Expression analysis of vernalization and day-length response genes in barley (*Hordeum vulgare* L.) indicates that *VRNH2* is a repressor of *PPDH2* (*HvFT3*) under long days

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Abstract

The response to vernalization and the expression of genes associated with responses to vernalization (*VRNH1*, *VRNH2*, and *VRNH3*) and photoperiod (*PPDH1*, *PPDH2*) were analysed in four barley (*Hordeum vulgare* L.) lines: 'Alexis' (spring), 'Plaisant' (winter), SBCC058, and SBCC106 (Spanish inbred lines), grown under conditions of vernalization and short days (VSD) or no vernalization and long days (NVLD). The four genotypes differ in *VRNH1*. Their growth habits and responses to vernalization correlated with the level of expression of *VRNH1* and the length of intron 1. 'Alexis' and 'Plaisant' behaved as expected. SBCC058 and SBCC106 showed an intermediate growth habit and flowered relatively late in the absence of vernalization. *VRNH1* expression was induced by cold for all genotypes. Under VSD, *VRNH1* expression was detected in the SBCC genotypes later than in 'Alexis' but earlier than in 'Plaisant'. *VRNH2* was repressed under short days while *VRNH1* expression increased in parallel. *VRNH3* was detected only in 'Alexis' under NVLD, whereas it was not expressed in plants with the active allele of *VRNH2* (SBCC058 and 'Plaisant'). Under VSD, *PPDH2* was expressed in 'Alexis', SBCC058, and SBCC106, but it was only expressed weakly in 'Alexis' under NVLD. Further analysis of *PPDH2* expression in two barley doubled haploid populations revealed that, under long days, *HvFT3* and *VRNH2* expression levels were related inversely. The timing of *VRNH2* expression under a long photoperiod suggests that this gene might be involved in repression of *PPDH2* and, indirectly, in the regulation of flowering time through an interaction with the day-length pathway.

Keywords: barley, gene expression, landraces, Mediterranean conditions, photoperiod, vernalization.

Introduction

The classic model of the genetic control of **vernalization** in barley (Takahashi and Yasuda, 1971) is based on three loci, *Sh*/*sh*, Sh_2/sh_2, and Sh_3/sh_3, among which epistatic relationships exist. Candidate genes for these three loci in barley have been proposed. *HvBM5A* (which corresponds to *TmAP1* or *WAP1* in wheat) was identified as a candidate for *VRNH1,* which is a synonym of Sh_2 (Danyluk *et al*., 2003; Trevaskis *et al*., 2003; Yan *et al.,* 2003). *VRNH1* promotes the transition of the apex from the vegetative to the reproductive stage. The locus is always expressed at high basal levels in plants that have spring (dominant) alleles (Trevaskis *et al*., 2006). In winter varieties that are responsive to vernalization, *VRNH1* expression is repressed until the plants are exposed to low temperatures (von Zitzewitz *et al*., 2005; Sasani *et al*., 2009). Allelic diversity at *VRNH1* has been described, mostly in relation to deletions within the first intron (Fu *et al*., 2005; Cockram *et al*., 2007; Szűcs *et al*., 2007). These deletions presumably cause variation in the levels of *VRNH1* expression in plants that have not been vernalized (Hemming *et al.,* 2009) and hence lead to different flowering times (Trevaskis *et al.,* 2003; von Zitzewitz *et al*., 2005).

A cluster of three genes, *ZCCT-H*, was identified as a candidate for *VRNH2*, which is synonymous with *Sh* (Yan *et al*., 2004). *VRNH2* acts as a repressor of flowering and delays flowering in plants that have not been vernalized (Takahashi and Yasuda, 1971; Yan *et al.,* 2004; Karsai *et al.,* 2005).

The allelic variation at the *VRNH2* locus seems to be of the presence/absence type, although there is still debate over which of the three *ZCCT-H* genes is functionally responsible (Dubcovsky *et al*., 2005; Trevaskis *et al*., 2006, Szűcs *et al*., 2007). The spring *VRNH2* allele is associated with a deletion of the three genes of the *ZCCT-H* cluster (Yan *et al*., 2004; Karsai *et al*., 2005; von Zitzewitz *et al*., 2005). Studies have found that in cereals *VRN2* expression is repressed by short days and by a high level of *VRN1* expression (Loukoianov *et al.,* 2005; Trevaskis *et al.,* 2006), which explains the long-known interaction between these two genes (Tranquilli and Dubcovsky, 2000).

HvFT1 is a candidate gene for *VRNH3* (*Sh3*; Yan *et al*., 2006). It is homologous to the *FLOWERING LOCUS T* (*FT*) gene of Arabidopsis (Turck *et al*., 2008). In Arabidopsis, *FT* promotes flowering and is activated by long days (Corbesier *et al*., 2007). In cereals, *FT* also promotes flowering during long days (Yan *et al*., 2006; Faure *et al*., 2007; Hemming *et al*., 2008). In winter varieties, *VRNH3* is only expressed after prolonged exposure to low temperatures (Yan *et al.,* 2006; Hemming *et al*., 2008). The role of *FT1* might extend beyond vernalization and it has been proposed to integrate the vernalization and day-length flowering pathways in cereals (Hemming *et al*., 2008; Distelfeld *et al*., 2009).

With regard to genes that are involved in responses to **photoperiod**, Laurie *et al*. (1994, 1995) identified two genes with large effects, *PPDH1* and *PPDH2*. *PPDH1* confers sensitivity to long photoperiod, i.e. the dominant or sensitive allele induces earlier flowering with long days. Turner *et al*. (2005) identified *HvPRR7,* a *pseudo-response regulator* gene, as a candidate for *PPDH1*, and proposed a diagnostic single nucleotide polymorphism (SNP) that differentiated

between alleles that conferred sensitivity and insensitivity to long photoperiod. The dominant *PPDH1* allele might accelerate flowering by up-regulation of *HvFT1* (Hemming *et al.*, 2008), which is mediated by the activity of *CONSTANS* (Turner *et al.*, 2005).

PPDH2 affects flowering under conditions with a short photoperiod (Laurie *et al.*, 1995). Recently, *HvFT3* has been identified as a candidate gene for *PPDH2* (Faure *et al.*, 2007; Kikuchi *et al.*, 2009). Two alleles have been described: a dominant functional allele, which is frequently present in spring varieties, and a recessive nonfunctional allele, which is mostly present in winter varieties (Faure *et al.*, 2007).

These five genes that are involved in the responses to vernalization and different day-lengths are the major players in the pathways that determine flowering time in barley and other cereals. These pathways, albeit not yet elucidated fully, are rich in interactions between the genes themselves and in responses to environmental cues (Greenup *et al.*, 2009; Shimada *et al.*, 2009; Higgins *et al.*, 2010). The results of these interactions are complex phenotypic responses, which are aimed at the promotion of flowering when optimal environmental conditions are present. Hence, the genes involved in this system should be studied concurrently because their responses might depend on the allelic configurations of the other genes.

Materials and Methods

Plant material

Four genotypes of barley (*Hordeum vulgare* L.) were chosen to assess differences in the expression of the five major genes involved in responses to temperature and day-length: SBCC058 and SBCC106 (inbred lines derived from landraces; belong to the Spanish Barley Core Collection, SBCC; Igartua *et al*. 1998), the French winter cultivar 'Plaisant' ('Ager' × 'Nymphe'), and the German spring cultivar 'Alexis' (Br.1622 × 'Triumph'). The genotypes studied exhibit differences in the length of the first intron of the *VRNH1* gene, as well as in some of the other major genes involved in the control of responses to vernalization and sensitivity to day-length (Table 1).

Doubled haploid lines from two different barley crosses ('Alexis' × 'Pané', Cuesta-Marcos *et al.,* 2008a, and 'Beka' × 'Mogador', Cuesta-Marcos *et al.,* 2008b, Table 1) were used to validate some of the results.

