

The Genetic Control of Reproductive Development under High Ambient Temperature^{1[OPEN]}

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Ambient temperature has a large impact on reproductive development and grain yield in temperate cereals. However, little is known about the genetic control of development under different ambient temperatures. Here, we demonstrate that in barley (*Hordeum vulgare*), high ambient temperatures accelerate or delay reproductive development depending on the photoperiod response gene *PHOTOPERIOD1* (*Ppd-H1*) and its upstream regulator *EARLY FLOWERING3* (*HvELF3*). A natural mutation in *Ppd-H1* prevalent in spring barley delayed floral development and reduced the number of florets and seeds per spike, while the wild-type *Ppd-H1* or a mutant *Hvelf3* allele accelerated floral development and maintained the seed number under high ambient temperatures. High ambient temperature delayed the expression phase and reduced the amplitude of clock genes and repressed the floral integrator gene *FLOWERING LOCUS T1* independently of the genotype. *Ppd-H1*-dependent variation in flowering time under different ambient temperatures correlated with relative expression levels of the *BARLEY MADS*-box genes *VERNALIZATION1* (*HvVRN1*), *HvBM3*, and *HvBM8* in the leaf. Finally, we show that *Ppd-H1* interacts with regulatory variation at *HvVRN1*. *Ppd-H1* only accelerated floral development in the background of a spring *HvVRN1* allele with a deletion in the regulatory intron. The full-length winter *Hvvrn1* allele was strongly down-regulated, and flowering was delayed by high temperatures irrespective of *Ppd-H1*. Our findings demonstrate that the photoperiodic and vernalization pathways interact to control flowering time and floret fertility in response to ambient temperature in barley.

Climate models predict that an increase in global average temperature will have large impacts on crop yield (Lobell et al., 2011). High temperatures are particularly critical during plant reproductive development and affect flowering time, flower fertility, and seed set. To sustain high crop yields under changing climatic conditions, it is important to understand the genetic basis of plant development in response to ambient temperature.

Temperature-dependent flowering is regulated by the vernalization and ambient temperature pathways. Whereas vernalization requires long periods of cold during the winter, the ambient temperature pathway modulates flowering in response to short-term temperature changes (Wigge, 2013). Research in the model

plant *Arabidopsis* (*Arabidopsis thaliana*), a facultative long-day (LD) plant, has demonstrated that the temperature and photoperiod pathways interact to control reproductive development. For example, high temperature accelerates flowering and overcomes the delay in flowering commonly observed under short photoperiods in *Arabidopsis* (Balasubramanian et al., 2006). Early flowering in response to high temperature was correlated with an increase in the expression of the floral integrator gene *FLOWERING LOCUS T* (*FT*) independently of day length (Halliday et al., 2003; Balasubramanian et al., 2006). The *FT* protein acts as a long-distance signal (florigen) that conveys the information to induce flowering from leaves to the shoot meristem (Corbesier et al., 2007; Jaeger and Wigge, 2007; Kobayashi and Weigel, 2007; Mathieu et al., 2007; Tamaki et al., 2007). In addition, recent studies have identified *EARLY FLOWERING3* (*ELF3*), a repressor of light signals to the circadian clock as an essential component of ambient temperature response (Thines and Harmon, 2010). *ELF3* forms together with *ELF4* and *LUX ARRHYTHMO* (*LUX*), the so-called "evening complex" (EC) that functions as a night-time repressor of gene expression in the circadian clock of *Arabidopsis* (Nusinow et al., 2011; Herrero et al., 2012). The circadian clock is an autonomous oscillator that produces endogenous biological rhythms with a period of about 24 h and consists of at least three interlocking feedback loops. The core loops comprise (1) the inhibition of EC

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genes by CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY) late at night, (2) the inhibition of *PSEUDO RESPONSE REGULATOR* (PRR) genes by the EC early at night, and (3) the inhibition of *LHY/CCA1* by TIMING OF CAB EXPRESSION1/PRR1 in the morning (Pokhilko et al., 2012).

Several independent studies have recently found that elevated temperatures, specifically during dark periods, inhibit the activity of the EC by an unknown mechanism (Box et al., 2015; Mizuno et al., 2014; Thines et al., 2014; Raschke et al., 2015), leading to increased expression of *PHYTOCHROME-INTERACTING FACTOR4* (*PIF4*; Koini et al., 2009). *PIF4* binding to the promoter of *FT* and consequent transcriptional activation of *FT* is promoted by an improved chromatin accessibility through temperature-dependent histone modifications at the *FT* promoter (Kumar and Wigge, 2010; Kumar et al., 2012). However, high temperature also accelerated flowering in *pif4* mutants under long photoperiods, suggesting that a *PIF4*-independent thermoresponsive flowering pathway acts through components of the photoperiod pathway (Koini et al., 2009; Press et al., 2016).

In addition, the MADS-box genes *SHORT VEGETATIVE PHASE*-like (*SVP*), *FLOWERING LOCUS C* (*FLC*), and *FLOWERING LOCUS M* (*FLM*; *MAF1*) play a role in the thermosensory regulation of flowering in Arabidopsis (Balasubramanian et al., 2006; Lee et al., 2007; Gu et al., 2013). Loss of function of either *SVP* or *FLM* results in partial temperature-insensitive early flowering (Balasubramanian et al., 2006; Lee et al., 2007, 2013; Posé et al., 2013). Moreover, *FLM* is subject to temperature-dependent alternative splicing (Balasubramanian et al., 2006; Sureshkumar et al., 2016) resulting in two major splice forms, that either facilitate or inhibit *SVP* dependent repression of *FT*, and the floral homeotic genes *SUPPRESSOR OF OVEREXPRESSION1* and *SEPALLATA* (Posé et al., 2013). Interestingly, a structural polymorphism in the first intron of *FLM* affects its expression, splicing, and also regulates flowering predominantly at lower ambient temperatures (Lutz et al., 2015). Such structural polymorphisms within the first intron are typical within the family of MADS-box transcription factor genes and play an important role for expression variation and possibly adaptation to different environments across different species (Hong et al., 2003; Distelfeld et al., 2009; Schauer et al., 2009; Yoo et al., 2011).

While flowering-time control in response to temperature is well described in Arabidopsis, little is known about the genetic determinants of ambient temperature response in cereal grasses (Bullrich et al., 2002; Appendino and Slafer, 2003; Lewis et al., 2008; Hemming et al., 2012). In barley (*Hordeum vulgare*), a complex interplay between day length and temperature in the regulation of flowering has been reported. Under LD conditions, barley plants accelerated reproductive development at 25°C compared with 15°C, whereas the opposite was the case under short days (SDs) (Hemming et al., 2012). In contrast to Arabidopsis, the transcript

