

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

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1 **Abstract**

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

24

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

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4

5 **Introduction**

6 The classic model of the genetic control of **vernalization** in barley (Takahashi
7 and Yasuda, 1971) is based on three loci, *Sh/sh*, *Sh₂/sh₂*, and *Sh₃/sh₃*, among
8 which epistatic relationships exist. Candidate genes for these three loci in barley
9 have been proposed. *HvBM5A* (which corresponds to *TmAP1* or *WAP1* in wheat)
10 was identified as a candidate for *VRNH1*, which is a synonym of *Sh₂* (Danyluk *et*
11 *al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003). *VRNH1* promotes the transition
12 of the apex from the vegetative to the reproductive stage. The locus is always
13 expressed at high basal levels in plants that have spring (dominant) alleles
14 (Trevaskis *et al.*, 2006). In winter varieties that are responsive to vernalization,
15 *VRNH1* expression is repressed until the plants are exposed to low temperatures
16 (von Zitzewitz *et al.*, 2005; Sasani *et al.*, 2009). Allelic diversity at *VRNH1* has
17 been described, mostly in relation to deletions within the first intron (Fu *et al.*,
18 2005; Cockram *et al.*, 2007; Szűcs *et al.*, 2007). These deletions presumably cause
19 variation in the levels of *VRNH1* expression in plants that have not been
20 vernalized (Hemming *et al.*, 2009) and hence lead to different flowering times
21 (Trevaskis *et al.*, 2003; von Zitzewitz *et al.*, 2005).

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

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

10 *HvFT1* is a candidate gene for *VRNH3* (*Sh3*; Yan *et al.*, 2006). It is
11 homologous to the *FLOWERING LOCUS T (FT)* gene of Arabidopsis (Turck *et*
12 *al.*, 2008). In Arabidopsis, *FT* promotes flowering and is activated by long days
13 (Corbesier *et al.*, 2007). In cereals, *FT* also promotes flowering during long days
14 (Yan *et al.*, 2006; Faure *et al.*, 2007; Hemming *et al.*, 2008). In winter varieties,
15 *VRNH3* is only expressed after prolonged exposure to low temperatures (Yan *et*
16 *al.*, 2006; Hemming *et al.*, 2008). The role of *FT1* might extend beyond
17 vernalization and it has been proposed to integrate the vernalization and day-
18 length flowering pathways in cereals (Hemming *et al.*, 2008; Distelfeld *et al.*,
19 2009).

20 With regard to genes that are involved in responses to **photoperiod**, Laurie *et al.*
21 (1994, 1995) identified two genes with large effects, *PPDH1* and *PPDH2*.
22 *PPDH1* confers sensitivity to long photoperiod, i.e. the dominant or sensitive
23 allele induces earlier flowering with long days. Turner *et al.* (2005) identified
24 *HvPRR7*, a *pseudo-response regulator* gene, as a candidate for *PPDH1*, and
25 proposed a diagnostic single nucleotide polymorphism (SNP) that differentiated

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

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

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

21

22 **Materials and Methods**

23 *Plant material*

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

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

13 *Plant growth conditions*

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

1 (Zadoks *et al.*, 1974), was recorded. The experiment was continued for a total of
2 150 days. Two plants were tested for each genotype and treatment.

3 For studies of gene expression, plants of ‘Plaisant’, SBCC058, SBCC106,
4 and ‘Alexis’ were grown in pots in Zaragoza (Spain), in a sunlit glasshouse at 19
5 $\pm 1^\circ\text{C}$, with a 16-h light/8-h dark photoperiod. Ten days after sowing, when the
6 plants had reached the two-leaf stage (stage 12 of the Zadoks scale), the pots were
7 assigned to one of two groups of the same size and transferred to two growth
8 chambers. Each group was exposed to a distinct experimental treatment. One was
9 a vernalization treatment (VSD), for which the plants were grown at $7\pm 1^\circ\text{C}$ under
10 a short photoperiod (8-h light/16-h dark) and a low level of light intensity (12
11 $\mu\text{mol m}^{-2} \text{s}^{-1}$). The second set of plants was grown under conditions of no
12 vernalization and long days (NVLD) at $22 \pm 1^\circ\text{C}$ and a photoperiod of 16-h
13 light/8-h dark with a high level of light intensity ($220 \mu\text{mol m}^{-2} \text{s}^{-1}$). We intended
14 to include SBCC106 in the same experiment, but a seed identification error was
15 detected and prevented the use of the results obtained. SBCC106 was later sown
16 and grown for ten days under the same conditions in the glasshouse until the two-
17 leaf stage, together with SBCC058 and ‘Plaisant’, but in this case the plants were
18 only subjected to the VSD treatment. Hereafter, the experiment that included
19 ‘Alexis’, SBCC058, and ‘Plaisant’ will be referred to as Experiment 1 (or Exp1)
20 and the later experiment that included SBCC058, SBCC106, and ‘Plaisant’ will be
21 referred to as Experiment 2 (or Exp2). In Exp1, two samples were obtained per
22 genotype for each sampling time, which resulted in two biological replicates.
23 Each sample consisted of two plants that were harvested and pooled. In Exp2,
24 three individual plants per sampling time and genotype were harvested, and were
25 treated as three biological replicates. Harvesting took place on day 0 (just before

1 transfer from the greenhouse to the growth chambers), and after 7, 14, 21, 28, and
2 35 days of each treatment. An additional sampling at day 42 was carried out in
3 Exp2. In all the experiments, plants were harvested in the middle of the light
4 period.