Plant growth conditions

The vernalization requirement of 'Plaisant', SBCC058 and 'Alexis' was evaluated at the Martonvásár (Hungary) phytotron, in accordance with the procedures described by Karsai *et al*. (2004). SBCC106 was not included in this experiment, but three landraces with the same genotype as SBCC106 in terms of *VRNH1*/*VRNH2* were included. Vernalization was applied in 15-day increments, for a total of four treatments that ranged from no vernalization (0 days) to 45 days of vernalization at 3ºC, under a short-day regime (8-h light/16-h dark) and low light intensity (12 ± 1 μmol m^{-2} s^{-1}). After vernalization (or 14 days after germination for the samples not subjected to vernalization), the seedlings were transferred to a regime with long days (16-h light) and a high level of light intensity (340 ± 22 μmol m^{-2} s^{-1}) at 18ºC. For each plant, the number of days to heading, which corresponds to developmental phase 49 on the Zadoks scale

(Zadoks *et al.*, 1974), was recorded. The experiment was continued for a total of 150 days. Two plants were tested for each genotype and treatment.

For studies of gene expression, plants of 'Plaisant', SBCC058, SBCC106, and 'Alexis' were grown in pots in Zaragoza (Spain), in a sunlit glasshouse at 19 ± 1ºC, with a 16-h light/8-h dark photoperiod. Ten days after sowing, when the plants had reached the two-leaf stage (stage 12 of the Zadoks scale), the pots were assigned to one of two groups of the same size and transferred to two growth chambers. Each group was exposed to a distinct experimental treatment. One was a vernalization treatment (VSD), for which the plants were grown at 7±1°C under a short photoperiod (8-h light/16-h dark) and a low level of light intensity (12 μmol m^{-2} s^{-1}). The second set of plants was grown under conditions of no vernalization and long days (NVLD) at 22 ± 1ºC and a photoperiod of 16-h light/8-h dark with a high level of light intensity (220 μmol m^{-2} s^{-1}). We intended to include SBCC106 in the same experiment, but a seed identification error was detected and prevented the use of the results obtained. SBCC106 was later sown and grown for ten days under the same conditions in the glasshouse until the two-leaf stage, together with SBCC058 and 'Plaisant', but in this case the plants were only subjected to the VSD treatment. Hereafter, the experiment that included 'Alexis', SBCC058, and 'Plaisant' will be referred to as Experiment 1 (or Exp1) and the later experiment that included SBCC058, SBCC106, and 'Plaisant' will be referred to as Experiment 2 (or Exp2). In Exp1, two samples were obtained per genotype for each sampling time, which resulted in two biological replicates. Each sample consisted of two plants that were harvested and pooled. In Exp2, three individual plants per sampling time and genotype were harvested, and were treated as three biological replicates. Harvesting took place on day 0 (just before

transfer from the greenhouse to the growth chambers), and after 7, 14, 21, 28, and 35 days of each treatment. An additional sampling at day 42 was carried out in Exp2. In all the experiments, plants were harvested in the middle of the light period.

For the gene expression analysis of the DH lines ('Alexis' × 'Pané' and 'Beka' × 'Mogador'), plants were grown in pots that contained soil in a sunlit glasshouse at a temperature of 19 ± 1ºC, with long days (16-h light/8-h dark). Seven days after sowing, the pots were transferred to a growth chamber, where they were grown under conditions of no vernalization under a long photoperiod (NVLD; 16-h light/8-h dark), at 22 ± 1ºC and with a high level of light intensity, (220 μmol m^{-2} s^{-1}). Plants were harvested after ten days of treatment. At harvest, two samples were collected per genotype. Each sample consisted of the pooled leaf tissue of two plants per genotype, to reduce the effects of individual variation.

RT-PCR and real-time PCR analysis

RNA was extracted from 100 mg of tissue with TRIzol® Reagent (Invitrogen) and treated with DNAse (DNAse I Recombinant, RNAse-free; Roche) to remove possible DNA contamination. An oligo $(dT)_{20}$ primer (Invitrogen) was used to prime the synthesis of first-strand complementary DNA (cDNA) from 1 μl of RNA (2.25 μg of total RNA), using SuperScript III Reverse Transcriptase (Invitrogen) in accordance with the manufacturer's instructions. A single reverse transcription (RT) reaction was carried out for each RNA sample.

Primers for *VRNH1, VRNH2*, and *Actin* were designed in accordance with Trevaskis *et al*. (2006); primers for *VRNH3* in accordance with Yan *et al*. (2006); and primers for *PPDH1* in accordance with Hemming *et al*. (2008). For *PPDH2*,

the forward primer was designed in accordance with Kikuchi et *al*. (2009) and the reverse primer in accordance with Faure *et al*. (2007). In all cases, the same primers were used for semiquantitative PCR and quantitative real-time PCR (qRT-PCR). Each primer pair amplified cDNA-specific DNA products.

Semiquantitative PCR

Semiquantitative PCR was performed in a GeneAmp® PCR System 2700 (Applied Biosystems). Cycling conditions were 4 min at 94ºC, followed by cycles of 30 s denaturation at 94ºC, 30 s annealing at 55ºC, and 30 s elongation at 72ºC for *Actin* (30 cycles), *VRNH1* (30 cycles), and *VRNH2* (35 cycles). For *PPDH1* (30 cycles) and *PPDH2* (35 cycles), the annealing temperature was 57ºC, whereas for *VRNH3* (35 cycles), the annealing temperature was set at 60ºC. The enzyme used was Platinum® Taq DNA Polymerase (Invitrogen), in accordance with the manufacturer's instructions. The PCR products were visualized on agarose gels.

Real-time PCR quantification

This was performed for samples obtained for each treatment at 0, 7, 21, and 35 days for Exp1. In Exp2, real-time PCR quantification was undertaken for groups of samples taken at 21 and 35 days. Amplifications were carried out in 20-µl reactions that included 10 µl of SYBR Green Quantimix Easy SYG Kit (Biotools, Madrid, Spain), 0.3 µM each primer, 4 mM $MgCl_2$, and 4 µl of cDNA, which corresponded to ~ 89 ng of total RNA.

Reactions were run on an ICycler iQ^{TM} (BioRad). Cycling conditions were 6 min at 95ºC, followed by 40 cycles of 30 s at 95ºC, 30 s at 60ºC, and 50 s at 72ºC for *VRNH1*, *VRNH2*, *VRNH3, Actin*, and *PPDH1*. For *PPDH2*, the annealing temperature was 58ºC. This was followed by a melting curve program

(55–95ºC), which involved incremental temperature increases of 0.5ºC with a hold for 10 s at each temperature. Fluorescence data were acquired during the 72ºC step and during the melting curve program. Three identical reactions (technical repeats) were performed per sample, for each cDNA-primer combination in each run. *Actin* expression levels were also quantified in the same run. Two biological repeats were carried out in Exp1 and three in Exp2. All experiments showed similar trends in separate biological repeats.

Expression levels were calculated using the ICycler iQ^{TM} software package (BioRad). The expression of the genes at each time point was normalized to the expression of *Actin*. The amplification efficiencies of each primer set were calculated.

Sequencing of HvFT3 (PPDH2)

Polymorphisms in *HvFT3 (PPDH2*) were ascertained by sequencing. Primers were designed to amplify overlapping fragments on the basis of the sequence from the cultivar 'Morex' (AB476614; Supplementary file 3).

GenBank accession numbers for the *HvFT3* nucleotide sequences described in this manuscript are as follows: 'Alexis', HM133570; 'Beka', HM133571; 'Pane', HM133572; and SBCC058, HM133573.

Statistical analysis

Statistical analysis of the differences in relative expression between genotypes and treatment times was carried out using the ANOVA procedure in SAS (SAS Institute, 1998). The variable used for the analysis was ΔC_T (C_T actin – C_T target gene) for each treatment and genotype, at each sampling time. This variable was preferred over the more commonly used $2^{\Delta CT}$ because of the concerns expressed

by Yuan *et al*. (2006) over its use for statistical analysis. These concerns were namely that the target variable for statistical analysis should be based directly on the C_T value, because this parameter is influenced directly by the treatment, concentration, and the nature of the sample itself (in our case, the different genotypes). The ANOVA model included biological replication, genotype, sampling time (0, 7, 21, and 35 days for Exp1; 21 and 35 days for Exp2), and genotype-by-time interactions for each treatment (VSD and NVLD for Exp1, VSD only for Exp2) separately. Genotypes and treatments were considered as fixed factors. The variability due to biological repeats and their interaction with the other factors was used as the error term to test time and genotype, as well as their interaction. Each value included in the analysis was the average of three technical repeats, to protect against slight fluctuations in reading and small pipetting errors. Differences between genotypes at each sampling time were calculated for each gene using orthogonal contrasts between each pair of genotypes.

