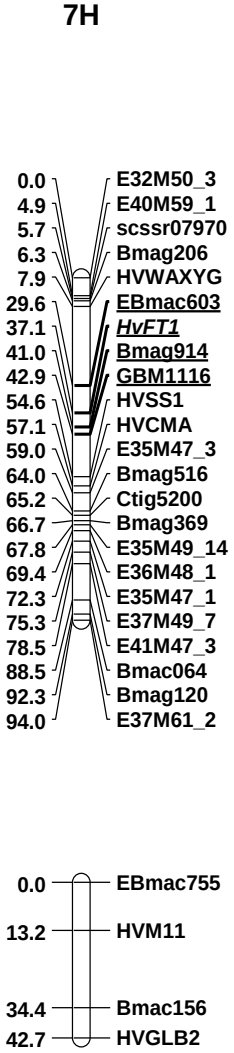

Figure 3. *Genetic map of two linkage groups corresponding to chromosome 7H of the population Beka x Logan. Underlined are those new markers incorporated to the previous map.*

The population had been evaluated in four more trials, besides those included in the previous publication (19). QTL analysis for heading date was carried out again, with the new information and it showed three major QTL on linkage groups 2 (Ctig4047, 1HL), 3 (Ctig1158, 2H), and 10 (HvFT1, 7H) where the early allele was provided by Logan. There were other three minor QTL on linkage groups 5 (3H), 7 (HvM30, 5H), and 10 (7H) where the early allele was that of parental Beka (Figure 4).

The three major QTL were coincident with the position of known flowering time genes, i.e. *HvELF3* on 1H, *HvCEN* on 2H and *HvFT1* on 7H. Logan contributed the earlier allele in the three cases.