5 For the gene expression analysis of the DH lines ('Alexis' × 'Pané' and
6 'Beka' × 'Mogador'), plants were grown in pots that contained soil in a sunlit
7 glasshouse at a temperature of $19 \pm 1^\circ\text{C}$, with long days (16-h light/8-h dark).
8 Seven days after sowing, the pots were transferred to a growth chamber, where
9 they were grown under conditions of no vernalization under a long photoperiod
10 (NVLD; 16-h light/8-h dark), at $22 \pm 1^\circ\text{C}$ and with a high level of light intensity,
11 ($220 \mu\text{mol m}^{-2} \text{s}^{-1}$). Plants were harvested after ten days of treatment. At harvest,
12 two samples were collected per genotype. Each sample consisted of the pooled
13 leaf tissue of two plants per genotype, to reduce the effects of individual variation.

14 *RT-PCR and real-time PCR analysis*

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

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

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

5 *Semiquantitative PCR*

6 Semiquantitative PCR was performed in a GeneAmp® PCR System 2700
7 (Applied Biosystems). Cycling conditions were 4 min at 94°C, followed by cycles
8 of 30 s denaturation at 94°C, 30 s annealing at 55°C, and 30 s elongation at 72°C
9 for *Actin* (30 cycles), *VRNH1* (30 cycles), and *VRNH2* (35 cycles). For *PPDH1*
10 (30 cycles) and *PPDH2* (35 cycles), the annealing temperature was 57°C, whereas
11 for *VRNH3* (35 cycles), the annealing temperature was set at 60°C. The enzyme
12 used was Platinum® Taq DNA Polymerase (Invitrogen), in accordance with the
13 manufacturer's instructions. The PCR products were visualized on agarose gels.

14 *Real-time PCR quantification*

15 This was performed for samples obtained for each treatment at 0, 7, 21, and 35
16 days for Exp1. In Exp2, real-time PCR quantification was undertaken for groups
17 of samples taken at 21 and 35 days. Amplifications were carried out in 20- μ l
18 reactions that included 10 μ l of SYBR Green Quantimix Easy SYG Kit (Biotools,
19 Madrid, Spain), 0.3 μ M each primer, 4 mM MgCl₂, and 4 μ l of cDNA, which
20 corresponded to ~ 89 ng of total RNA.

21 Reactions were run on an ICycler iQ™ (BioRad). Cycling conditions were
22 6 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 60°C, and 50 s at
23 72°C for *VRNH1*, *VRNH2*, *VRNH3*, *Actin*, and *PPDH1*. For *PPDH2*, the
24 annealing temperature was 58°C. This was followed by a melting curve program

1 (55–95°C), which involved incremental temperature increases of 0.5°C with a hold
2 for 10 s at each temperature. Fluorescence data were acquired during the 72°C
3 step and during the melting curve program. Three identical reactions (technical
4 repeats) were performed per sample, for each cDNA-primer combination in each
5 run. *Actin* expression levels were also quantified in the same run. Two biological
6 repeats were carried out in Exp1 and three in Exp2. All experiments showed
7 similar trends in separate biological repeats.

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

12 *Sequencing of HvFT3 (PPDH2)*

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

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

19 *Statistical analysis*

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

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

16

17 **Results**

18 *Flowering time in response to vernalization*

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

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

3 *Differences in gene expression*

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

16 *VRNH1 (HvBM5)*

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

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

8 *VRNH2 (HvZCCTa, b)*

9 Under the VSD treatment, *VRNH2* expression in ‘Plaisant’, SBCC106, and
10 SBCC058 was low and decreased with time, so that it had almost disappeared at
11 35 days (Figs. 3 and 5). The decrease in *VRNH2* expression contrasted with the
12 increase in *VRNH1* expression in ‘Plaisant’, SBCC058 (Figs. 2B, 2C, and 3), and
13 SBCC106 (Fig. 5). Under the NVLD treatment, *VRNH2* expression for ‘Plaisant’
14 and SBCC058 increased after 7 days, was sustained, and then decreased slightly
15 until the end of the experiment (Figs. 2C and 3).

16 *VRNH3 (HvFTL)*

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

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

3 *PPDH1 (HvPRR7)*

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

13 *PPDH2 (HvFT3)*

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

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

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

14 *Sequencing of HvFT3 in ‘Alexis’ and SBCC058*

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

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

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

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

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

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

1 be detected in some DH lines that did carry *VRNH2*, but in which the level of
2 *VRNH2* expression was apparently lower (DH lines 412 and 414). Indeed, lines
3 without *VRNH2* exhibited the highest level of expression of *HvFT3* (DH lines
4 416, 424, 427, and 429). This suggests that *VRNH2* might play a role in the down-
5 regulation of *HvFT3* (Fig. 6). There seemed to be no relationship between the
6 genotype for *PPDH1* and the expression of *HvFT3*. Finally, expression of *VRNH3*
7 was detected in only a few DH lines of the ‘Alexis’ × ‘Pané’ population, namely,
8 those that carried the spring allele of *VRNH1* from ‘Alexis’ or in which *VRNH2*
9 was absent (Fig. 6).

10

11 **Discussion**

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

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

1 to differences in function (Fu *et al.*, 2005; Cockram *et al.*, 2007; Szűcs *et al.*,
2 2007).

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

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

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

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

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

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

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

6 It has been proposed that genotypes that carry the *VRNHI* allele found in
7 SBCC058, even in the presence of *VRNH2*, should be classified agronomically as
8 ‘spring’ varieties with a reduced requirement for vernalization (Cockram *et al.*,
9 2007; Szűcs *et al.*, 2007), whereas the SBCC106 allele confers a strict winter
10 habit, with a requirement for full vernalization (Cockram *et al.* 2007). The results
11 of our study provide evidence that indicates that lines SBCC106 (*VRNHI-6*) and
12 SBCC058 (*VRNHI-4*) exhibit patterns of expression of *VRNHI* that are
13 intermediate between those of the varieties with habits of winter and spring
14 growth. The intermediate nature of the vernalization response of SBCC058 was
15 confirmed recently using a different set of materials (Casao *et al.*, 2010). In this
16 previous study, the introgression of the SBCC058 *VRNHI* allele into a winter-type
17 background reduced but did not cancel the vernalization requirement of the
18 winter-type cultivar.