Results

Flowering time in response to vernalization

The lines studied differed in their responses to vernalization (Fig. 1). For all genotypes except 'Alexis', the length of time to flowering decreased as the period of exposure to low temperature increased. 'Alexis' was completely unaffected by exposure to the cold, regardless of the length of the cold period. The three SBCC106-like lines showed very consistent results. The period of cold treatment required for these three lines and SBCC058 to flower early was no more than 30 days. Without vernalization, SBCC058 flowered 28 days later than 'Alexis' and

the genotypes similar to SBCC106 60 days later than ‘Alexis’, whereas ‘Plaisant’ did not reach this stage during the experimental period (150 days).

Differences in gene expression

We analysed the expression patterns of three lines (‘Plaisant’, ‘Alexis’, and SBCC058) under VSD and NVLD treatments (Exp1) and that of SBCC106 under VSD conditions (Exp2). Gene expression was assessed by qRT-PCR at every other sampling time (Figs. 2 and 4) and by semiquantitative PCR at all sampling times (Figs. 3 and 5). For each gene, the number of cycles performed for the semiquantitative PCR was set in accordance with the qRT-PCR results and corresponded to the point at which the differences in expression among genoytpes could be differentiated best. Differences among genotypes and sampling times were detected for *VRNH1*, *VRNH2*, *VRNH3*, and *PPDH2* for the VSD treatment, and for all the genes for the NVLD treatment (Figs. 2–5). Of the five genes studied, the level of expression of *VRNH1* was the highest. The genes with the lowest expression levels were *VRNH3* and *PPDH2*.

VRNH1 (HvBM5)

The expression of *VRNH1* was much higher in ‘Alexis’ than in the other genotypes for both treatments (Figs. 2A and 3). There was no expression of *VRNH1* in SBCC058, SBCC106, or ‘Plaisant’ at day 0. Under the VSD treatment, *VRNH1* expression increased gradually (Figs. 2B–5), first in SBCC058 (around day 7) and then in ‘Plaisant’ (around day 35), with the level for SBCC106 being between the other two (Figs. 4A and 5). Expression in ‘Plaisant’ remained significantly lower than that in SBCC106 and SBCC058 until day 35 (Fig. 4A).

Under the NVLD treatment, *VRNH1* expression in SBCC058 also increased with time, but at a lower rate than for the VSD treatment (Figs. 2B and 3). In contrast, *VRNH1* expression in 'Plaisant' was undetectable with the NVLD treatment for the entire duration of the experiment (Figs. 2B and 3). Expression of *VRNH1* in SBCC058 was always significantly lower than that in 'Alexis' and significantly higher than that in 'Plaisant' for the later sampling times (days 21 and 35, Fig. 3).

VRNH2 (HvZCCTa, b)

Under the VSD treatment, *VRNH2* expression in 'Plaisant', SBCC106, and SBCC058 was low and decreased with time, so that it had almost disappeared at 35 days (Figs. 3 and 5). The decrease in *VRNH2* expression contrasted with the increase in *VRNH1* expression in 'Plaisant', SBCC058 (Figs. 2B, 2C, and 3), and SBCC106 (Fig. 5). Under the NVLD treatment, *VRNH2* expression for 'Plaisant' and SBCC058 increased after 7 days, was sustained, and then decreased slightly until the end of the experiment (Figs. 2C and 3).

VRNH3 (HvFT1)

All genotypes carried the same recessive allele in *VRNH3* (Table 1); however, differences in expression were detected. The expression of *VRNH3* was very low under the VSD treatment for all genotypes in both experiments and for all sampling times. Indeed, no expression was apparent in the semiquantitative PCR gels (Figs. 3 and 5) after 35 cycles. By qRT-PCR, *VRNH3* expression could only be detected in 'Alexis' at the last sampling time (Fig. 2D). Under NVLD conditions, *VRNH3* could already be detected in 'Alexis' after 7 days, whereas

SBCC058 and ‘Plaisant’ exhibited hardly any expression of this gene (Figs. 2D and 3).

PPDH1 (HvPRR7)

qRT-PCR did not detect any significant differences in *PPDH1* expression among genotypes and sampling times for the VSD treatment (Fig. 2E), nor were there apparent differences among the four genotypes in the semiquantitative assays (Figs. 3 and 5), even though ‘Alexis’ carries a different allele to the other three genotypes. In contrast, we observed significant differences among genotypes under the NVLD conditions (Fig. 2E). Overall, in Exp1, *PPDH1* expression increased over time for ‘Alexis’ and ‘Plaisant’, but not for SBCC058. At day 35, ‘Alexis’ and ‘Plaisant’ exhibited significantly higher transcript levels than SBCC058 (Figs. 2E and 3).

PPDH2 (HvFT3)

In the case of *PPDH2*, we found differences among genotypes and times for both treatments (Figs. 2F–5). Differences can be explained partly by the presence of *ppdH2* (the nonfunctional allele) in ‘Plaisant’, which caused the absence of transcripts in this variety. All the other lines analysed in Exp1 and Exp2 had the same functional allele (Table 1). Differences between sampling times stemmed mostly from the fact that the expression at day 0 was almost zero, as compared with later sampling times (in genotypes other than ‘Plaisant’). *PPDH2* exhibited a higher level of expression under VSD conditions than under the NVLD treatment (Figs. 2F-5). The levels of *PPDH2* expression in ‘Alexis’ and SBCC058 were very similar, with transcripts being detected after just 7 days of VSD treatment and the levels then increasing slightly with time (Figs. 2F and 3). In contrast,

expression was detected later in SBCC106 in the VSD treatment in Exp2, at 21 days, as compared with 'Alexis' and SBCC058 (Figs. 4B and 5). Moreover, *PPDH2* expression in SBCC106 did not reach the level attained in SBCC058 at 35 days (Fig. 4B). For the NVLD treatment, *PPDH2* expression was detected only in 'Alexis' (Figs. 2F and 3). No expression was found in SBCC058 even though it carries the same allele as 'Alexis'. After 35 days, when 'Alexis' had already flowered, the level of *PPDH2* transcripts in 'Alexis' had decreased again.

To investigate the differences in the expression of *PPDH2* among the genotypes that carried functional alleles ('Alexis' and SBCC058), we carried out two different experiments: (i) we sequenced *HvFT3* (*PPDH2*) in several genotypes (including 'Alexis' and SBCC058) that have the functional allele of this gene and (ii) we analysed the expression profile of *PPDH2* in two different doubled haploid populations under NVLD conditions.

Sequencing of HvFT3 in 'Alexis' and SBCC058

The differences in expression of *HvFT3* (*PPDH2*) between 'Alexis' and SBCC058 in Exp1 were apparently due to differences in regulation, because these lines both carry putatively functional alleles. To ensure that the difference in expression pattern was not due to sequence polymorphisms, which might produce functional changes, we sequenced 1,922 bp of the *HvFT3* gene. In addition to 'Alexis' and SBCC058, we also sequenced the gene from the Spanish cultivar 'Pané' (SBCC167) and from the French spring cultivar 'Beka' (SBCC169). All of these carry the functional allele of *HvFT3*.

The sequences obtained for the four genotypes were the same and 99% identical to that of cultivar 'Morex' (AB476614) (Supplementary file 3). The only observed polymorphism within the coding sequence, after comparison with

‘Morex’, was in exon 3. This SNP does not produce a change in the amino acid sequence. Therefore, the differences observed in the *HvFT3* expression profiles of the studied lines were not caused by polymorphisms in the coding sequence of the gene.