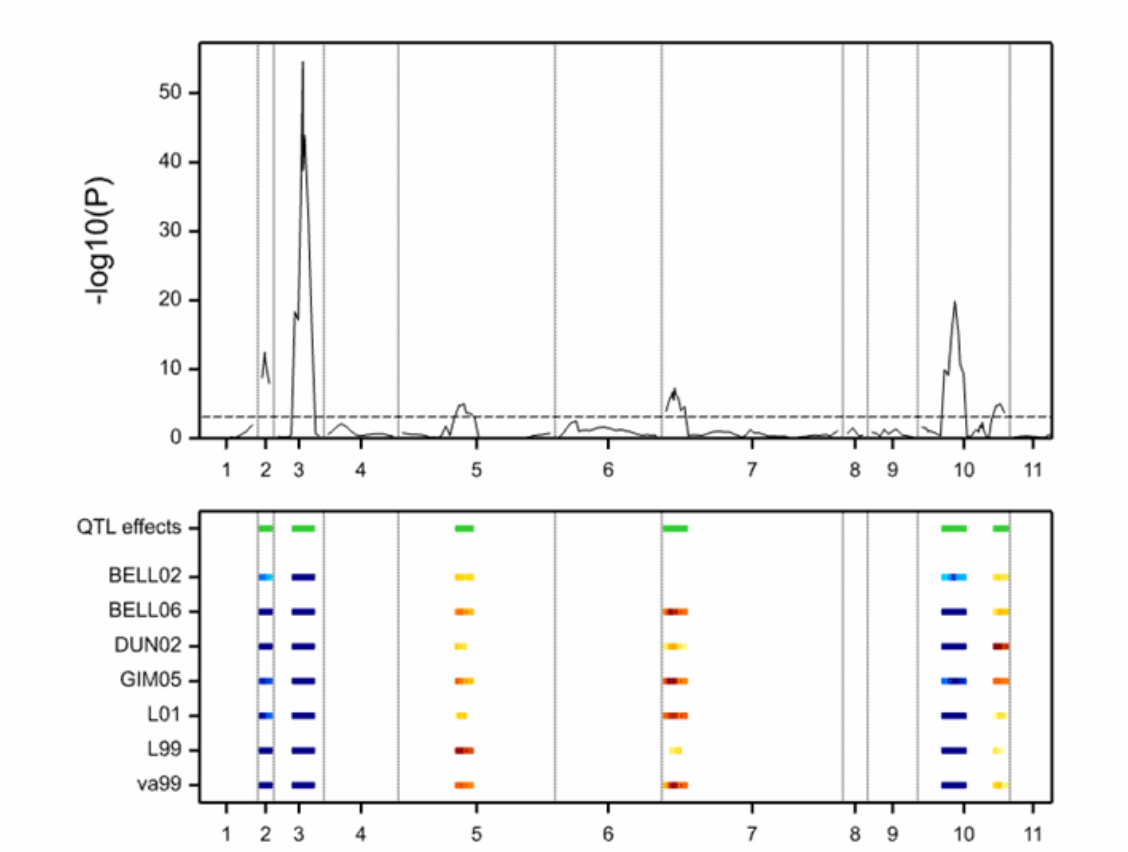

Figure 4. *Multi-environment QTL analysis for days to flowering for seven field experiments carried out with 102 doubled haploid lines and the parents of the population Beka x Logan. The peaks above the threshold (dashed line) indicate presence of QTL significantly affecting the trait. In the lower part of the figure, field trials are coded with the name of the location (Bell – Bell-lloch, Dun – Dundee, Gim – Gimenells, L – Lleida, Va – Valladolid) and two digits for the year; the coloured dashes indicate the extent of the QTL and its direction: blue means that the early allele came from Logan, yellow-brown from Beka, with intensity proportional to the size of the effect.*

Analysis of variance revealed clear effects of *HvCEN* (Ctig1158), *HvELF3* (Ctig4047), *HvFT1* and HvM30 across all trials. There was just one interaction of *HvCEN* (Ctig1158) and *HvELF3* (Ctig4047) and also two significant triple interactions, both involving *HvCEN* and *HvELF3* (not shown).

4. OBJECTIVES:

The aim of this project is to:

- Analyze the expression of the genes: *HvFT1*, *HvCEN* and *HvELF3*, in the parents and selected doubled haploid lines of the Beka x Logan population.
- To find out whether the expression of the genes is related to the time to flowering of the lines.
- To test whether the expression of a given gene has influence on the others.

5. MATERIALS AND METHODS:

5.1. Plant material:

Doubled haploid lines were obtained from the F1 of the Beka x Logan cross by another culture. Beka is a top-quality malting variety. It is well adapted to Mediterranean environment and extensively cultivated in Spain, while the malting variety Logan is a North American two-row cultivar. (19)

Beka and Logan carry the same *VrnH1-1* allele, both are null for *VrnH2,* have the recessive allele *ppdH1* and carry the dominant *PpdH2* allele. The parents of this population differ in *HvELF3* (Logan has a mutant allele, with many polymorphisms in the coding region); *HvCEN* (Beka has the haplotype III, typical of spring cultivars, whereas Logan has the haplotype I, found also in cultivar Morex); and *HvFT1* (Beka has 2 copies of the gene, late promoter, and TC in the first intron; Logan has 1 copy of the gene, early promoter and TC in the first intron).

Besides the parents, eight DH lines with contrasting alleles at these genes were selected for this study (Table 1). The eight DH lines have in common the Beka allele for HvM30, related with early flowering.

Table 1. *Haplotypes for markers closest to major flowering time genes for selected doubled haploid (DH) lines of the population Beka x Logan.*

HvELF3 **(Ctig4047)**	***HvCEN*** **(Ctig1158)**	**HvM30**	***HvFT1***	**Genotype**
A	A	A	**A**	Beka
B	B	B	**B**	Logan
A	A	A	**A**	2814
A	A	A	**B**	2785
A	B	A	**A**	2796
A	B	A	**B**	2799
B	A	A	**A**	2775
B	A	A	**B**	2779
B	B	A	**A**	2771
B	B	A	**B**	2820

5.2. Plant growth conditions:

Glasshouse experiments were planted on October 15th. Seeds of each genotype were sown in three pots, and every pot was placed in a tray. Therefore, each tray had a pot of each genotype.