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

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

7 *Expression analysis of vernalization and photoperiod genes*

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

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

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

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

15 *Correlation between VRNH2 expression and HvFT3 repression*

16 In our study, we also analysed *HvFT3*, the candidate gene for *PPDH2* (Faure *et al.*
17 *et al.*, 2007; Kikuchi *et al.*, 2009). Under conditions with a short photoperiod,
18 *HvFT3* was expressed in all the genotypes that carried the active allele. There are
19 numerous reports that describe the effect of this gene under conditions of short
20 days (Laurie *et al.* 1995; Faure *et al.*, 2007; Karsai *et al.*, 2008; Kikuchi et al.,
21 2009). *HvFT3* was also expressed weakly under conditions of a long photoperiod
22 in ‘Alexis’. This result was unexpected, but confirms similar observations by
23 Faure *et al.* (2007) and Kikuchi *et al.* (2009) in the spring cultivars ‘Triumph’ and
24 ‘Morex’, respectively. The lack of expression of *HvFT3* in SBCC058 during the
25 NVLD treatment must have been caused by a mechanism of repression that is

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

4 A possible role of *HvFT3* in the determination of flowering time is also
5 supported by a previous QTL analysis on the population ‘Beka’ × ‘Mogador’
6 (Cuesta-Marcos *et al.*, 2008b). We found that *HvFT3* (Supplemental file 2)
7 corresponded to a major QTL that affected flowering under short days in the field
8 and in glasshouse experiments; this QTL was detected in earlier studies and also
9 found previously to be associated with *HvFT3* (Laurie *et al.*, 1995; Faure *et al.*,
10 2007; Kikuchi *et al.*, 2009). However, previously we also found a QTL with a
11 peak at *HvTF3* under conditions of long days in a glasshouse, although its
12 influence on flowering time under these conditions was weaker than that during
13 short days (Cuesta-Marcos *et al.*, 2008b). This result seems to be consistent with
14 the expression pattern of *HvFT3* that was detected under conditions of long days
15 in ‘Alexis’. Karsai *et al.* (2008) also found an effect of *PPDH2* under conditions
16 of a long photoperiod in the ‘Dicktoo’ × ‘Morex’ population.

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

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

11 The interaction of *VRNH2* with two of the representatives of the *FT* gene
12 family suggests the possibility of similar mechanisms of action for both *HvFT1*
13 and *HvFT3*. This hypothesis would be consistent with the results of Kikuchi *et al.*
14 (2009). In this previous study, it was hypothesized that *HvFT3* functions
15 indirectly to promote flowering and that its activity can be modulated by
16 photoperiod signals, even with a short photoperiod. An important role of *VRNH2*
17 in the promotion to flowering has been proposed recently by Distelfeld and
18 Dubcovsky (2010), although they acknowledge that its function has not been
19 elucidated completely. In this study, we suggest that *VRNH2*, a gene shown
20 previously to act as a repressor of *HvFT1*, also appears to act as a repressor of
21 *HvFT3*. By this mechanism, the pattern of *HvFT3* expression with respect to day
22 length might be determined fully or partially by *VRNH2*: *VRNH2* is not expressed
23 under conditions of short days, therefore, *HvFT3* is expressed. However, *VRNH2*
24 is expressed under conditions of long days and represses the expression of *HvFT3*.
25 In barley varieties in which the *VRNH2* locus is deleted, *HvFT3* is expressed

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

5

6 Supplementary material

7 File S1 - Mapping of *HvFT3* in the ‘Beka’ × ‘Mogador’ population

8 File S2 - QTL analysis for traits related to flowering time in the ‘Beka’ ×
9 ‘Mogador’ population

10 File S3 – Sequencing of *HvFT3*

11

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References

Casao MC, Igartua E, Karsai I, Bhat PR, Cuadrado N, Gracia MP, Lasa JM, Casas AM. 2010. Introgression of an intermediate VRNH1 allele leads to reduced vernalization requirement without affecting freezing tolerance. *Molecular Breeding* DOI 10.1007/s11032-010-9497-y.

Casas AM, Yahiaoui S, Ciudad FJ, Molina-Cano JL, Karsai I, Meszaros K, Lasa JM, Gracia MP, Hayes PM, Igartua E, Szűcs P. 2008. *Vrn-H1* and *Vrn-*

H2 allelic diversity in barley may explain specific adaptation to the Mediterranean environments. In: Cereal science and technology for feeding ten billion people: Genomics era and beyond. Eds: J.L. Molina Cano, P. Christou, A. Graner, K. Hammer, N. Jouve, B. Keller, J.M. Lasa, W. Powell, C. Royo, P. Shewry, A.M. Stanca. Zaragoza, CIHEAM/IRTA. *Options Méditerranéennes*, Serie A, No. 81, pp.105-109.

Cockram J, Chiapparino E, Taylor SA, Stamati K, Donini P, Laurie DA, O'Sullivan DM. 2007. Haplotype analysis of vernalization loci in European barley germplasm reveals novel *VRN-H1* alleles and a predominant winter *VRN-H1/VRN-H2* multi-locus haplotype. *Theoretical and Applied Genetics* **115**, 993-1001.