level of the barley homolog of *FT* was not influenced by temperature, and no clear candidate genes for the integration of thermal signals into the flowering-time pathways have been identified so far (Hemming et al., 2012). Barley is a facultative LD plant and is characterized by winter and spring growth habits as determined by natural variation at the two vernalization genes, *VERNALIZATION1* (*HvVRN1*; *HvBM5a*) and *HvVRN2* (Yan et al., 2003, 2004; Trevaskis et al., 2006). Winter types accelerate flowering after a prolonged period of cold (vernalization), whereas spring barley does not respond to vernalization. The MADS-box gene *HvVRN1* is characterized by a series of different deletions and insertions in the first regulatory intron, which has been linked to differences in vernalization response and flowering behavior (Hemming et al., 2009). Photoperiod response, rapid flowering under LDs, is determined by natural variation at the *PHOTOPERIOD-H1* (*Ppd-H1*) gene, which is homologous to the *PRR* genes of the circadian clock in Arabidopsis (Turner et al., 2005). The wild-type allele is prevalent in winter barley, while a natural mutation in the conserved CCT domain of *Ppd-H1* causes a delay in flowering under LDs and is predominant in spring barley from cultivation areas with long growing seasons (Turner et al., 2005; von Korff et al., 2006, 2010; Wang et al., 2010). *Ppd-H1* induces flowering under LDs by up-regulating *HvFT1*, the barley homolog of *FT* in Arabidopsis (Turner et al., 2005; Campoli et al., 2012b). *Ppd-H1* is repressed during the night by *HvELF3*, *HvLUX1*, and *PHYTOCHROME C*, and mutations in these genes result in a day-neutral up-regulation of *HvFT1* and early flowering (Faure et al., 2012; Zakhrebekova et al., 2012; Campoli et al., 2013; Pankin et al., 2014). Consequently, the major vernalization and photoperiod response genes are known in barley, whether these also play a role for thermoresponsive flowering is not known.

In Arabidopsis, commonly used macroscopic indicators of reproductive phase change or floral transition are time to bolting or rosette leaf number under the first open floral bud (Pouteau and Albertini, 2009). Under optimal conditions, floral transition, bolting, and flowering are well correlated in Arabidopsis. In barley, most stages of reproductive development, including flowering, occur within the leaf sheath and can therefore only be scored upon dissection of the shoot. Waddington et al. (1983) developed a quantitative scale for barley and wheat development based on the morphogenesis of the shoot apex and carpels. This scale is based on the progression of the most advanced floret primordium and carpel of the inflorescence. The enlargement of the apical dome at Waddington stage (W) 1.0 represents an apex that is transitioning to a reproductive state and indicates the end of the vegetative phase. The emergence of the first floret primordia on the shoot apex at the double ridge stage (W2.0) specifies a reproductive shoot apical meristem (SAM). At the stamen primordium stage (W3.5), the first floral organ primordia differentiate, and stem elongation initiates. In barley, the induction of floret primordia on the

inflorescence continues until the awn primordium stage (W5.0). Anthesis and pollination of the most advanced floret occurs at the last stage of the Waddington scale (W10.0). This last step can be scored macroscopically because it is marked by the emergence of awn tips from the top of the leaf sheath (heading). Most commonly, flowering is scored as heading in barley. However, the different phases of shoot apex development differ in their sensitivity to environmental cues and are controlled by different genetic factors, so that floral transition and flowering may not be correlated and separated in time by many weeks (Digel et al., 2015). Variation in the timing of different developmental phases in turn affects the number of floret primordia, fertile flowers, and seeds per spike (Digel et al., 2015). To better understand the effects of temperature on development, it is therefore important to investigate the effects of environmental and genetic variation on individual phases of shoot apex development.

The objective of present study was to elucidate the genetic control of reproductive development under high ambient temperature in barley. We show that high ambient temperature delays the phase and reduces the amplitude of clock gene expression. Further, we demonstrate that under high ambient temperature, flowering time and seed number are controlled by interactions between *Ppd-H1* and *HvVRN1* and correlate with expression levels of the *BARLEY MADS*-box genes *HvBM3* and *HvBM8* in the leaf. These findings provide new insights into the genetic and molecular control of flowering time and inflorescence development under high ambient temperature in barley.

RESULTS

High Ambient Temperature Delays Reproductive Development and Reduces Seed Set in Spring Barley

To examine the effect of high ambient temperature on flowering in barley, we scored the spring barley genotypes Bowman and Scarlett for days to flowering under control (20°C/16°C) and high temperatures (28°C/24°C) in LDs. These genotypes carry a mutated *ppd-H1* allele, a functional *HvELF3* allele, and a spring *HvVRN1* allele and therefore do not respond to vernalization and are late flowering under LDs. Flowering was significantly delayed in both Bowman and Scarlett under high as compared to control temperatures (Fig. 1A). In addition, high temperature reduced floret and seed number per spike in both genotypes (Fig. 1, B and C). The total number of florets and seeds per spike were reduced in Bowman by 19% and 34% and in Scarlett by 30% and 74%, respectively, at high compared to control temperatures (Fig. 1, B and C). Under short-day condition (8 h light/16 h dark), Bowman and Scarlett plants never flowered neither under control nor under high ambient temperature conditions (data not shown).

The effect of increased temperature on floral development was evaluated by monitoring the progression

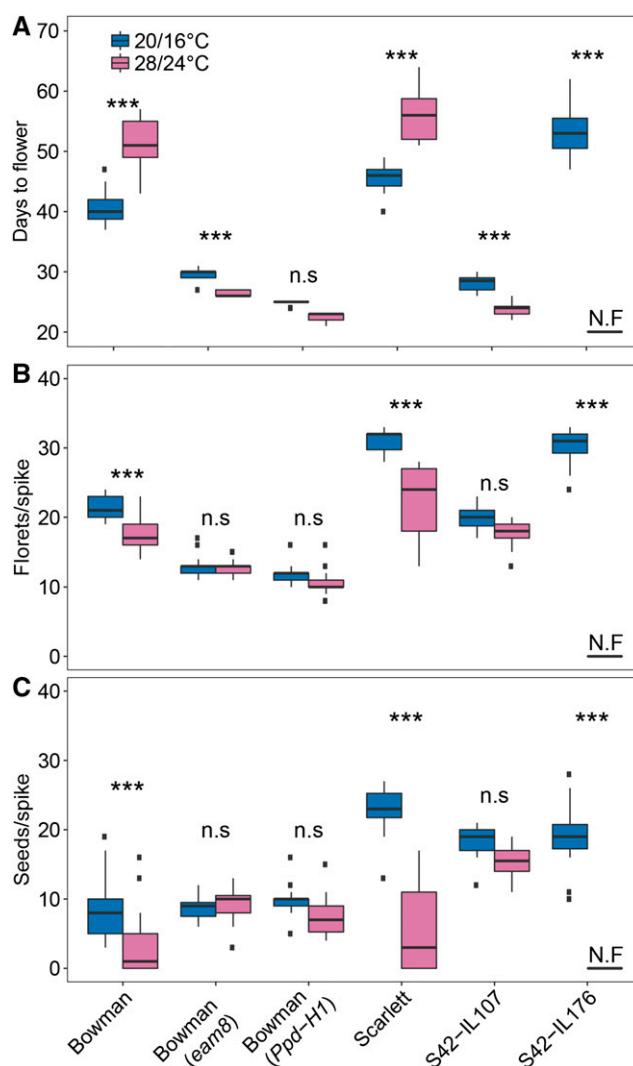


Figure 1. High ambient temperature affects flowering time, floret, and seed number per main spike in barley. Days to flower (A), the number of florets (B), and the number of seeds per main spike (C) under control (blue; 20°C/16°C, day/night) and high ambient temperatures (pink; 28°C/24°C, day/night) in the spring barley varieties Bowman and Scarlett and the derived ILs Bowman(*eam8*) (*Hvelf3*), Bowman(*Ppd-H1*), S42-IL107 (*Ppd-H1*), and S42-IL176 (*Hvvrn1*). Flowering time, floret, and seed number were recorded for 20 plants per genotype and treatment under LDs (16 h light/8 h night). N.F. indicates nonflowering plants. Statistical differences were calculated by a two-factorial ANOVA and a post-hoc Tukey's multiple comparison test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; n.s. = nonsignificant.