Plants were grown under controlled conditions: Glasshouse had an average temperature of 20.5ºC. Supplementary lighting was provided to assure a long-day photoperiod (16h light, 8h dark).

As the objective of the study was to compare the expression of the genes under identical conditions, it was important to guarantee that all pots received the same amount of water during irrigation, as well as exposure to light and cooling was equal. The position of the pots in the tray was often changed to avoid the influence of environmental factors on the expression of genes. Phenotypic observations were time to flag leaf expansion and date of awns appearance. Dates were registered as shown in Figure 5.

To sample, we chose plants with a similar growth. Leaf segments from the last expanded leaf were harvested in 2 different dates:

- 23 days after sowing (7-Nov)
- 38 days after sowing (22-Nov)

The genes under study are affected by the circadian rhythm. For this reason, samples were taken 14 hours after the beginning of the light period. In the first date, two plants from every pot, from trays one and three were sampled, while in the second date, the plants used came from trays two and three. For expression analyses we used at least three biological replicates for each data point.

5.3 RNA isolation:

For homogenization we used Mixer Mill model MM400, Retsch. The isolation was carried out following the NucleoSpin® RNA Plant kit (Macherey-Nagel), at room temperature. (Table 2)

As RNA is very sensitive to temperature, the samples were kept frozen at -80ºC. To assure the isolation was performed successfully, quality was visualized by agarose gel electrophoresis, and concentration of RNA was measured using Nanodrop Spectrophotometer.

Table 2. *RNA isolation protocol.*

1 Homogenize sample	Grind up under liquid N_2
2 Lyse cells	Add 450 µL Buffer RA1
3 Filtrate lysate	Load NucleoSspin filter. Centrifuge 5min, 11000 rpm
4 Adjust RNA binding conditions	Add 400 µL ethanol 70% to the lysate
5 Bind RNA	Load lysate in the NucleoSpin RNA Plant Column and centrifuge 30 s, 11000 rpm

6 Desalt silica membrane	Add 350 µL MDB and centrifuge 1 min, 11000 rpm
7 Digest DNA	Add 95 µL rDNase reaction mixture Incubate at room temperature for 15 min
8 Wash and dry silica membrane	1st wash: Add 200 µL Buffer RAW2, centrifuge 30 s, 11000 rpm 2nd wash: Add 600 µL Buffer RA3, centrifuge 30 s, 11000 rpm 3rd wash: Add 250 µL Buffer RA3, centrifuge 2 min, 11000 rpm
9 Elute RNA	Add 20 µL RNase-free H_2O Centrifuge 1 min, 11000 rpm Add 10 µL RNase – free H_2O, centrifuge 1 min

5.4 cDNA synthesis:

cDNA was synthesized starting from 1µg of total RNA and using the SuperScript III reverse transcriptase enzyme (Invitrogen).

Table 3. *cDNA synthesis protocol*

RNA(1 µg) + H_2O	12 µL
50 µM Oligo dT_{20}	1 µL
10 mM dNTPs	1 µL
Centrifuge Incubate at 65ºC for 15 min. Incubate in ice for 2min Centrifuge	
5x Buffer RT	4 µL
0,1M DTT	1 µL
SuperScript III	1 µL
Total volume	20 µL

The concentration of single stranded DNA was measured in Nanodrop.

5.5 PCR quantification:

Quantification was achieved using ABI7500 Real-Time PCR System (Applied Biosystems) and SYBRGreen Flourescence Chemistry (2X Power SYBR Green Supermix, Thermo Fisher Scientific) was used. To calculate relative mRNA abundance we used as housekeeping gene *Actin*, and the three genes under study (*HvFT1*, *HvELF3* and *HvCEN*). For each biological replicate, we employed two technical repeats.