Corbesier L, Vincent C, Jang S, Fornara F, Fan Q, Searle I, Giakountis A, Farrona S, Gissot L, Turnbull C, Coupland G. 2007. FT protein movement contributes to long-distance signaling in floral induction of Arabidopsis. *Science* **316**, 1030-1033.

Cuesta-Marcos A, Casas AM, Yahiaoui S, Gracia MP, Lasa JM, Igartua E. 2008a. Joint analysis for heading date QTL in small interconnected barley populations. *Molecular Breeding* **21**, 383-399.

Cuesta-Marcos A, Igartua E, Ciudad FJ, Codesal P, Russell JR, Molina-Cano JL, Moralejo M, Szűcs P, Gracia MP, Lasa JM, Casas AM. 2008b. Heading date QTL in a spring x winter barley cross evaluated in Mediterranean environments. *Molecular Breeding* **21**, 455-471.

Danyluk J, Kane NA, Breton G, Limin AE, Fowler DB, Sarhan F. 2003. *TaVRT-1*, a putative transcription factor associated with vegetative to reproductive transition in cereals. *Plant Physiology* **132**, 1849-1860.

Distelfeld A, Li C, Dubcovsky J. 2009. Regulation of flowering in temperate cereals. *Current Opinion in Plant Biology* **12**, 178-184.

Distelfeld A, Dubcovsky J. 2010. Characterization of the maintained vegetative phase deletions from diploid wheat and their effect on *VRN2* and *FT* transcript levels. *Molecular Genetics and Genomics* **283**, 223-232.

Dubcovsky J, Chen C, Yan L. 2005. Molecular characterization of the allelic variation at the *VRN-H2* vernalization locus in barley. *Molecular Breeding* **15**, 395-407.

Faure S, Higgins J, Turner A, Laurie DA. 2007. The *FLOWERING LOCUS T*-like gene family in barley *Hordeum vulgare*.. *Genetics* **176**, 599-609.

Fu D, Szűcs P, Yan L, Helguera M, Skinner J, Hayes P, Dubcovsky J. 2005. Large deletions within the first intron of the *VRN-1* are associated with spring growth habit in barley and wheat. *Molecular Genetics and Genomics* **273**, 54-65.

- Greenup A, Peacock WJ, Dennis ES, Trevaskis B.** 2009. The molecular biology of seasonal flowering-responses in *Arabidopsis* and the cereals. *Annals of Botany* **103**, 1165–1172.
- Hemming MN, Peacock WJ, Dennis ES, Trevaskis B.** 2008. Low-temperature and daylength cues are integrated to regulate *FLOWERING LOCUS T* in barley. *Plant Physiology* **147**, 355–366.
- Hemming MN, Fieg S, Peacock WJ, Dennis ES, Trevaskis B.** 2009. Regions associated with repression of the barley *Hordeum vulgare*. *VERNALIZATION1* gene are not required for cold induction. *Molecular Genetics and Genomics* **282**, 107–117.
- Higgins JA, Bailey PC, Laurie DA.** 2010. Comparative genomics of flowering time pathways using *Brachypodium distachyon* as a model for the temperate grasses. *PLoS One* **5**, e10065.
- Igartua E, Gracia MP, Lasa JM, Medina B, Molina-Cano JL, Montoya JL, Romagosa I.** 1998. The Spanish barley core collection. *Genetic Resources & Crop Evolution* **45**, 475-481.
- Karsai I, Hayes PM, Kling J, Matus IA, Mészáros K, Láng L, Bedő Z, Sato K.** 2004. Genetic variation in component traits of heading date in *Hordeum vulgare* subsp. *spontaneum* accessions characterized in controlled environments. *Crop Science* **44**, 1622–1632.
- Karsai I, Szűcs P, Mészáros K, Filichkina T, Hayes PM, Skinner JS, Láng L, Bedő Z.** 2005. The *VRN-H2* locus is a major determinant of flowering time in a facultative × winter growth habit barley *Hordeum vulgare* L.. mapping population. *Theoretical and Applied Genetics* **110**, 1458-1466.
- Karsai I, Szűcs P, Kószegi B, Hayes PM, Casas A, Bedő Z, Veisz O.** 2008. Effects of photo and thermo cycles on flowering time in barley: a genetical phenomics approach. *Journal of Experimental Botany* **59**, 2707-2715.
- Kikuchi R, Kawahigashi H, Ando T, Tonooka T, Handa H.** 2009. Molecular and functional characterization of PEBP genes in barley reveal the diversification of their roles in flowering. *Plant Physiology* **149**, 1341–1353.
- Laurie DA, Pratchett N, Bezant JH, Snape JW.** 1994. Genetic analysis of a photoperiod response gene on the short arm of chromosome 2 (2H) of *Hordeum vulgare* (barley). *Heredity* **72**, 619-627.
- Laurie DA, Pratchett N, Bezant JH, Snape JW.** 1995. RFLP mapping of five major genes and eight quantitative trait loci controlling flowering time in a winter x spring barley *Hordeum vulgare* L. cross. *Genome* **38**, 575-585.
- Loukoianov A, Yan L, Blechl A, Sanchez A, Dubcovsky J.** 2005. Regulation of *VRN-1* vernalization genes in normal and transgenic polyploid wheat. *Plant Physiology* **138**, 2364-2373

Oliver SN, Finnegan EJ, Dennis ES, Peacock WJ, Trevaskis B. 2009. Vernalization-induced flowering in cereals is associated with changes in histone methylation at the *VERNALIZATION1* gene. *Proceedings of the National Academy of Sciences, USA* **106**, 8386–8391.

Roberts EH, Summerfield RJ, Cooper JP, Ellis RH. 1988. Environmental-control of flowering in barley (*Hordeum vulgare* L). I. Photoperiod limits to long-day responses, photoperiod-insensitive phases and effects of low-temperature and short-day vernalization. *Annals of Botany* **62**, 127-144.