Regulation of HvFT3 expression under conditions that do not typically induce its expression

The expression of *HvFT3* detected in ‘Alexis’ under a long photoperiod was unexpected, because this gene has been thought to respond only to short days. We sought to confirm the response of this gene to conditions that had been thought not to induce its expression (NVLD treatment) in a different set of plant materials. The doubled haploid populations ‘Alexis’ × ‘Pané’ (Cuesta-Marcos *et al*. 2008a) and ‘Beka’ × ‘Mogador’ (Cuesta-Marcos *et al*. 2008b) segregate at *VRNH1* and *VRNH2*. Therefore, they can be used to assess the possible effects of genes involved in the response to vernalization on *HvFT3*. In the first population, ‘Pané’ has all five alleles for the genes involved in responses to vernalization and photoperiod that are carried by SBCC058 (therefore, the population segregates for *VRNH1*, *VRNH2*, and *PPDH1*; Table 1). In the second population, ‘Beka’ carries the functional *HvFT3* allele, whereas ‘Mogador’ has the nonfunctional allele, the same as ‘Plaisant’. Thus, this population segregates for *VRNH1*, *VRNH2*, and *HvFT3* (Table 1), although we only chose lines with the functional allele of *HvFT3* for this experiment.

In ‘Pané’, *HvFT3* was not transcribed, whereas ‘Alexis’ and ‘Beka’ showed high levels of *HvFT3* expression (Fig. 6). Expression of *HvFT3* was not detected in DH lines that carried the functional allele but showed high levels of expression of *VRNH2* (DH lines 385 and 426). However, *HvFT3* expression could

be detected in some DH lines that did carry *VRNH2*, but in which the level of *VRNH2* expression was apparently lower (DH lines 412 and 414). Indeed, lines without *VRNH2* exhibited the highest level of expression of *HvFT3* (DH lines 416, 424, 427, and 429). This suggests that *VRNH2* might play a role in the down-regulation of *HvFT3* (Fig. 6). There seemed to be no relationship between the genotype for *PPDH1* and the expression of *HvFT3*. Finally, expression of *VRNH3* was detected in only a few DH lines of the 'Alexis' × 'Pané' population, namely, those that carried the spring allele of *VRNH1* from 'Alexis' or in which *VRNH2* was absent (Fig. 6).

Discussion

Expression of VRNH1 is responsible for a gradation in the vernalization requirements of barley

'Alexis' and 'Plaisant' presented flowering behaviours, responses to vernalization, and expression patterns for flowering genes that were in accordance with expectations for typical varieties with spring and winter growth habits, respectively. Typically, spring cereal varieties do not require a cold period prior to heading, whereas in winter varieties, it is an essential prerequisite for flowering (Roberts *et al.,* 1988). However, SBCC106 and SBCC058 displayed intermediate responses. This situation resembles the gradation of vernalization requirements described by Takahashi and Yasuda (1971), which was associated with an allelic series of what is now known as *VRNH1*. The phenology of the four lines studied seems to be associated with the respective *VRNH1* alleles. Polymorphism at *VRNH1* has been described by several authors, and in some cases has been related

to differences in function (Fu *et al*., 2005; Cockram *et al*., 2007; Szűcs *et al*., 2007).

The cultivar 'Plaisant', with a winter growth habit, carries an allele of *VRNH1* with a full-length intron 1. This cultivar flowers very late in the absence of vernalization and expression of *VRNH1* is undetectable in plants that have not been vernalized, as reported for other winter cultivars by Trevaskis *et al*. (2003), von Zitzewitz *et al*. (2005), and Hemming *et al*. (2009). As expected (von Zitzewitz *et al.,* 2005; Trevaskis *et al*., 2006), cold treatment induced the expression of *VRNH1* and decreased the time to flowering.

SBCC106 and SBCC058, with an intermediate growth habit, carry alleles of *VRNH1* with deletions of ~0.5 kb (*VRNH1-6* in Hemming *et al.,* 2009) and ~4 kb (*VRNH1-4*, *ibid*) in intron 1, respectively. These deletions have been reported previously: *VRNH1-4* in cultivars 'Albacete' and 'Calicuchima-sib' (von Zitzewitz *et al*., 2005, Szűcs *et al*., 2007) and *VRNH1-6* in cultivar 'Express' (Cockram *et al*., 2007). SBCC106 and SBCC058 flowered relatively late in the absence of vernalization, whereas cold treatment induced increased *VRNH1* expression and decreased the time to flowering.

The small deletion (~0.5 kb) in intron 1 that is carried by cultivar SBCC106 is sufficient to enable the detection of *VRNH1* expression in plants that have not been vernalized and are grown under a short photoperiod (Hemming *et al*., 2009). The low level of *VRNH1* expression associated with this allele appears to be sufficient to allow flowering in the absence of vernalization, after a long vegetative period, as already reported by Hemming *et al*. (2009).

'Alexis', a spring cultivar, carries a *VRNH1* allele with a very large deletion in intron 1 (~9 kb), which was first described in cultivar 'Triumph' (von Zitzewitz *et al*., 2005) and corresponds to allele *VRNH1-3* reported by Hemming *et al.* (2009). This cultivar flowered early in the absence of vernalization and exhibited high levels of *VRNH1* expression in plants that had not been vernalized. Cold treatment was found to induce an increase in *VRNH1-3* expression levels, although flowering time did not change significantly, as reported by Hemming *et al*. (2009).

In general, the growth habits of these four barley lines and their responses to vernalization were correlated with their level of expression of *VRNH1* and the size of the deletion in intron 1. The larger the deletion, the higher the levels of *VRNH1* transcript in plants both with and without vernalization, and the earlier the plants tended to flower. This is consistent with data presented for several *VRNH1* alleles by other researchers (Cockram *et al*., 2007; Szűcs *et al*., 2007; Hemming *et al*., 2009). However, this is the first report that shows increased expression of the *VRNH1-6* allele in response to vernalization. In addition, the duration of the cold treatment required to trigger *VRNH1* expression differed for the four alleles studied. 'Alexis', the spring cultivar, exhibited high expression from the very beginning, whereas SBCC058, SBCC106, and 'Plaisant', in this order, exhibited increasingly long lag periods until expression was detected.

Oliver *et al*. (2009) showed that, in barley, as in Arabidopsis, flowering induced by vernalization is associated with epigenetic changes at the *VRNH1* gene that promote an active chromatin state. In this earlier study, two cultivars of barley were used, a winter type ('Sonja'), with a full-length intron 1, and a spring type ('Morex'), which carries a large deletion in the first intron of *VRNH1*

(*VRNH1-1*). It was suggested that regions of the first intron that are present in the winter cultivar could be important for the repression of *VRNH1* before vernalization. A similar mechanism might also be responsible for the differing behaviour of the *VRNH1* alleles studied here, which are characterized by differences at intron 1.

It has been proposed that genotypes that carry the *VRNH1* allele found in SBCC058, even in the presence of *VRNH2*, should be classified agronomically as 'spring' varieties with a reduced requirement for vernalization (Cockram *et al*., 2007; Szűcs *et al*., 2007), whereas the SBCC106 allele confers a strict winter habit, with a requirement for full vernalization (Cockram *et al*. 2007). The results of our study provide evidence that indicates that lines SBCC106 (*VRNH1-6*) and SBCC058 (*VRNH1-4*) exhibit patterns of expression of *VRNH1* that are intermediate between those of the varieties with habits of winter and spring growth. The intermediate nature of the vernalization response of SBCC058 was confirmed recently using a different set of materials (Casao *et al*., 2010). In this previous study, the introgression of the SBCC058 *VRNH1* allele into a winter-type background reduced but did not cancel the vernalization requirement of the winter-type cultivar.

Thus, as other researchers have suggested, different *VRNH1* alleles are associated with different growth habits and flowering times. We propose that polymorphism of *VRNH1* polymorphism can be used as the basis for the adaptation of cultivars to enable them to grow in particular regions. SBCC058 and SBCC106 are representative of the two main *VRNH1*/*VRNH2* haplotypes found in a large class of Spanish barleys (Casas *et al*., 2008). In fact, out of the 159 landraces represented in the Spanish Barley Core Collection, 47 carry the

VRNH1/*VRNH2* haplotype of SBCC058 and 93 carry the same haplotype as SBCC106. This latter haplotype has been found at very low frequencies in European barley germplasm (5C+Z in Cockram *et al*., 2007). These *VRNH1* alleles found in Spanish barleys could confer advantages that enable adaptation to the Mediterranean climate that predominates in the Iberian Peninsula, with winters that are milder than those in more northerly latitudes.