of the main shoot apex (MSA) in Bowman and Scarlett plants grown at 20°C/16°C and 28°C/24°C according to the Waddington scale (Waddington et al., 1983). Microscopic dissection of the MSA revealed that high temperature did not have a strong effect on floral transition (W2.0), but greatly delayed the late reproductive phase of inflorescence development (after W3.5) both in Bowman and Scarlett (Fig. 2, A and C). In summary, high ambient temperature primarily delayed inflorescence development and reduced the number of

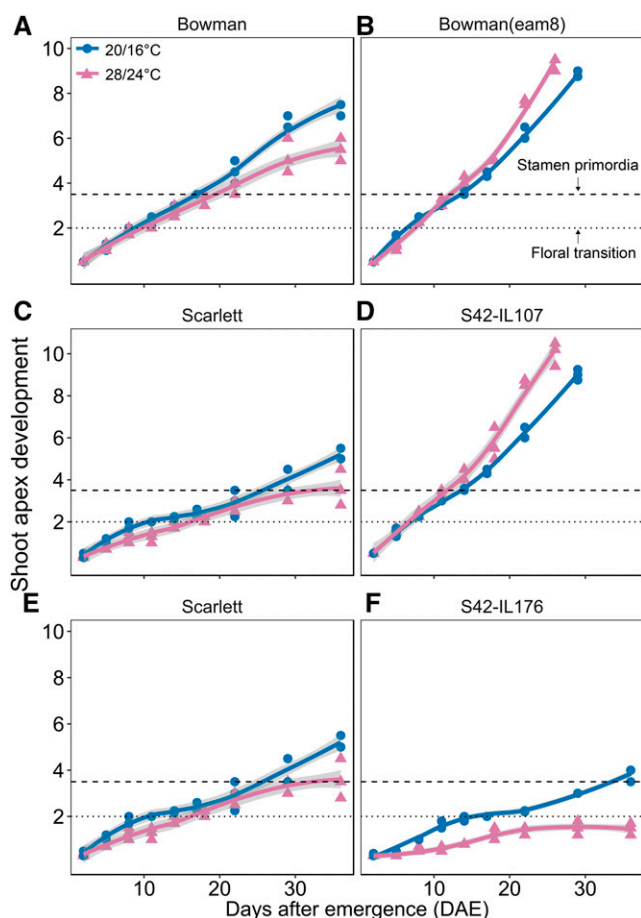


Figure 2. High ambient temperature affects MSA development in barley. Microscopic development of the MSA was scored under control (blue; 20°C/16°C, day/night) and high ambient (pink; 28°C/24°C, day/night) temperatures every 3 d according to the Waddington scale (Waddington et al., 1983). MSA development was delayed under high compared to control temperature in Bowman (A) and Scarlett (C and E), accelerated in Bowman(*eam8*) (B) and S42-IL107 (D), and further delayed floral transition in S42-IL176 (F). Three or four plants per genotype were dissected at each time point in each treatment under LDs (16 h light/8 h night). Statistical differences ($P < 0.05$) were calculated using a polynomial regression model at 95% confidence interval (Loess smooth line).

seeds per spike in the spring barley genotypes Bowman and Scarlett.

High Ambient Temperature Accelerates Flowering Time in Genotypes with a Nonfunctional *Hvelf3* Allele and a Dominant *Ppd-H1* Allele

In *Arabidopsis*, the circadian clock and photoperiod pathways modulate ambient temperature responses to regulate flowering. Therefore, we further characterized reproductive development in introgression lines (ILs) with a nonfunctional *Hvelf3* or dominant *Ppd-H1* alleles under control and high ambient temperatures. *HvELF3* is a component of the EC in *Arabidopsis* and represses

Ppd-H1 expression in the night in barley (Faure et al., 2012). Therefore, the barley *Hvelf3* mutant genotype is characterized by high expression of *Ppd-H1* during the night (Faure et al., 2012). The IL Bowman(*eam8*) carrying a nonfunctional *Hvelf3* allele in the background of Bowman and the ILs S42-IL107 and Bowman(*Ppd-H1*) with the wild-type *Ppd-H1* gene in the background of Scarlett and Bowman were analyzed along with the parental genotypes for flowering time, floret fertility, and seed set. In addition, the microscopic development of the MSA was evaluated in Scarlett, Bowman, S42-IL107, and Bowman(*eam8*) under control and high ambient temperatures.

Microscopic dissection of the MSA revealed that in contrast to Bowman with a delayed development under high temperatures, Bowman(*eam8*) showed an accelerated MSA development at 28°C/24°C compared to 20°C/16°C (Fig. 2B). As a result, Bowman(*eam8*) plants flowered on average 5 d earlier at high compared to control temperatures (Fig. 1A).

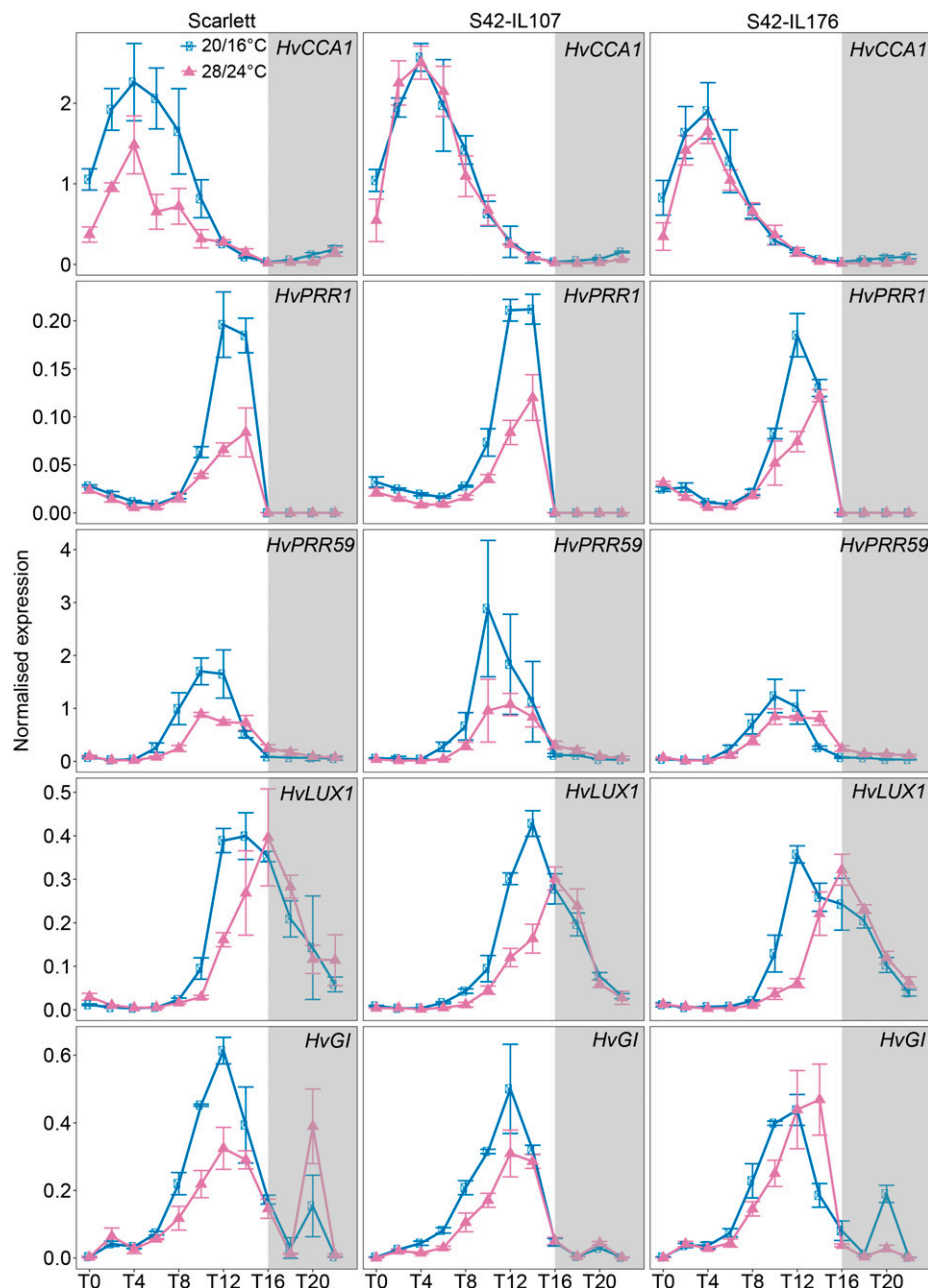
Since *HvELF3* might control flowering time through its downstream target *Ppd-H1*, we evaluated if variation at *Ppd-H1* mediated the flowering response under high ambient temperature. In contrast to the parental lines, S42-IL107 and Bowman(*Ppd-H1*) plants flowered on average 7 and 2 d earlier under high ambient compared to control temperatures (Fig. 2D). The dissection of the MSA in Scarlett and S42-IL107 revealed that high ambient temperature accelerated in particular the phase of stem elongation and inflorescence development (Fig. 2D). In addition, the ANOVA for floret and seed number revealed a significant interaction between *Ppd-H1* and *HvELF3* with temperature (Supplemental Table S1). High ambient temperatures caused a larger reduction in floret and seed number in Bowman and Scarlett than in the ILs Bowman(*eam8*), S42-IL107, and Bowman(*Ppd-H1*) (Fig. 1, B and C; Supplemental Table S1).