SAS Institute. 1998. SAS/STATTM User's Guide Release 6.03 Edition. 1028. SAS Institute Inc., Cary, NC.

Sasani S, Hemming MN, Oliver SN, Greenup A, Tavakkol-Afshari R, Mahfoozi S, Poustini K, Sharifi HR, Dennis ES, Peacock WJ, Trevaskis B. 2009. The influence of vernalization and daylength on expression of flowering-time genes in the shoot apex and leaves of barley *Hordeum vulgare*.. *Journal of Experimental Botany* **60**, 2169–2178.

Shimada S, Ogawa T, Kitagawa S, Suzuki T, Ikari C, Shitsukawa N, Abe T, Kawahigashi H, Kikuchi R, Handa H, Murai K. 2009. A genetic network of flowering-time genes in wheat leaves, in which an *APETALA1/FRUITFULL*-like gene, *VRN1*, is upstream of *FLOWERING LOCUS T*. *The Plant Journal* **58**, 668-681.

Szűcs P, Skinner JS, Karsai I, Cuesta-Marcos A, Haggard KG, Corey AE, Chen THH, Hayes PM. 2007. Validation of the *VRN-H2/VRN-H1* epistatic model in barley reveals that intron length variation in *VRN-H1* may account for a continuum of vernalization sensitivity. *Molecular Genetics and Genomics* **277**, 249-261.

Takahashi R, Yasuda S. 1971. Genetics of earliness and growth habit in barley. In: Nilan RA ed. *Barley Genetics II*. Washington State University Press, pp 388-408.

Tranquilli G, Dubcovsky J. 2000. Epistatic interaction between vernalization genes *Vrn-A^{m1}* and *Vrn-A^{m2}* in diploid wheat. *Journal of Heredity* **91**, 304-306.

Trevaskis B, Bagnall DJ, Ellis MH, Peacock WJ, Dennis ES. 2003. MADS box genes control vernalization-induced flowering in cereals. *Proceedings of the National Academy of Science, USA*, **100**, 13099-13104.

Trevaskis B, Hemming MN, Peacock WJ, Dennis ES. 2006. *HvVRN2* responds to daylength, whereas *HvVRN1* is regulated by vernalization and developmental status. *Plant Physiology* **140**. 1397–1405.

Turck F, Fornara F, Coupland G. 2008. Regulation and Identity of Florigen: FLOWERING LOCUS T moves center stage. *Annual Review of Plant Biology* **59**, 573-594.

Turner A, Beales J, Faure S, Dunford RP, Laurie DA. 2005. The pseudo-response regulator *Ppd-H1* provides adaptation to photoperiod in barley. *Science* **310**, 1031-1034.

von Zitzewitz J, Szűcs P, Dubcovsky J, Yan L, Pecchioni N, Francia E, Casas A, Chen THH, Hayes PM, Skinner JS. 2005. Molecular and structural characterization of barley vernalization genes. *Plant Molecular Biology* **59**, 449–467.

Yan L, Loukoianov A, Tranquilli G, Helguera M, Fahima T, Dubcovsky J. 2003. Positional cloning of wheat vernalization gene *VRN1*. *Proceedings of the National Academy of Sciences, USA* **100**, 6263-6268.

Yan L, Loukoianov A, Blechl A, Tranquilli G, Ramakrishna W, SanMiguel P, Bennetzen JL, Echenique V, Dubcovsky J. 2004. The wheat *VRN2* gene is a flowering repressor down-regulated by vernalization. *Science* **303**, 1640-1644.

Yan L, Fu D, Li C, Blechl A, Tranquilli G, Bonafede M, Sanchez A, Valarik M, Dubcovsky J. 2006. The wheat and barley vernalization gene *VRN3* is an orthologue of *FT*. *Proceeding of the National Academy of Sciences, USA* **103**, 19581-19586.

Yuan JS, Reed A, Chen F, Stewart CN. Jr 2006. Statistical analysis of real-time PCR data. *BMC Bioinformatics* **7**, 85.

Zadoks JC, Chang TT, Konzak CF. 1974. A decimal code for the growth stages of cereals. *Weed Research* **14**, 415-421

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	<i>VRNH1</i> ^{1*}	<i>VRNH2</i> ²	<i>VRNH3</i> ³	<i>PPDH1</i> ⁴	<i>PPDH2</i> ⁵
‘Plaisant’	<i>vrnh1</i>	<i>VRNH2</i>	<i>vrnH3</i>	<i>PPDH1</i>	<i>ppdH2</i>
SBCC106	<i>VRNH1-6</i>	<i>VRNH2</i>	<i>vrnH3</i>	<i>PPDH1</i>	<i>PPDH2</i>
SBCC058	<i>VRNH1-4</i>	<i>VRNH2</i>	<i>vrnH3</i>	<i>PPDH1</i>	<i>PPDH2</i>
‘Alexis’	<i>VRNH1-3</i>	<i>vrnH2</i>	<i>vrnH3</i>	<i>ppdH1</i>	<i>PPDH2</i>
Pané	<i>VRNH1-4</i>	<i>VRNH2</i>	<i>vrnH3</i>	<i>PPDH1</i>	<i>PPDH2</i>
Beka	<i>VRNH1-1</i>	<i>vrnH2</i>	<i>vrnH3</i>	<i>ppdH1</i>	<i>PPDH2</i>
Mogador	<i>vrnh1</i>	<i>VRNH2</i>	<i>vrnH3</i>	<i>ppdH1</i>	<i>ppdH2</i>

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'-atccattggtgtgtggtca-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 (3°C, 8-h light). Plants were grown in a phytotron at 18°C, 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 $2^{\Delta C_T}$, where ΔC_T is $(C_{T \text{ Actin}} - C_{T \text{ target gen}})$, 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 $2^{\Delta C_T}$, where ΔC_T is ($C_{T \text{ Actin}} - C_{T \text{ target gen}}$), 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.