Table 4. *qPCR protocol.*

cDNA (250 ng/ µL)	1 µL
H_2O	4 µL
2x Syber Green	10 µL
Mix Primers F+R, 2 µM each	5 µL
Total volume	20 µL

Table 5. *Primer efficiencies*

Actin	0,97
HvFT1	0,86
HvCEN	0,96
HvELF3	0,95

The threshold cycle (Ct), at the beginning of the exponential phase, was recorded. We used the average of the two technical repeats for calculations.

Results were treated with the ΔCt method, taking into account the efficiency (E) of the primers. We used the next equation, were the "Ct" is the average Ct for the gene, and the "gene" can be any of the three genes in the study: *HvFT1*, *HvCEN* and *HvELF3*.

$$\frac{(1+Eact)^{Ct\ act}}{(1+Egene)^{Ct\ gene}}$$

5.6. Statistical analysis

Results were compared in Excel.

Analysis of variance for differences among marker alleles and their interactions in field and greenhouse experiments were performed using mixed models procedures implemented with REML (residual maximum likelihood), in package Genstat 15.0 (23). Markers were considered fixed effects, environments in the field experiments and replicates in the greenhouse experiments were consider random.

6. RESULTS:

HvFT1 has been described as floral integrator of photoperiod and vernalization. The expression of the gene has been related with early flowering, so cultivars that flower earlier are expected to have higher expression of *HvFT1*. The first association of *HvFT1* haplotype with differences in flowering time was reported by Yan et al (6), predicting that cis regulatory sites on the promoter and first intron might be important for transcriptional regulation. Recent studies describe however, incoherent results for haplotype-phenotype relation. They suggest that not only the haplotype is important for the growth habit, but also the copy number of *HvFT1*. (9)

6.1 Phenotypic observations:

Logan is earlier than Beka, so plants with Logan alleles are supposed to flower before those with Beka alleles (19). The time to flag leaf expansion and awn emergence, support the above affirmation. Logan's flag leaf was expanded two days before that of Beka, and also the awns appeared earlier (Figure 5). There was a relation between the genetic constitution of the doubled haploid lines and their phenotypes; those with a majority of Logan alleles flowered before those with a majority of Beka alleles Besides, all lines with the Logan allele in Ctig4047 (2775, 2779, 2771, 2820) were the earliest to flower.