Expression analysis of vernalization and photoperiod genes

Expression analyses might help to explain the causes that underlie the variety of phenotypic responses that are observed with respect to vernalization. As far as we know, this is the first study that has examined the time course of expression of the five major genes that are associated with responses to vernalization and photoperiod in barley simultaneously.

The interactions among *VRNH1*, *VRNH2*, and *VRNH3* form a feedback regulatory loop, which means that modification of the transcript levels of any one of these genes affects the transcript levels of the others (Distelfeld *et al*., 2009). This model predicts that, under the conditions that are prevalent during winter after sowing in Autumn (low temperature and short photoperiod), *VRNH2* is repressed (by a lack of long days) and *VRNH1* is expressed increasingly as the number of cold days increases. This was confirmed by the results described herein for this kind of genotypes (‘Plaisant’, SBCC106, and SBCC058). In general, the expression of *VRNH2* was accompanied by almost complete absence of *VRNH1* expression under a long photoperiod. These results agree with those reported by Yan *et al*. (2006). However, in SBCC058, simultaneous expression of these two genes under the conditions of NVLD was detected, but at levels that might indicate a rise in *VRNH1* and the beginning of a decrease in *VRNH2* expression.

VRNH3 expression was not detected in any of the lines under the VSD treatment because the conditions did not induce the expression of this gene, as expected (long days are required). Slight expression of *VRNH3* could only be detected in 'Alexis' after 35 days (Fig. 2). A low level of expression of *VRNH3* was also observed under short days (12-h light) by Kikuchi et al. (2009) in the spring cultivar 'Morex'.

Under conditions with a long photoperiod, expression of *VRNH3* was not detected in genotypes in which *VRNH2* was present, as predicted by the feedback model and shown experimentally by Hemming *et al*. (2008). *VRNH3* expression was detected only in 'Alexis', although its level was low. 'Alexis' has a genotype that conveys insensitivity to long photoperiod, and high levels of *VRNH3* expression have only been reported in the literature for genotypes with an active *PPDH1* allele (Turner *et al*., 2005; Faure *et al*., 2007; Hemming *et al*., 2008; Kikuchi *et al*., 2009).

Correlation between VRNH2 expression and HvFT3 repression

In our study, we also analysed *HvFT3*, the candidate gene for *PPDH2* (Faure *et al*., 2007; Kikuchi *et al*., 2009). Under conditions with a short photoperiod, *HvFT3* was expressed in all the genotypes that carried the active allele. There are numerous reports that describe the effect of this gene under conditions of short days (Laurie *et al*. 1995; Faure *et al*., 2007; Karsai *et al*., 2008; Kikuchi et al., 2009). *HvFT3* was also expressed weakly under conditions of a long photoperiod in 'Alexis'. This result was unexpected, but confirms similar observations by Faure *et al*. (2007) and Kikuchi *et al*. (2009) in the spring cultivars 'Triumph' and 'Morex', respectively. The lack of expression of *HvFT3* in SBCC058 during the NVLD treatment must have been caused by a mechanism of repression that is

absent in ‘Alexis’, because both genotypes share the same *HvFT3* allele (Table 1) and no differences were detected between them in terms of the nucleotide sequence.

A possible role of *HvFT3* in the determination of flowering time is also supported by a previous QTL analysis on the population ‘Beka’ × ’Mogador’ (Cuesta-Marcos *et al*., 2008b). We found that *HvFT3* (Supplemental file 2) corresponded to a major QTL that affected flowering under short days in the field and in glasshouse experiments; this QTL was detected in earlier studies and also found previously to be associated with *HvFT3* (Laurie *et al*., 1995; Faure *et al*., 2007; Kikuchi *et al*., 2009). However, previously we also found a QTL with a peak at *HvTF3* under conditions of long days in a glasshouse, although its influence on flowering time under these conditions was weaker than that during short days (Cuesta-Marcos *et al*, 2008b). This result seems to be consistent with the expression pattern of *HvFT3* that was detected under conditions of long days in ‘Alexis’. Karsai *et al*. (2008) also found an effect of *PPDH2* under conditions of a long photoperiod in the ‘Dicktoo’ × ‘Morex’ population.

By further analysis of *HvFT3* expression in DH lines of two barley populations, we found that, under conditions of long days, the expression levels of *HvFT3* and *VRNH2* were related inversely (Fig. 6). This suggests a possible role of *VRNH2* in the down-regulation of *HvFT3* expression and, indirectly, in the regulation of flowering time through an interaction with the pathway that affects responses to day length. Expression of *HvFT3* under conditions of a long photoperiod was detected in ‘Alexis’ and some DH lines, but not in SBCC058 or ‘Pané’. *VRNH2* (absent in ‘Alexis’) was expressed in SBCC058 under these conditions. Therefore, *VRNH2* expression might repress *HvFT3* expression in

SBCC058. Interaction of *VRNH2* with the photoperiod pathway has already been described by Hemming *et al*. (2008). They reported an interaction between *VRNH2, VRNH3*, and *PPDH1* under a long photoperiod, such that deletion of *VRNH2* was associated with expression of *VRNH3* and early flowering only when combined with the *PPDH1* allele that conveys sensitivity to a long photoperiod. They concluded that *VRNH2* counteracts the effects of *PPDH1* to prevent flowering before vernalization. Our results illustrate that *VRNH2* also offsets the effects of *PPDH2* under conditions of a long photoperiod. In the absence of *VRNH2*, expression of *PPDH2* and *VRNH3* could be observed in plants with both alleles of *PPDH1*.

The interaction of *VRNH2* with two of the representatives of the *FT* gene family suggests the possibility of similar mechanisms of action for both *HvFT1* and *HvFT3*. This hypothesis would be consistent with the results of Kikuchi *et al*. (2009). In this previous study, it was hypothesized that *HvFT3* functions indirectly to promote flowering and that its activity can be modulated by photoperiod signals, even with a short photoperiod. An important role of *VRNH2* in the promotion to flowering has been proposed recently by Distelfeld and Dubcovsky (2010), although they acknowledge that its function has not been elucidated completely. In this study, we suggest that *VRNH2*, a gene shown previously to act as a repressor of *HvFT1*, also appears to act as a repressor of *HvFT3*. By this mechanism, the pattern of *HvFT3* expression with respect to day length might be determined fully or partially by *VRNH2*: *VRNH2* is not expressed under conditions of short days, therefore, *HvFT3* is expressed. However, *VRNH2* is expressed under conditions of long days and represses the expression of *HvFT3*. In barley varieties in which the *VRNH2* locus is deleted, *HvFT3* is expressed

under conditions of long days. However, *HvFT3* is expressed at a lower level under long days than under short days, which suggests that a promoter of *HvFT3* is activated more strongly under conditions of short days or there is an additional repressor activity under conditions of long days.

Supplementary material

File S1 - Mapping of *HvFT3* in the 'Beka' × 'Mogador' population

File S2 - QTL analysis for traits related to flowering time in the 'Beka' × 'Mogador' population

File S3 – Sequencing of *HvFT3*

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Table 1. Genotypes for the genes associated with responses to vernalization and photoperiod in the cultivars and lines under study.