Taken together, high ambient temperature affected inflorescence development and flowering time in a *HvELF3*- and *Ppd-H1*-dependent manner. Quantitative variation in the reduction of seed number under high ambient temperatures was dependent on *HvELF3* and *Ppd-H1*.

Variation at *HvVRN1* Affects Reproductive Development under High Ambient Temperature

Natural variation in the length of the first regulatory intron of *HvVRN1* has a strong effect on vernalization response in barley. Therefore, we examined whether this variation also affected the response to ambient temperature variation in barley. For this purpose, we compared the development of Scarlett with that of S42-IL176. Scarlett carries a spring *HvVRN1* allele with a deletion in the first regulatory intron, whereas S42-IL176 carries an introgression of the full-length winter *Hvvrn1* allele. Although high ambient temperature delayed reproductive development in both genotypes,

Figure 3. Diurnal expression patterns of circadian clock genes in Scarlett, S42-IL107, and S42-IL176 under control and high ambient temperatures. Diurnal expression of circadian clock genes was assayed every 2 h for 24 h under control (blue; 20°C/16°C, day/night) and high ambient (pink; 28°C/24°C, day/night) temperatures under LDs (16 h light/8 h night). Gray boxes indicate nights. Error bars indicate $\pm$ SD of three biological replicates.



the effect was more pronounced in S42-IL176, which did not undergo floral transition and did not flower until 160 d after emergence (DAE) when the experiment was stopped (Fig. 2F, 1A). Consequently, the full-length intron of *Hvvrn1* was correlated with a strong delay in floral transition under high ambient temperatures. In order to assess if variation at *HvVRN1* was also associated with inflorescence development in response to ambient temperature, we shifted Scarlett and S42-IL176 plants from 20°C/16°C to 28°C/24°C only after floral transition (W2.0). Under these conditions, the IL with the winter *Hvvrn1* allele also showed a strong delay in inflorescence development under high ambient temperatures compared to control conditions

(Supplemental Fig. S1B). Flowering was delayed by about 2 weeks under 28°C/24°C compared to 20°C/16°C. However, S42-IL176 plants were able to produce flowers and seeds, when the temperature treatment was started after floral transition (Supplemental Fig. S1C).

High Ambient Temperature Affects the Expression of Clock Genes

To further characterize the *Ppd-H1*-, *HvELF3*-, and *HvVRN1*-dependent effects of high temperature on barley development, we analyzed the expression of

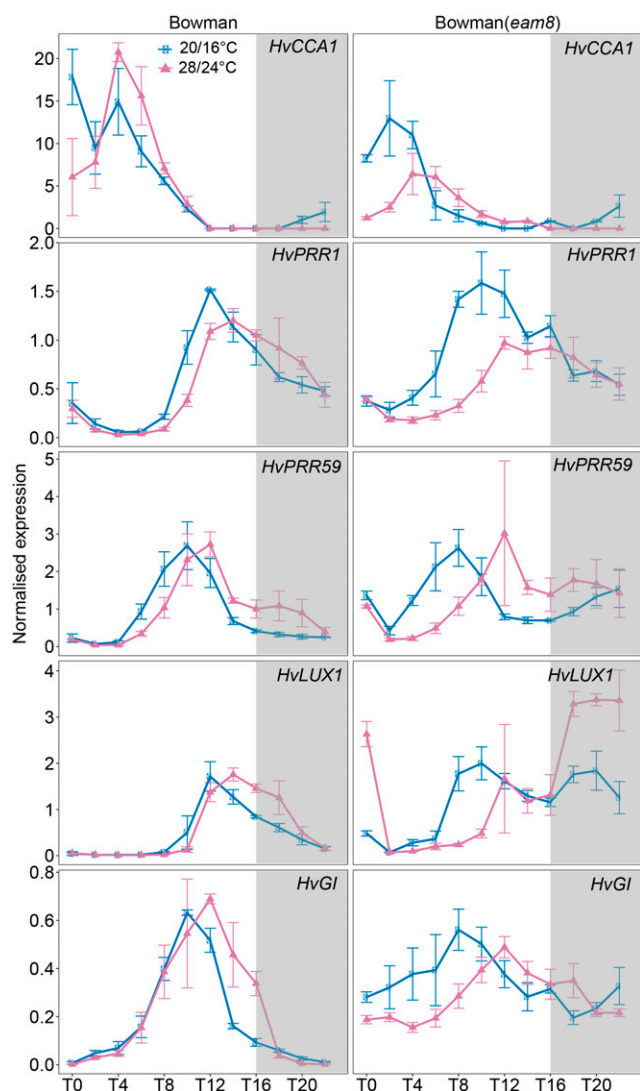


Figure 4. Diurnal expression of circadian clock genes in Bowman and Bowman(*eam8*) under control and high ambient temperatures. Diurnal expression of circadian clock genes was assayed every 2 h for 24 h under control (blue; 20°C/16°C, day/night) and high ambient (pink; 28°C/24°C, day/night) temperatures under LDs (16 h light/8 h night) are shown. Gray boxes indicate nights. Error bars indicate $\pm$ SD of three biological replicates.

barley genes from the circadian clock, photoperiod, and vernalization response pathways in the parental and ILs. Because the barley clock is plastic under abiotic stresses, we first tested the effects of high ambient temperature on variation in the diurnal pattern of clock gene expression. Under control conditions, the circadian clock genes showed a diurnal pattern of expression with clock genes peaking at different times of the day, corroborating previous results (Campoli et al., 2012b; Habte et al., 2014). The expression phase of clock genes did not differ between the parental lines Scarlett, and the ILs S42-IL107 and S42-IL176, suggesting that *Ppd-H1* and

HvVRN1 did not affect diurnal clock oscillations. By contrast, the expression phase and shape of clock genes were significantly different between Bowman and Bowman(*eam8*). The expression phase of the clock genes in Bowman(*eam8*) was advanced by 2 h. The expression peaks were less defined and broader in Bowman(*eam8*) than in Bowman. Moreover, Bowman(*eam8*) exhibited higher levels of *Ppd-H1* expression at most time points during the day compared to Bowman. Consequently, the loss-of-function mutation in *HvELF3* affected the diurnal pattern of clock gene expression and caused a strong increase in *Ppd-H1* expression independent of the ambient temperature.