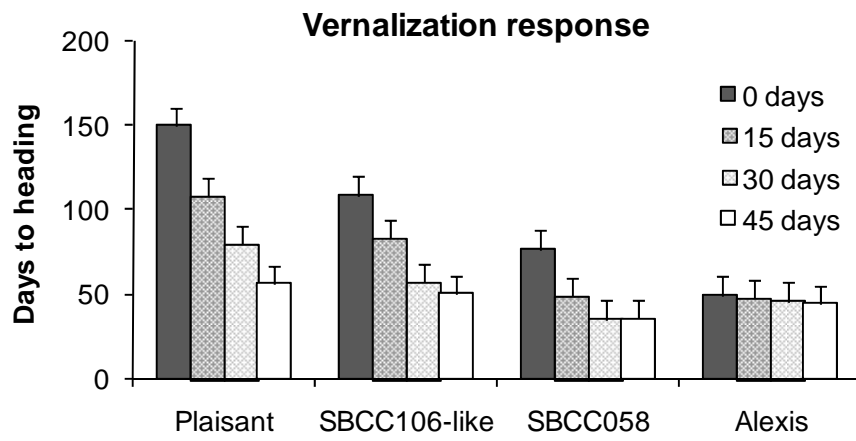


Figure 2.

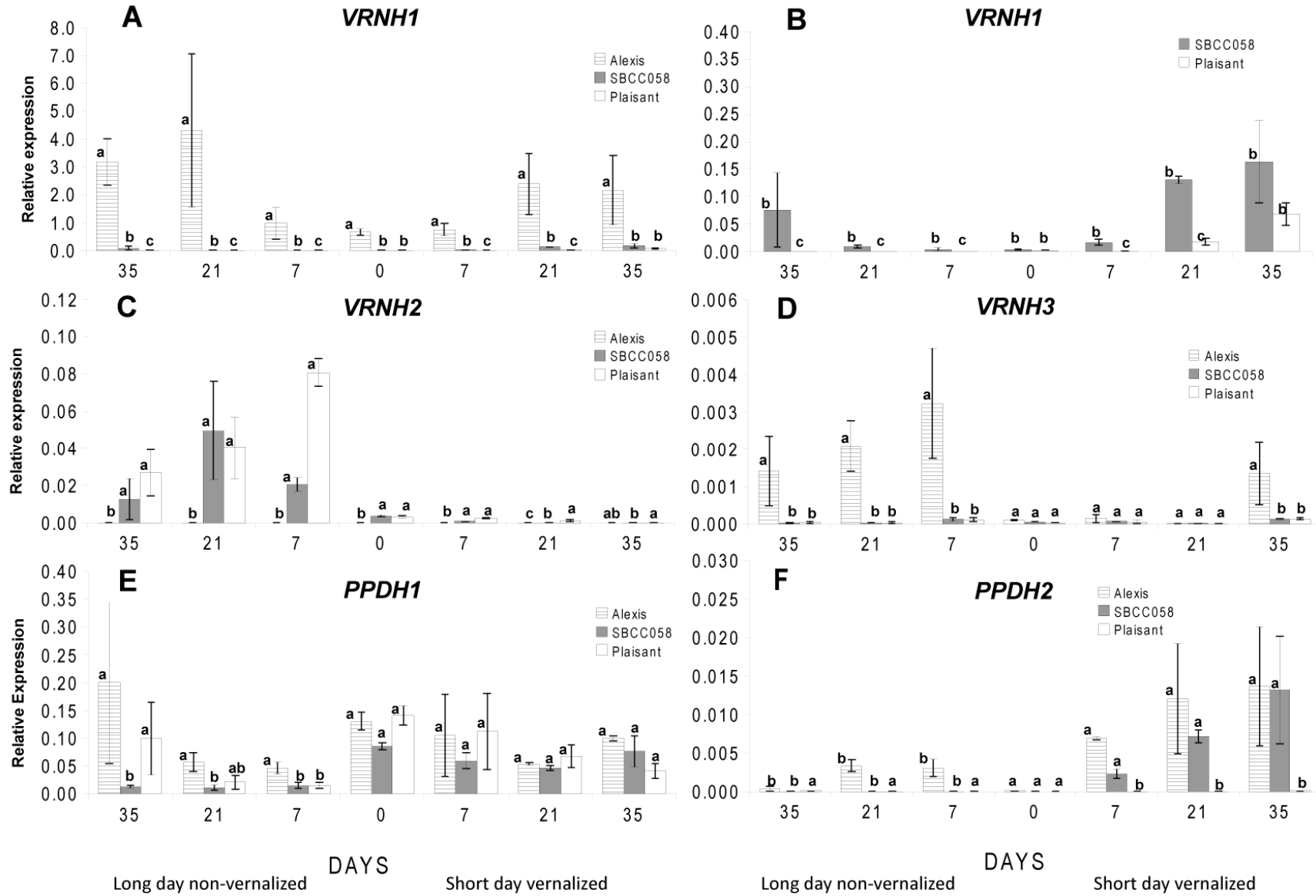


Figure 3.