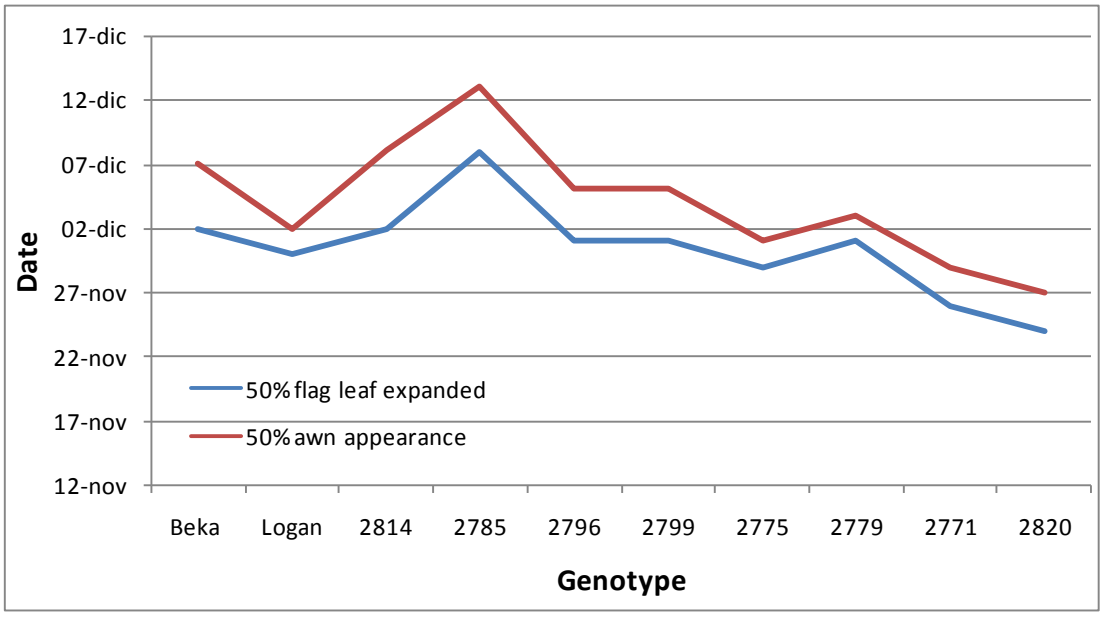

Figure 5. *Days to flag leaf expansion and awns appearance.*

6.2 Expression of *HvFT1*

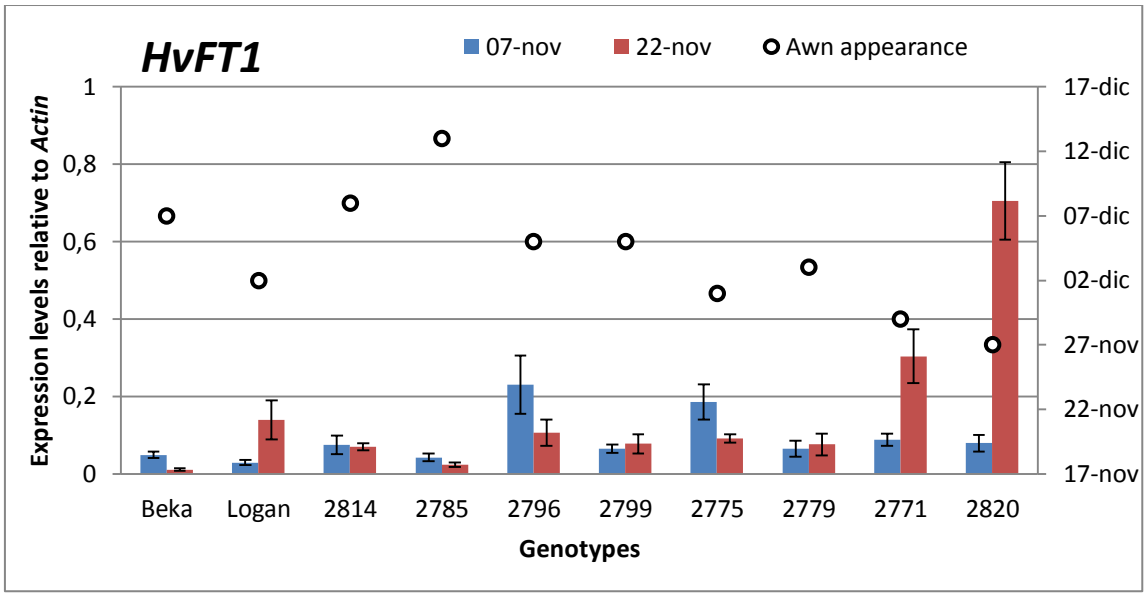

Figure 6. *Expression of HvFT1 normalized to Actin*.

Analyzing the expression of *HvFT1* (figure 6) in the second sampling, plants with higher expression of *HvFT1* flowered earlier, in particular DH lines 2771 and 2820. Those plants that flowered later showed lower *HvFT1* expression.

There is no apparent relation between the expression of *HvFT1* and the allele they carry.