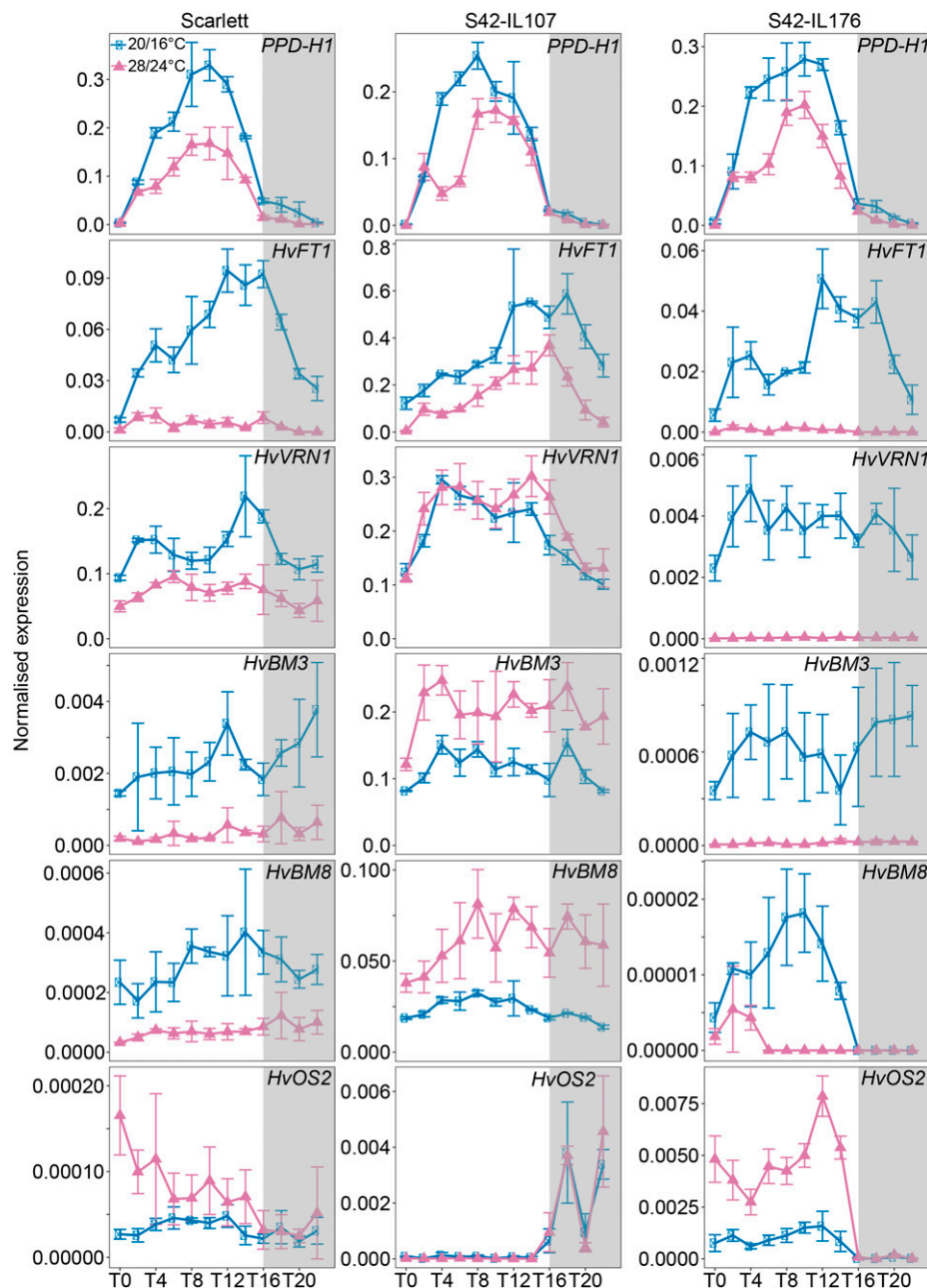
High ambient temperatures caused a decrease in the expression of clock genes, as seen for most clock genes in Scarlett and for *HvCCA1* and *HvPRR1* in Bowman (Figs. 3 and 4). Furthermore, the expression phase of clock genes was delayed by 4 h under high ambient temperature compared to control conditions in Scarlett and Bowman. This reduction in expression amplitude and the shift in the expression phase were also observed in all ILs, suggesting that temperature affected the phase of clock gene expression independently of the genotype.

High Ambient Temperature Reduces Expression of Flowering Time Genes

As the clock genes are putative upstream regulators of flowering-time genes, we investigated whether the temperature-dependent changes in clock gene expression correlated with changes in the expression of flowering-time genes. As observed for the clock genes, most flowering-time regulators showed a significantly lower expression under high ambient temperature. *Ppd-H1* exhibited a reduction in expression in Scarlett, Scarlett-derived ILs, and Bowman(*eam8*), but not in Bowman under high ambient temperature. The expression levels of *HvCO1*, the barley homolog of the major Arabidopsis photoperiod response gene *CONSTANS*, were reduced, and the peak expression was delayed by approximately 4 h under high ambient temperature in Scarlett and Scarlett-derived ILs (Supplemental Fig. S4). While in Bowman *HvCO1* expression peaked at dusk (T16) under control temperature, it showed an expression peak in the night at T20 under high ambient temperature (Supplemental Fig. S5). This suggested that *HvCO1* expression was controlled by the clock and a temperature-dependent phase shift of clock genes. However, no consistent changes in the level and peak time of *HvCO1* expression were observed in Bowman and Bowman(*eam8*) (Supplemental Fig. S5).

The expression levels of *HvFT1*, a putative target of *Ppd-H1*, were significantly down-regulated under high temperature in all genotypes. In addition, *HvFT1* expression levels were overall significantly different between genotypes with higher transcript abundance in

Figure 5. High ambient temperature affects the expression of flowering-time genes in Scarlett, S42-IL107, and S42-IL176 under control and high ambient temperatures. Diurnal expression of flowering-time genes was assayed every 2 h for 24 h under control (blue; 20°C/16°C, day/night), and high ambient (pink; 28°C/24°C, day/night) temperatures under LDs (16 h light/8 h night) are shown. Gray boxes indicate nights. Error bars indicate $\pm$ SD of three biological replicates.



S42-IL107 and Bowman(*eam8*) and lower transcript levels in S42-IL176 compared to the parental lines (Figs. 5 and 6).