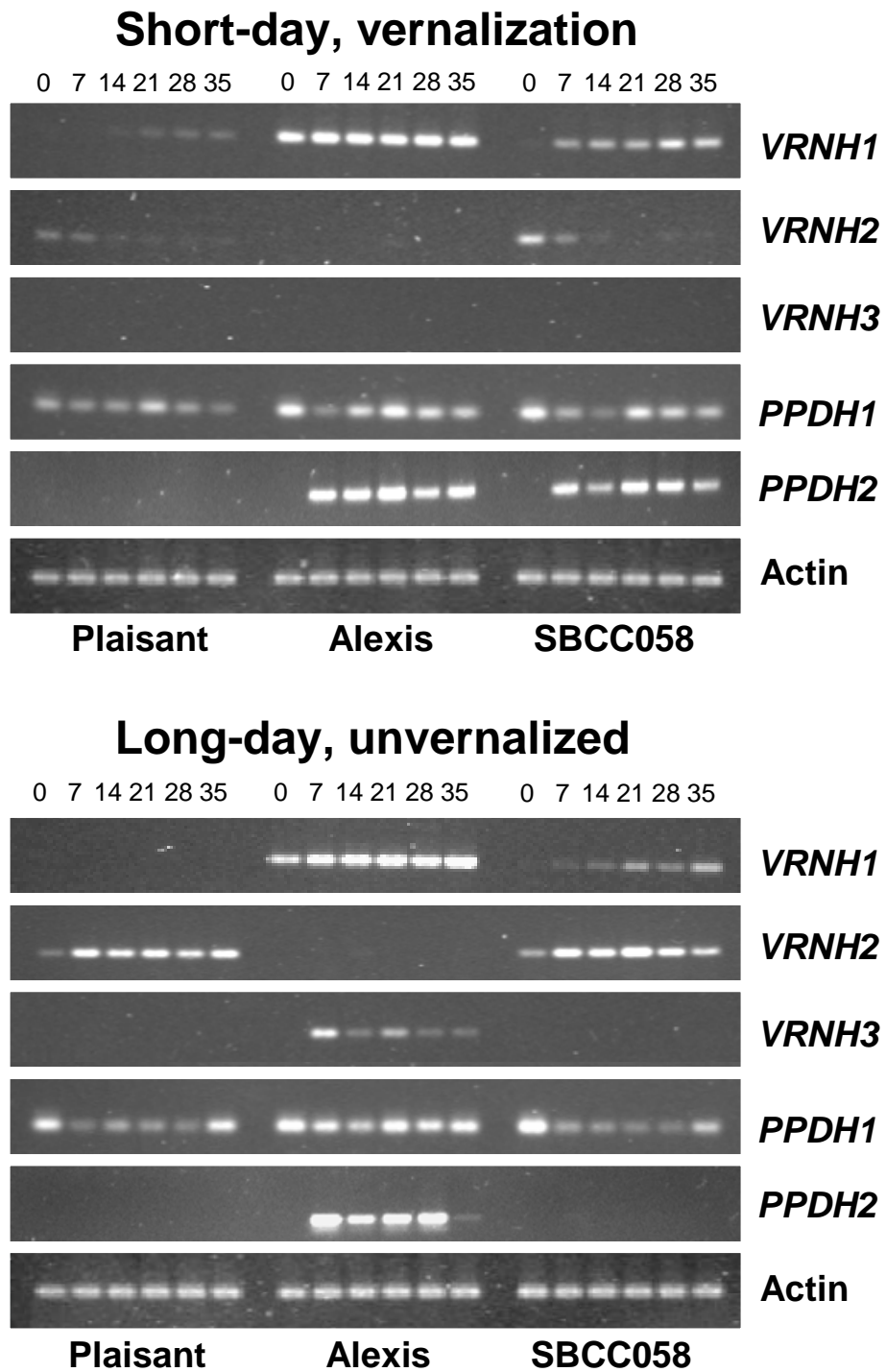


Figure 4.

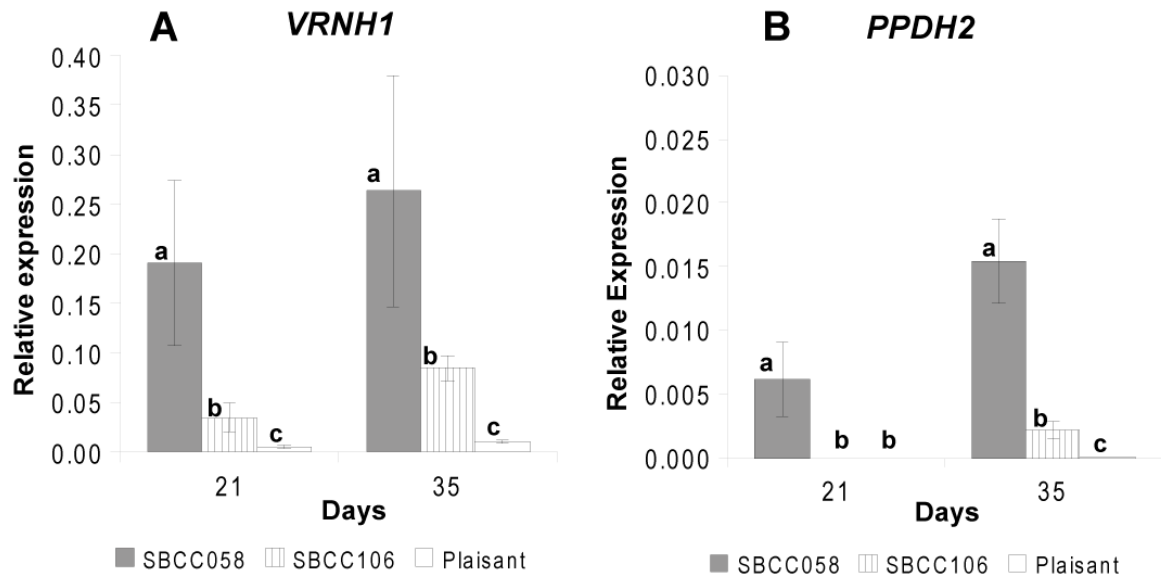


Figure 5.