Vernalization and photoperiod genes					
Cultivar or line	***VRNH1*[1*]**	***VRNH2*[2]**	***VRNH3*[3]**	***PPDH1*[4]**	***PPDH2*[5]**
'Plaisant'	*vrnh1*	*VRNH2*	*vrnH3*	*PPDH1*	*ppdH2*
SBCC106	*VRNH1-6*	*VRNH2*	*vrnH3*	*PPDH1*	*PPDH2*
SBCC058	*VRNH1-4*	*VRNH2*	*vrnH3*	*PPDH1*	*PPDH2*
'Alexis'	*VRNH1-3*	*vrnH2*	*vrnH3*	*ppdH1*	*PPDH2*
Pané	*VRNH1-4*	*VRNH2*	*vrnH3*	*PPDH1*	*PPDH2*
Beka	*VRNH1-1*	*vrnH2*	*vrnH3*	*ppdH1*	*PPDH2*
Mogador	*vrnh1*	*VRNH2*	*vrnH3*	*ppdH1*	*ppdH2*

[1] Alleles based on the size of intron 1, in accordance with Hemming *et al*. (2009)

[2] Presence/absence of *HvZCCT*, in accordance with Karsai *et al*. (2005)

[3] Alleles based on two SNPs in intron 1, as reported by Yan *et al*. (2006)

[4] Alleles based on SNP22 of Turner *et al*. (2005)

[5] Alleles based on amplification of a 431-bp product using primers FT3.1F (5'-atccattggttgtgtggctca-3') and FT3.2R (5'-atctgtcaccaacctgcaca-3'), which amplify the entire region from exons 1 to 2 of the *HvFT3* gene ('Alexis', SBCC058, SBCC106, 'Pané' and 'Beka'). These primers give a null allele for 'Plaisant' and 'Mogador'. The allele from 'Plaisant' (*ppdH2*) was amplified using the F4/R1 primers reported by Kikuchi *et al*. (2009). *HvFT3* was localized on the long arm of chromosome 1H in the 'Beka' × 'Mogador' mapping population (File S1), which matches the location of a QTL for response to a short photoperiod (File S2).

Figure legends

Figure 1. Days from sowing to flowering of four barley lines ('Plaisant', SBCC106-like, SBCC058, and 'Alexis'; mean of two replications) after 0, 15, 30, or 45 days of vernalization (3ºC, 8-h light). Plants were grown in a phytotron at 18ºC, 16-h light. Error bar (LSD 11.19 days, P=0.05).

Figure 2. Relative expression levels of *VRNH1* (A, B), *VRNH2* (C), *VRNH3* (D), *PPDH1* (E), and *PPDH2* (F) assayed by qRT-PCR in three barley lines, grown under conditions of vernalization and short days (VSD) or no vernalization and long days (NVLD). B shows enlarged graphs of *VRNH1* expression for SBCC058 and 'Plaisant'. The results shown are normalized with respect to the level of the housekeeping gene *Actin* for each genotype and treatment. Samples were taken from plants that were 10 days old (time 0) or after 7, 21, and 35 days of growth under each treatment. The variable of relative gene expression shown is $2^{\Delta CT}$, where ΔC_T is ($C_{T\ Actin} - C_{T\ target\ gen}$), for each genotype and treatment. Error bars represent standard errors of the mean. For the sampling times, bars with the same letter are not significantly different at P=0.05 according to orthogonal contrasts performed for an ANOVA that included all sampling times and genotypes per treatment.

Figure 3. Semiquantitative PCR for *VRNH1* (30 cycles), *VRNH2* (35 cycles), *VRNH3* (35 cycles), *PPDH1* (30 cycles), *PPDH2* (35 cycles), and *Actin* (30 cycles) in three lines of barley vernalized under conditions of a short photoperiod (VSD), or grown without vernalization with long days (NVLD), over the course of five weeks (0, 7, 14, 21, 28, and 35 days).

Figure 4. Relative expression levels of *VRNH1* (A) and *PPDH2* (B), assayed by qRT-PCR in three barley lines, grown under vernalization and short-day

conditions (VSD). The results shown are normalized with respect to the housekeeping gene *Actin* for each genotype and treatment. Samples were taken after 21 or 35 days of growth. The variable of relative gene expression shown is $2^{\Delta CT}$, where ΔC_T is ($C_{T\ Actin} - C_{T\ target\ gen}$), for each genotype and treatment. Error bars represent standard errors of the mean. For each sampling time, bars with the same letter are not significantly different at P=0.05 according to orthogonal contrasts performed for an ANOVA that included all sampling times and genotypes per treatment.

Figure 5. Semiquantitative PCR for *VRNH1* (30 cycles), *VRNH2* (35 cycles), *VRNH3* (35 cycles), *PPDH1* (30 cycles), *PPDH2* (35 cycles), and *Actin* (30 cycles) in three lines of barley vernalized under conditions of a short photoperiod (VSD), in the course of six weeks (0, 7, 14, 21, 28, 35, and 42 days).

Figure 6. Semiquantitative PCR for *VRNH1* (30 cycles), *VRNH2* (35 cycles), *VRNH3* (35 cycles), *PPDH1* (30 cycles), *PPDH2* (35 cycles), and *Actin* (30 cycles) in DH lines of two barley mapping populations grown for 10 days without vernalization under conditions of a long photoperiod (NVLD). Genetic constitution of the parental lines was as follows: 'Alexis' (*VRNH1-3*, *vrnH2*, *PPDH2*, *ppdH1*), 'Pané' (*VRNH1-4*, *VRNH2*, *PPDH2*, *PPDH1*), 'Beka' (*VRNH1-1*, *vrnH2*, *PPDH2*, *ppdH1*), and 'Mogador' (*vrnH1*, *VRNH2*, *ppdH2*, *ppdH1*); *VRNH1* alleles are coded in accordance with Hemming *et al*. (2009). P, 'Pané' allele; A, 'Alexis' allele; B, 'Beka' allele; M, 'Mogador' allele.

Figure 1.

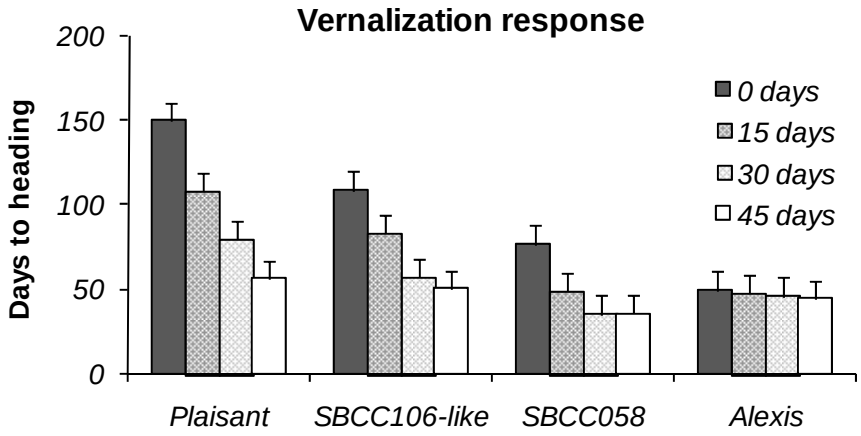

Vernalization response
Days to heading
200
150
100
50
0
0 days
15 days
30 days
45 days
Plaisant
SBCC106-like
SBCC058
Alexis

Figure 2.