6.3 Expression of *HvCEN*

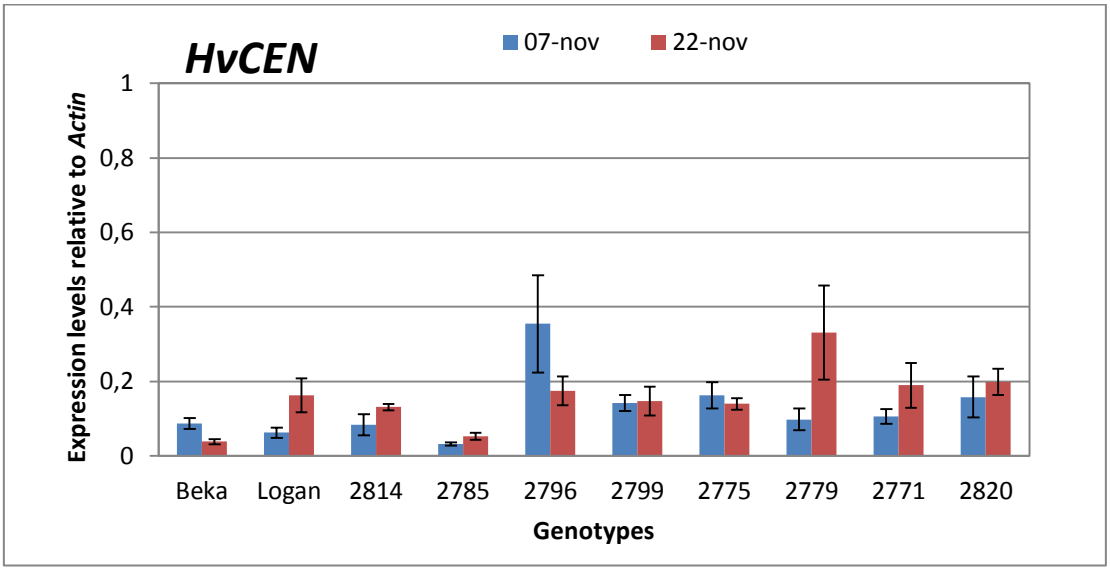

Figure 7. *Expression of HvCEN normalized to Actin.*

The expression of *HvCEN* was consistent considering the parents. Logan had more expression in the second sampling than Beka, and it also flowered earlier.

However, looking at the doubled haploid lines, expression pattern has no apparent link with the time to flowering. In the second sampling date, lines with more *HvCEN* mRNA levels do not always coincide with earlier plants.