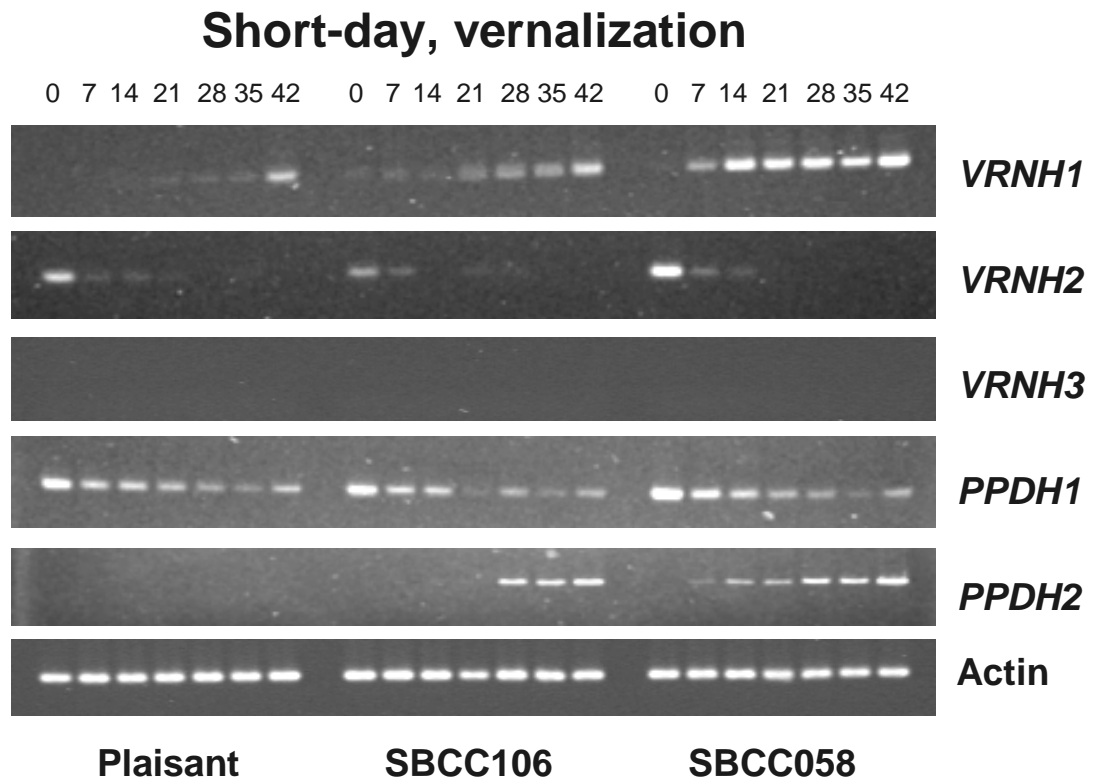
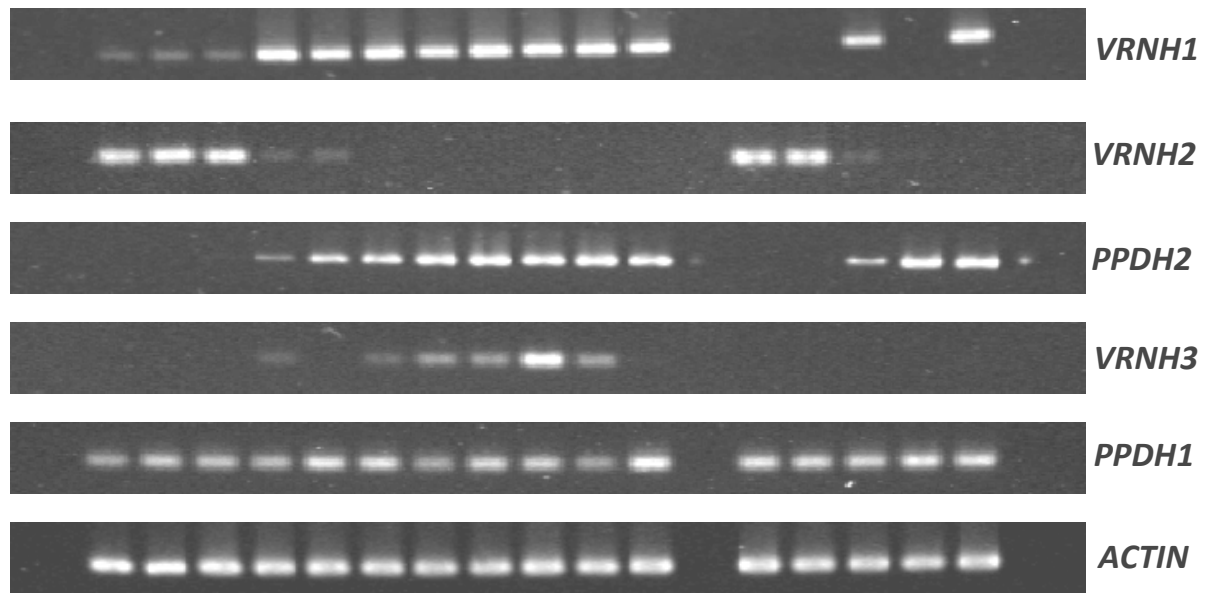


Figure 6.



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

¹Both ‘Alexis’ and ‘Pané’ carry the functional allele in *PPDH2*

²‘Alexis’, ‘Pané’, ‘Beka’, and ‘Mogador’ all carry the same recessive allele in *VRNH3*

³Both ‘Beka’ and ‘Mogador’ carry the allele that conveys insensitivity to long photoperiod in *PPDH1*