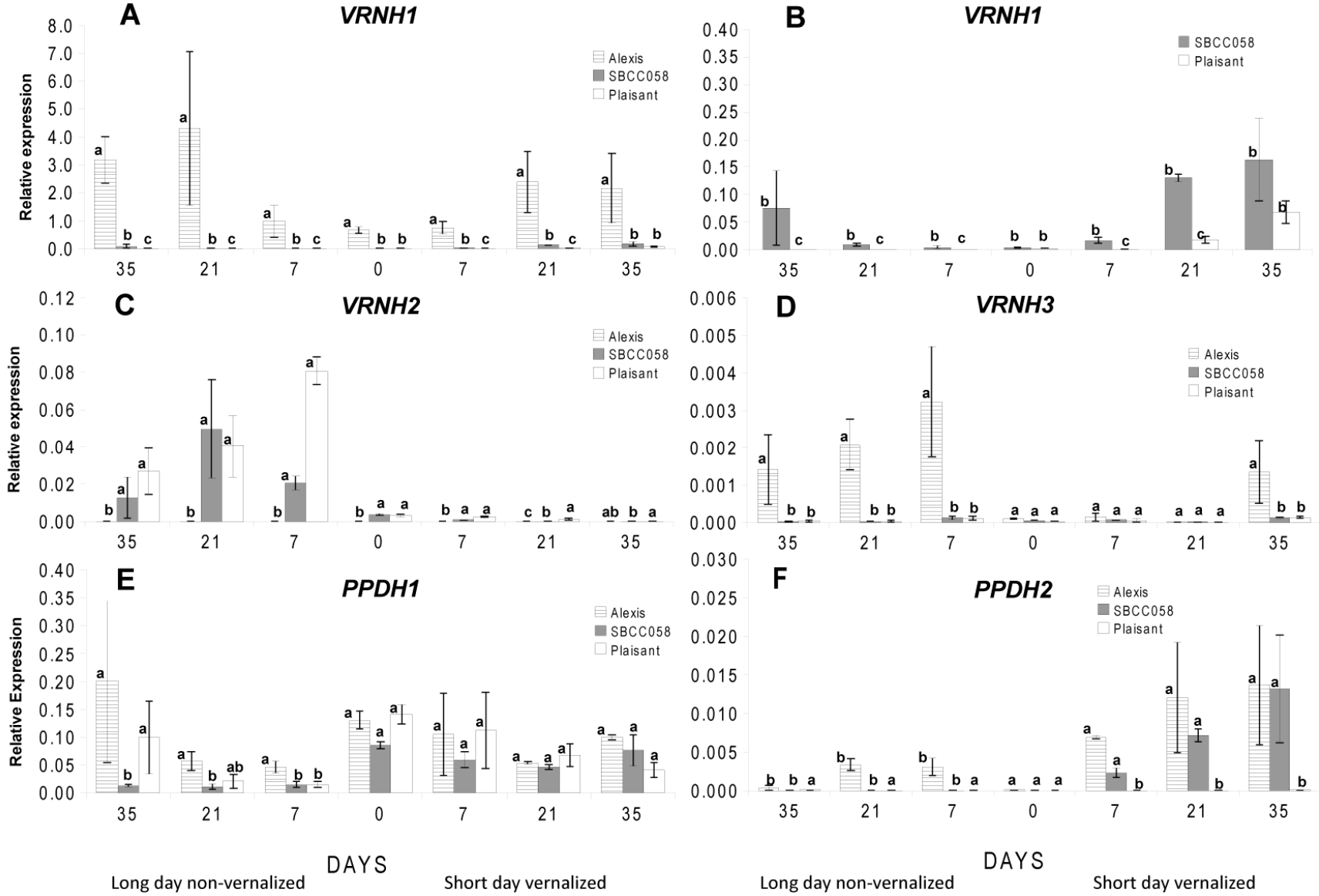

A
VRNH1
Relative expression
Alexis
SBCC058
Plaisant
B
VRNH1
SBCC058
Plaisant
C
VRNH2
Relative expression
D
VRNH3
E
PPDH1
Relative Expression
F
PPDH2
35 21 7 0 7 21 35
DAYS
Long day non-vernalized
Short day vernalized

Figure 3.

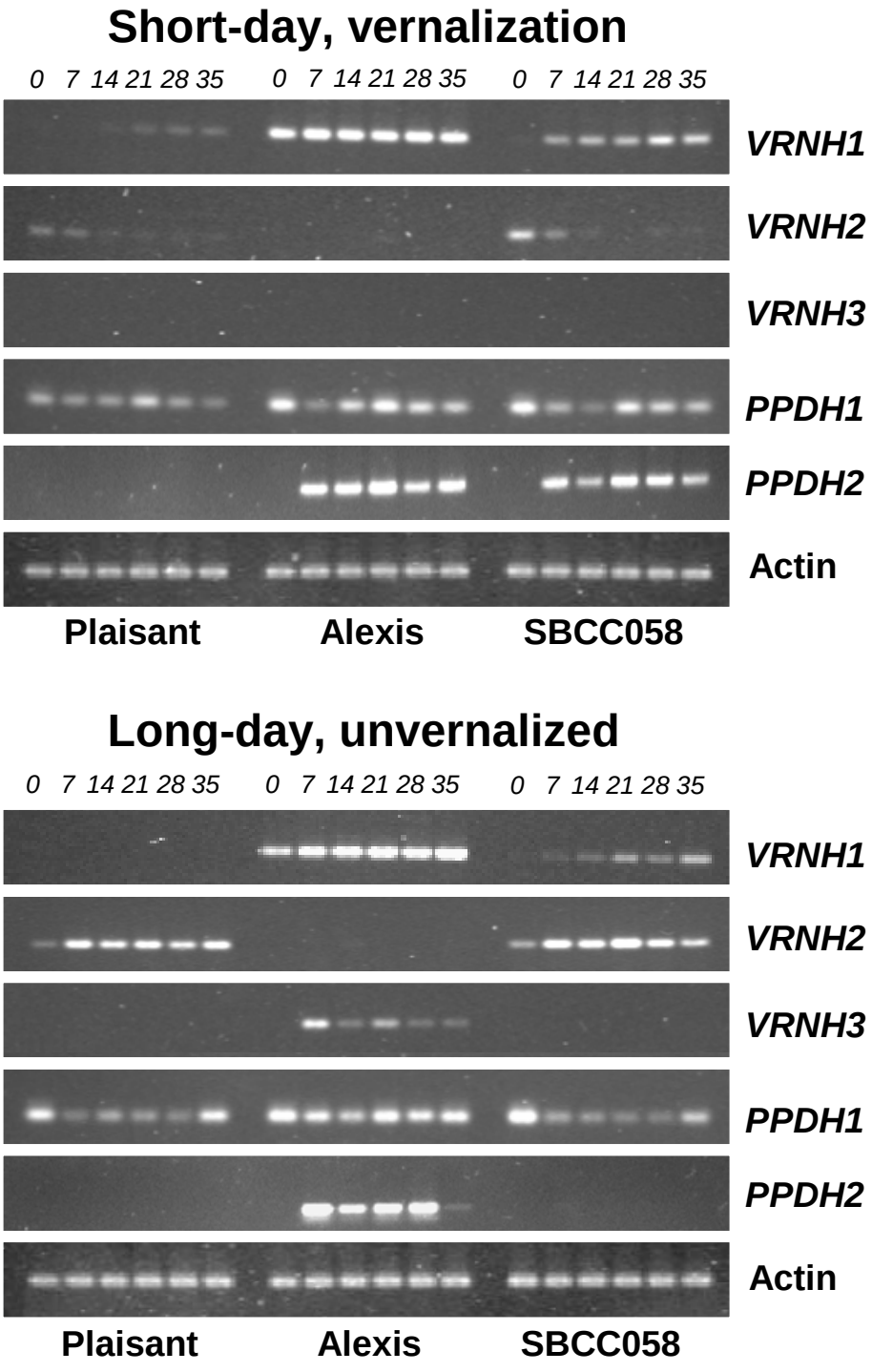

Short-day, vernalization
0 7 14 21 28 35 0 7 14 21 28 35 0 7 14 21 28 35
VRNH1
VRNH2
VRNH3
PPDH1
PPDH2
Actin
Plaisant
Alexis
SBCC058
Long-day, unvernalized
0 7 14 21 28 35 0 7 14 21 28 35 0 7 14 21 28 35
VRNH1
VRNH2
VRNH3
PPDH1
PPDH2
Actin
Plaisant
Alexis
SBCC058

Figure 4.