6.4 Expression of *HvELF3*

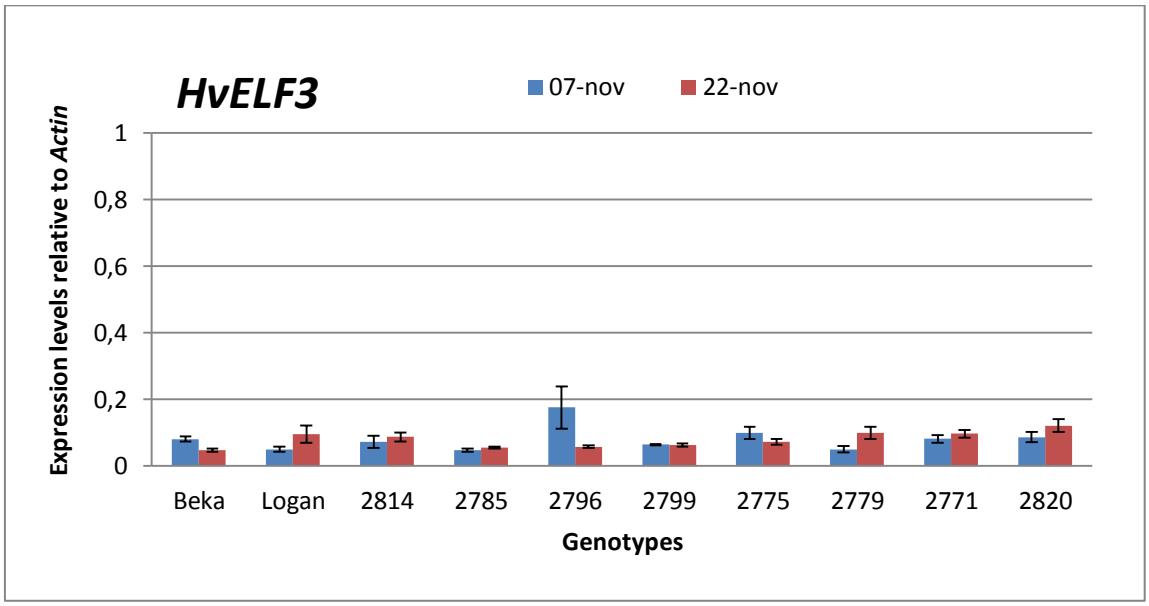

Figure 8. *Expression of HvELF3 normalized to Actin.*

The expression of *HvELF3* was very similar in all plants. No apparent difference can be suggested based on the Ctig4047 allele they carry.

We also tested possible interactions between the expressions of the different genes by analysis of variance. No differences were detected for any of the analyzed genes in the first sampling date. In the second sampling date, there were differences for the expression of *HvFT1*, according to the *HvELF3* and *HvCEN*. Plants with Logan alleles in those markers showed higher *HvFT1* expression. Also, two significant interactions were detected, one between the alleles of *HvELF3* and *HvCEN* (P=0.028) and another one between the alleles of *HvCEN* and HvFT1 (P=0.004). In both cases, plants with the Logan alleles in those markers showed higher *HvFT1* expression.

7. DISCUSSION:

The three analyzed genes in the project have been proposed to be good candidates for the QTLs detected and described before. In different researches, they have been reported to influence in the time to flower.

Nowadays, it can be assumed that the gene *HvFT1* responds to environmental signals and they affect flowering time. Polymorphisms affecting both the promoter and the intron have wide range of effects in flowering time; those that confer earliness development usually have higher levels of expression. However, as reported in Loscos et al. (12) we cannot consider this gene as an individual factor affecting flowering time, but we also have to bear in mind the genetic background.

Beka and Logan have different haplotypes, 139-142-TC y 135-146-TC and number of copies (2 vs 1), respectively. Plants with the Logan allele flower earlier than those with the Beka allele. Logan has a different promoter, similar to that of SBCC145 (22). This promoter clearly results in earlier flowering, as we saw in some landraces from the Spanish barley core collection (10) and also in the SBCC145 x Beatrix population (22). Beka also contributes something to earlier flowering on chromosome 5H, marker HvM30.

What we see in the expression analysis is that the highest expression of the gene *HvFT1* is found in the earliest plants (2771 and 2820). Nevertheless, the *HvFT1* alleles of these plants are one from Beka and the other one from Logan. If *HvFT1* was the main determinant of flowering, genotypes with the Logan allele for *HvFT1* gene were expected to flower before, these are: 2779, 2785, 2799 and 2820. As it can be seen on Figure 6, this does not occur, 2820 is the genotype that flowered the earliest, but the three others mentioned flowered later than genotypes with the *HvFT1* Beka allele. As we said before, it is important to consider the genetic background.

Besides that, it has been reported copy number variation in *HvFT1*, suggesting that can lead to higher expression in those genotypes that have more copies. Beka has two copies of the gene and Logan one. In the Beka x Mogador population, (12) there was a QTL and a slight higher *HvFT1* expression in plants with the Beka allele, with 2 copies of the gene, whereas Mogador has only one copy. In this study, the allele from Logan, with just one copy, but a different promoter, was associated with earlier flowering. Apparently, there is no evidence that copy number variation affects the expression of *HvFT1* in the Beka x Logan population.

Apart from *HvFT1*, the expression of two other genes is also analyzed. *HvELF3* and *HvCEN* have been considered the best candidates for two QTLs described in the previous results: Ctig4047 is on the long arm of chromosome 1H, very

close to Mat-a, or *HvELF3* (16). Ctig1158 maps near the centromere on 2H, very close to the position of Eam6, *HvCEN* (18).