The MADS-box genes *HvVRN1*, *HvBM3*, and *HvBM8* were also strongly down-regulated under high versus control temperatures. In S42-IL176, the expression levels of the winter *Hvvrn1* allele were 90-fold lower, while the expression levels of the spring *HvVRN1* allele in Scarlett were only 2-fold lower under high ambient compared to control temperature (Fig. 5). This suggested that the winter allele of *HvVRN1* was repressed by high ambient temperatures (Fig. 5). The expression patterns of *HvBM3* and *HvBM8* were comparable to

those of *HvVRN1* with a stronger temperature-dependent down-regulation in S42-IL176 compared to Scarlett. In contrast, S42-IL107 with a dominant *Ppd-H1* allele exhibited an up-regulation of *HvVRN1*, *HvBM3*, and *HvBM8* under high compared to control temperatures (Fig. 5). In Bowman(*eam8*), expression levels of the *HvBM* genes were approximately 10-fold higher compared to Bowman under control and high ambient temperature conditions. In addition, *HvVRN1* and *HvBM3* were only slightly down-regulated under high versus control temperatures, while expression of *HvBM8* was not significantly different between control and high-temperature conditions (Fig. 6). *HvOS2*, a

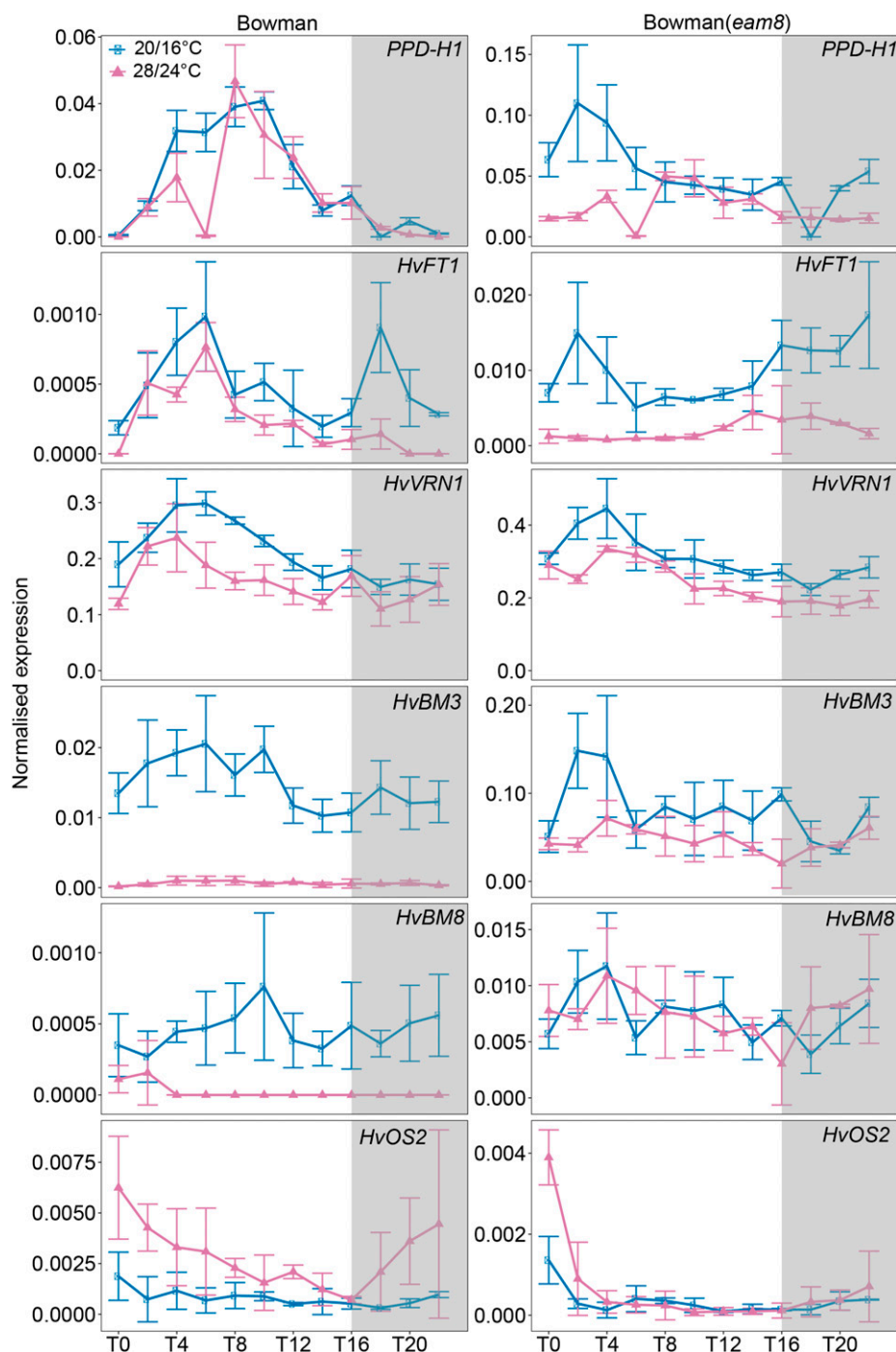


Figure 6. Diurnal expression of flowering-time genes in Bowman and Bowman(*eam8*) under control and high ambient temperatures. Diurnal expression of flowering-time genes sampled every 2 h for 24 h under control (blue; 20°C/16°C, day/night) and high ambient (pink; 28°C/24°C, day/night) temperatures under LDs (16 h light/8 h night) are shown. Gray boxes indicate nights. Error bars indicate $\pm$ SD of three biological replicates.

repressor of flowering and homolog of the major Arabidopsis vernalization gene *FLC* (Greenup et al., 2010, Ruelens et al., 2013), was up-regulated under high versus control temperatures and was controlled by *Ppd-H1*, *HvELF3*, and *HvVRN1*. Expression levels of *HvOS2* were up-regulated under high ambient temperature in Scarlett and Bowman, but very low during the day in S42-IL107 and Bowman(*eam8*) under both temperatures. *HvOS2* expression levels were further increased under high temperatures in S42-IL176 with the winter

Hvvrn1 allele and no detectable expression of *Hvvrn1*. *HvOS2* expression levels were consequently negatively correlated with *HvVRN1* expression and controlled by ambient temperature.

Variation at *Ppd-H1* and *HvELF3*, therefore, correlated with the temperature-dependent regulation of the MADS-box transcription factor genes. It is interesting to note, that in S42-IL107, the expression patterns of *HvFT1* and the *HvBM* genes were not correlated under the different temperature regimes, as the *HvBM* genes