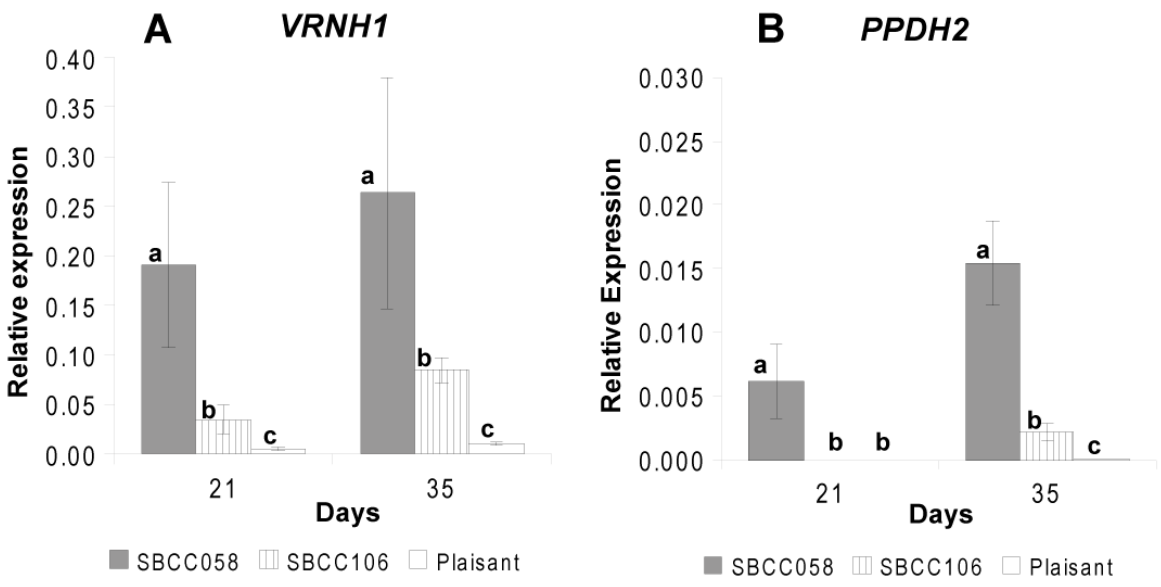

A
VRNH1
Relative expression
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
a
b
c
a
b
c
21
35
Days
SBCC058
SBCC106
Plaisant
B
PPDH2
Relative Expression
0.030
0.025
0.020
0.015
0.010
0.005
0.000
a
b
b
a
b
c
21
35
Days
SBCC058
SBCC106
Plaisant

Figure 5.

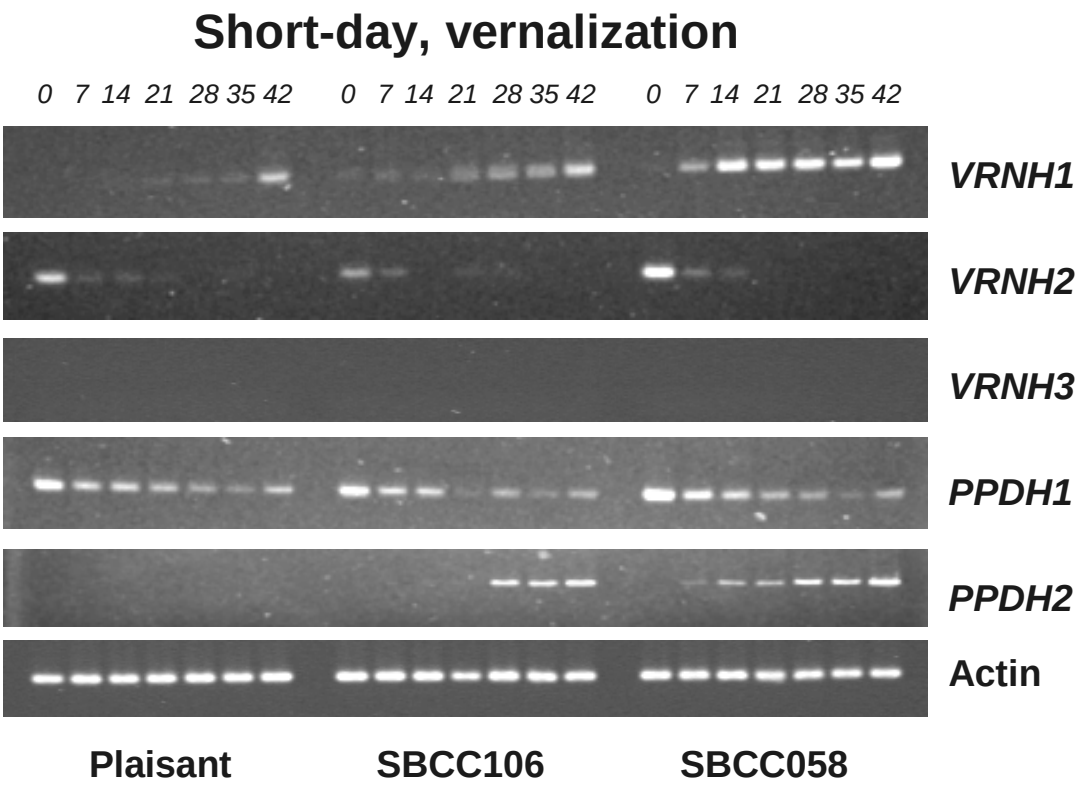
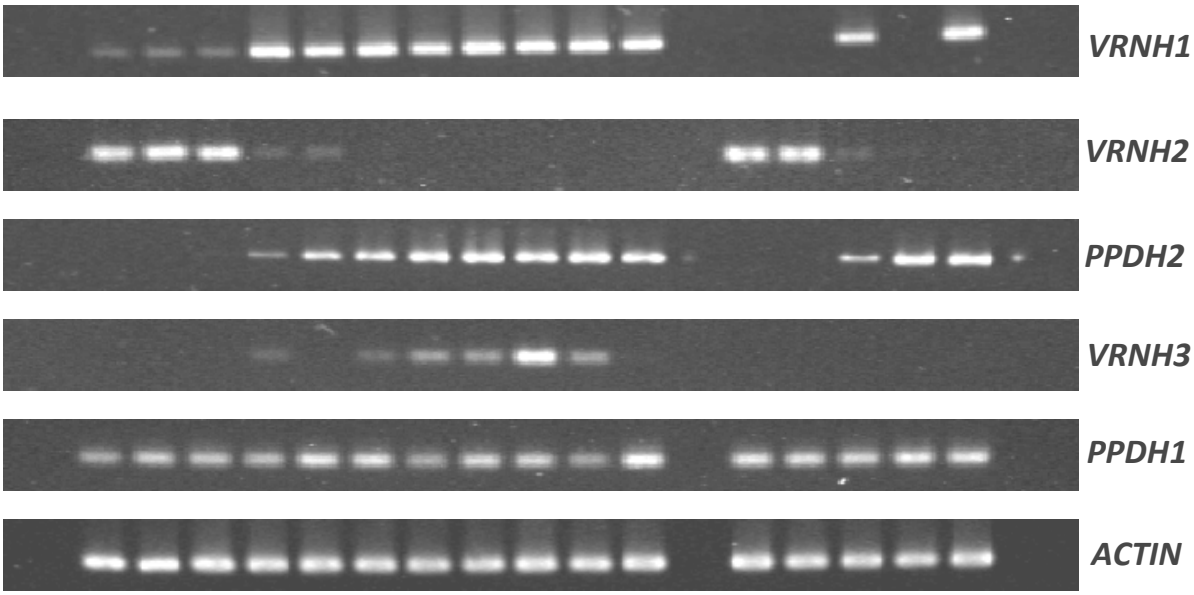

Short-day, vernalization
0 7 14 21 28 35 42 0 7 14 21 28 35 42 0 7 14 21 28 35 42
VRNH1
VRNH2
VRNH3
PPDH1
PPDH2
Actin
Plaisant
SBCC106
SBCC058

Figure 6.



	Pané	DH385	DH426	DH412	DH414	DH364	DH416	DH424	DH427	DH429	Alexis	Mogador	DH1855	DH1829	DH1819	Beka
VRNH1	P	P	P	A	A	A	P	A	A	A	A	M	M	B	M	B
VRNH2	P	P	P	P	P	P	A	A	A	A	A	M	M	M	B	B
PPDH2[1]	-	-	-	-	-	-	-	-	-	-	-	M	B	B	B	B
VRNH3[2]	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
PPDH1[3]	P	P	A	P	A	P	P	P	A	P	A	-	-	-	-	-

[1]Both 'Alexis' and 'Pané' carry the functional allele in *PPDH2*

[2]'Alexis', 'Pané', 'Beka', and 'Mogador' all carry the same recessive allele in *VRNH3*

[3]Both 'Beka' and 'Mogador' carry the allele that conveys insensitivity to long photoperiod in *PPDH1*