For the gene *HvCEN,* fourteen SNPs were identified that defined thirteen haplotypes. Logan carries haplotype I and Beka haplotype III of the gene (18). They differ in two SNPs within an intron and, both have the same amino acid in position 135, Ala. Apparently; the difference in nucleotide sequence does not explain the different flowering behavior.

Logan carries the same *HvCEN* allele as cultivar Morex. In the Steptoe x Morex population a QTL was detected on 2H, in the region of Eam6. It showed QTL x environment interaction, but the early allele seemed to be the one from Morex (21). In the Beka x Mogador population, Eam6 was the major determinant for flowering time in autumn sowings (17). The parents of that population carry the two contrasting haplotypes III (Beka) and II (Mogador), as reported by Comadran et al. (18). The Mogador allele of HvCEN (haplotype II) was associated with earlier flowering (12). The earliest plants in that study were those with this haplotype. A comparison of selected DH lines from this population showed a slight difference in flowering time and higher expression in plants that contained alleles from Beka in *HvFT1* and *HvCEN.* The alleles were different from those in Beka x Logan, so no comparison can be made. In any case, the genotypes in our study that carry Beka allele for both genes are 2814 and 2775, and they are not earlier than the others (Figure 5). On the other hand, in the second sampling date, an analysis of variance showed a significant interaction between *HvFT1* and *HvCEN* also in this population. Plants with the Logan allele in *HvCEN* had higher *HvFT1* expression than those with the Beka allele (2796 and 2799 vs 2814 and 2785; or 2771 and 2820 vs 2775 or 2779). Our results also suggest that *HvCEN*, or a closely linked gene, is the responsible of the 2H QTL.

The most evident relation between genotype and phenotype is when we compared the expression of *HvELF3* and the effect of both alleles in the time to flower. Many alleles has been described for *HvELF3*, the variety Mari has a 4-bp deletion in exon 2 (mat-a.8) and Early Russian (eam8.w) a C-to-T point mutation in exon 2, both of which would encode a truncated protein (15); a large rearrangement of the gene was reported in eam8-k (16) The mutations in this gene give early flowering and photoperiod insensitivity. Several authors have reported that mutant alleles showed higher *HvFT1* expression (7, 15, 16, and 20). Logan has an allele that differs from those reported. What we saw in the results (Figure 8) is that those plants that flowered first; 2771, 2775, 2779 and 2820 are those with the Logan allele for the gene *HvELF3* (marker Ctig4047). Those plants also have higher expression for *HvFT1*. These results suggest that the alleles of *HvELF3* may have influence on the expression of *HvFT1* and therefore in the time to flower, as it has been previously reported.

Expression of Hv*ELF3* is very similar in all plants, independently on the date they flowered. Maybe the expression levels do not have big influence in flowering, and the effects are due to the change in the protein sequence that affects the pathway somehow. *HvELF3* is a circadian clock gene that contributes to photoperiod-dependent flowering in plants. Wild type ELF3 represses the activity of core circadian clock genes and it is essential for maintaining correct diurnal expression patterns. A significant interaction was found between *HvELF3* and *HvCEN*. Future studies could investigate whether the proteins encoded by *HvELF3* and *HvCEN* are part of the same pathway.

In summary, what we can conclude is:

- We verified, as in previous studies, that *HvFT1* expression increase in plants that have an earlier development, although we could not demonstrate any connection between the alleles of this gene and the phenotype.
- Logan alleles for Ctig4047 and Ctig1158 have big influence in the time to flower, compared with the other alleles for the other genes, even if the expression pattern does not differ among plants.
- There are two significant interactions found between Ctig4047 x Ctig1158 and Ctig1158 x *HvFT1*, which have to be investigated in more detail.
- Our results suggest that *HvCEN* might be the gene responsible for the QTL identified close to Ctig1158, and that *HvELF3* is the gene under Ctig4047.

The work reported here is a small example of research in how some genes can affect time to flowering in barley, which has a big impact on yield. These researches are useful as long as they discover important breeding targets on barley that could be used by plant breeders to assure efficient crops in regions with very different environmental conditions.

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