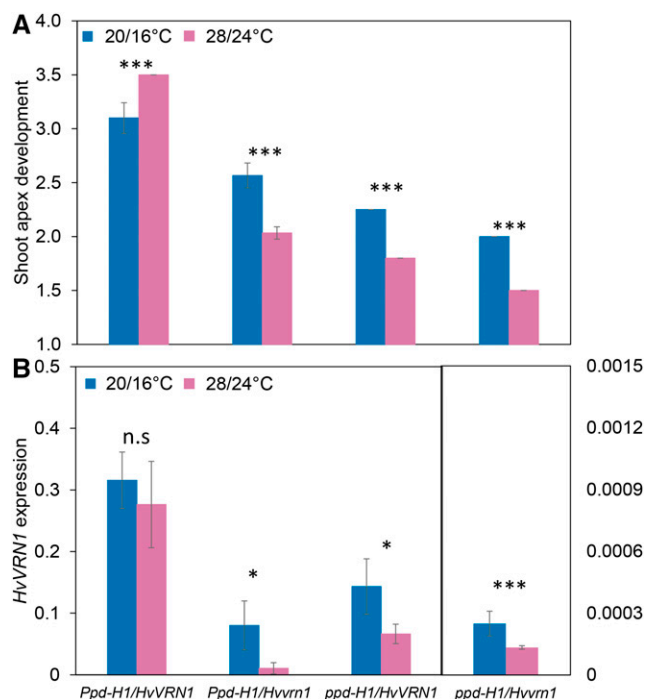


Figure 7. *Ppd-H1* and *HvVRN1* interact to control the development of the MSA under different ambient temperatures. Microscopic changes in MSA development were scored under control (blue; 20°C/16°C, day/night) and high ambient (pink; 28°C/24°C, day/night) temperatures under LDs (16 h light/8 h night) in F₃ families derived from a cross between the winter barley Igri and the spring barley Golden Promise. Selected F₃ families segregated for *Ppd-H1* and *HvVRN1* and were fixed for the spring alleles at *HvVRN2* (deleted) and *HvFT1*. Early MSA development was accelerated under high temperature in *Ppd-H1/HvVRN1* and delayed in *Ppd-H1/Hvvrn1*, *ppd-H1/HvVRN1*, and *ppd-H1/Hvvrn1*. Significant differences were determined by a two-way ANOVA and a Tukey HSD pairwise comparison test: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, n.s. = nonsignificant.

were up-regulated, but *HvFT1* was down-regulated under high compared to control temperatures. The expression patterns of the *HvBM* genes, but not of *HvFT1*, correlated with the differential flowering time in response to high ambient temperatures. Low expression of the *HvBM* genes under high temperatures in Scarlett and Bowman coincided with a delay in reproductive development, while accelerated inflorescence development in S42-IL107 correlated with an up-regulation of the *HvBM* genes under high ambient versus control temperatures. In Bowman(*eam8*) with accelerated development under high temperatures, the expression of *HvBM3* and *HvBM8* was strongly increased compared to Bowman and not very different between temperature regimes. In S42-IL176 with a winter *Hvvrn1* allele, a complete down-regulation of *HvFT1* and *HvBM* genes correlated with a strong delay in reproductive development, as this genotype did not undergo floral transition under high temperatures.

Taken together, the wild-type *Ppd-H1* and a loss-of-function *Hvelf3* allele correlated with an accelerated

development under high compared to control temperatures and a higher expression of *HvBM* genes under high compared to control temperatures. In addition, variation in the regulatory region of the first intron in *HvVRN1* controlled the expression of *HvVRN1* itself, of the related *HvBM* genes and reproductive development under high ambient temperatures.

Ppd-H1 and *HvVRN1* Interact to Control Inflorescence Development under High Ambient Temperatures

Our results showed that variation at *Ppd-H1* was correlated with the expression of *HvBM* genes including *HvVRN1* under high compared to control temperatures. Therefore, we examined if *Ppd-H1* and *HvVRN1* interacted to control reproductive development under different ambient temperatures. For this purpose, we analyzed MSA development and gene expression of *HvVRN1* in F₃ families selected from a cross between the winter barley variety Igri and the spring barley variety Golden Promise. The F₃ families were segregating for variation at *Ppd-H1* and *HvVRN1* but were fixed for the spring alleles at the other major flowering loci *HvVRN2* and *HvFT1*. Reproductive development was delayed under high ambient temperatures in F₃ plants with a spring *ppd-H1* allele irrespective of the *HvVRN1* allele as seen for Scarlett and S42-IL176. In addition, under high ambient temperature, the dominant *Ppd-H1* allele accelerated development in the background of a spring *HvVRN1* allele, as observed for S42-IL107. F₃ plants carrying a winter *Hvvrn1* allele and a wild-type *Ppd-H1* allele exhibited a delay in MSA development under high ambient temperature compared to control conditions (Fig. 7A). Consequently, *Ppd-H1* interacted with *HvVRN1* to control the development under high temperatures, where only plants with a dominant *Ppd-H1* and a spring *HvVRN1* allele showed an accelerated development under high versus control temperatures. The expression of the spring *HvVRN1* allele was not affected in the presence of a dominant *Ppd-H1* allele under high ambient versus control temperatures. However, the winter *Hvvrn1* allele was down-regulated in the *Ppd-H1* and *ppd-H1* backgrounds under high compared to control temperature (Fig. 7B). The winter *Hvvrn1* allele was stronger down-regulated than the spring *HvVRN1* allele as shown for Scarlett and S42-IL176. These results indicated that *Ppd-H1* interacts with *HvVRN1*, where a dominant *Ppd-H1* allele only accelerated floral development under high ambient temperature in the background of a spring *HvVRN1* allele.

DISCUSSION

Understanding how ambient temperature controls plant development and eventually grain yield in crop plants is gaining importance in the light of a predicted increase in average global temperatures. The circadian clock influences plant adaptation to different abiotic

stresses and controls many different output traits, including plant development. Furthermore, the circadian clock itself is altered in response to changing environmental conditions. For example, osmotic stress increased the amplitude and advanced the expression phase of clock genes in barley, and high salinity resulted in a lengthening of the circadian period in wheat (Erdei et al., 1998; Habte et al., 2014). We found that an increase in ambient temperature from 20°C/16°C to 28°C/24°C decreased expression levels and delayed the phase of clock gene expression. Although the clock is temperature-compensated and maintains an approximately 24 h period over a range of ambient temperatures (Pittendrigh, 1954; Gould et al., 2006; Salomé et al., 2010), previous studies have reported changes in the expression phase and amplitude of oscillator components under different temperatures. For example, in *Arabidopsis* peak expression levels of *CCA1* and *LHY* RNA rhythms increased in amplitude as temperatures decreased from 17°C to 12°C (Gould et al., 2006; Mizuno et al., 2014). Temperatures of above 30°C are considered as heat stress for temperate cereals (Barnabás et al., 2008). However, an induction of a stress response when increasing the temperature from 20°C to 28°C cannot be excluded. Therefore, the observed alterations in clock oscillations in this work may be related to changes in the level of stress-response hormones. In *Arabidopsis*, application of the stress hormone abscisic acid lengthened the period of the *Arabidopsis* clock (Hanano et al., 2006), probably through evolutionary conserved ABA-responsive elements (ABREs) ABREs present in the promoters of *TIMING OF CAB EXPRESSION1*, *LHY*, and *CCA1* (Bieniawska et al., 2008; Spensley et al., 2009; Picot et al., 2010; Habte et al., 2014). In addition, the heat shock transcription factor *HsfB2b* repressed transcription of *PRR7* at high temperatures and in response to drought (Kolmos et al., 2014). Salomé et al. (2010) and Kolmos et al. (2014) found that the *PRR* genes are important for the temperature compensation of the clock in *Arabidopsis*, as high temperature led to overcompensation and lengthening of the period in a *HsfB2b* overexpression line or double *prp7/9* mutant. In our study, the changes in clock gene expression under high ambient temperature were also observed in S42-IL107 and Bowman(*eam8*), suggesting that these temperature-mediated changes of the clock were not controlled by the *PRR* homolog *Ppd-H1* or its upstream regulator *HvELF3*. In addition, the down-regulation of all *PRR* genes under high ambient temperature suggested that the repressive EC consisting of *HvELF3*, *HvELF4*, and *HvLUX1* was not reduced in its activity under high temperature in barley as demonstrated for *Arabidopsis* (Mizuno et al., 2014).

Although the function of clock plasticity under different environmental conditions is not well understood, it may affect the expression of different clock output genes and related traits. We observed that the altered clock expression patterns correlated with changes in the diurnal expression patterns of flowering-time genes.

Similar to the reduction in the expression amplitudes of clock genes, the expression levels of the majority of flowering-time genes including *Ppd-H1* and its downstream target *HvFT1* were strongly reduced under high ambient temperatures. However, in contrast to the clock genes, temperature-dependent changes in the expression of flowering-time genes were controlled by *Ppd-H1*, *HvELF3*, and *HvVRN1*. *Ppd-H1* and *HvFT1* transcripts were reduced under high compared to the control temperatures in all genotypes. In contrast, relative expression patterns of *BARLEY MADS-box* (*BM*) genes *HvBM3*, *HvVRN1* (*HvBM5a*), and *HvBM8* were genotype and condition specific. While in Scarlett *HvVRN1*, *HvBM3*, and *HvBM8* were down-regulated, they were not down-regulated or even up-regulated under high versus control temperature in S42-IL107 and Bowman(*eam8*). This indicated that *Ppd-H1* and *HvELF3* controlled the relative expression levels of *BM* genes under different ambient temperature conditions. *HvBM3* and *HvBM8* are known targets of *Ppd-H1* under LD conditions and their expression patterns correlate with the development of the inflorescence (Digel et al., 2015, 2016). In this study, we show that the effect of *Ppd-H1* on *HvBM3* and *HvBM8* expression and flowering time was temperature dependent. Scarlett and Bowman with a mutated *ppd-H1* allele showed a relatively lower expression of *HvBM3*, *HvVRN1*, and *HvBM8* and a delay in floral development under high versus control temperatures. S42-IL107, with a wild type *Ppd-H1* allele, exhibited a relatively higher expression of *HvBM3*, *HvVRN1*, and *HvBM8* and was characterized by a faster inflorescence development under high versus control temperatures. Interestingly, functional variation at *Ppd-H1* also had a strong effect on the number of florets and seeds per main spike under high ambient temperatures. While in Scarlett and Bowman the mutated *ppd-H1* allele was correlated with a strong reduction of the number of seeds per main spike, S42-IL107 did not show a significant reduction in seed number under high temperatures. This suggested that *Ppd-H1* affected floret fertility and seed set under high ambient temperatures, possibly by controlling the rate of development of the inflorescence.

A previous study found that high ambient temperatures accelerated flowering time under LDs, but delayed development under SDs in a winter barley with a wild-type *Ppd-H1* allele (Hemming et al., 2012). The authors suggested an interaction between the photoperiod and thermosensitive pathway. Our study demonstrates that this interaction is mediated by *Ppd-H1*, which is functional under LDs, but not under SDs (Digel et al., 2015). Furthermore, we show that the effect of *Ppd-H1* on early reproductive development under high temperatures is dependent on *HvVRN1*. Only in the background of a spring *HvVRN1* allele or after up-regulation of *Hvvrn1* by vernalization, the wild-type *Ppd-H1* allele is capable of accelerating early reproductive development under high ambient temperatures.

Among the *BM* genes, *HvVRN1* has been extensively characterized for its role in vernalization response. The

winter *HvVRN1* allele is up-regulated by a prolonged exposure to cold to allow flowering after winter. Our results suggest that *HvVRN1* expression is negatively regulated by high ambient temperature, and this down-regulation of the winter *Hvvrn1* allele correlated with a strong delay in reproductive development. The full-length winter *Hvvrn1* allele in S42-IL176 was more strongly down-regulated by high ambient temperature compared to the spring *HvVRN1* allele with a deletion in the first intron. Interestingly, a recent study has revealed that natural variation in the first intron of the *MADS-box* gene *FLM* was responsible for differential temperature response in Arabidopsis (Lutz et al., 2015). Consequently, structural variation in related *MADS-box* transcription factors may play a role in temperature adaptation across different species. In Arabidopsis, high ambient temperature accelerates plant development and growth. However, different Arabidopsis ecotypes show substantial variation in the thermo-sensitive response mediated by natural variation at the vernalization gene and floral repressor *FLC*. High expression levels of *FLC* in autonomous pathway mutants functioned as a potent suppressor of thermal induction (Balasubramanian et al., 2006). *HvOS2*, the putative barley homolog of *FLC*, was up-regulated under high ambient temperature in a *HvVRN1*-dependent manner. The barley vernalization gene and floral inducer *HvVRN1*, in turn, was down-regulated by high temperature, and this correlated with a down-regulation of *HvBM3* and *HvBM8* and a delay in floral development. Different vernalization genes might, therefore, mediate thermo-sensitive flowering across different species.

CONCLUSION

Our study demonstrates that an interaction of *Ppd-H1* and *HvVRN1* controls reproductive development and the number of seeds per spike under high ambient temperatures. These genetic interactions between *Ppd-H1* and *HvVRN1* are important to consider for breeding barley better adapted to climate change.

MATERIALS AND METHODS

Plant Material, Growth Conditions, and Phenotyping

Flowering time, development of the SAM, flower fertility, and seed set were scored in the spring cultivars Bowman and Scarlett and four derived ILs. Bowman and Scarlett are characterized by a mutation in the CCT domain of *Ppd-H1* and the spring allele (*HvVRN1-1*; Hemming et al., 2009) at the vernalization response gene *HvVRN1*. The IL Bowman(*eam8.w*) carries a base pair mutation leading to a premature stop codon in *HvELF3*, orthologous to *ELF3* in Arabidopsis (*Arabidopsis thaliana*; Faure et al., 2012). Bowman(*Ppd-H1*) carries an introgression of the dominant *Ppd-H1* allele from wild barley (*Hordeum vulgare*; Druka et al., 2011). The Scarlett derived ILs S42-IL107 and S42-IL176 carry a dominant allele of *Ppd-H1* and a recessive winter *Hvvrn1* allele, respectively, both derived from wild barley (von Korff et al., 2006; Schmalenbach et al., 2008; Wang et al., 2010). In addition, development of the MSA and expression of *HvVRN1* were analyzed in selected F_3 families derived from a cross between the winter barley Igri and the spring barley Golden Promise. These F_3 families segregated for natural variation at *Ppd-H1* and *HvVRN1* and were fixed for the spring alleles at *HvFT1* and *VRN-H2* (locus deleted, Mulki et al., 2016).

For scoring of shoot apex development, flowering time, floret number, and seed number per spike seeds were stratified at 4°C for 5 d for even germination followed by a transfer to controlled growth chambers with day/night temperatures of 20°C/16°C or 24°C/28°C, a light intensity of ~300 μ M and long photoperiods (LD; 16 h light/8 h dark). Light and temperature were monitored throughout the experiments using WatchDog series 1000 light sensors. Plants were fertilized once per week, and trays were shuffled twice a week to normalize for position effects. Plants were either shifted to high ambient temperatures after stratification or after floral transition as determined by the formation of a double ridge SAM (Waddington et al., 1983). Experiments were replicated two or three times using different randomizations to minimize the environmental effects.

The MSA of three to four representative plants per genotype (Bowman, Bowman(*eam8*), Scarlett, S42-IL107, and S42-IL176) and treatment were dissected every 3 to 7 d starting from the third DAE until 36 DAE. At each time point, the developmental stage of the MSA was determined according to the quantitative scale of Waddington et al. (1983), which rates the development of the most advanced floret primordium. Images of apices were obtained using the Nikon imaging software and a stereo microscope (Nikon SMZ18) equipped with a digital camera (Nikon digital sight DS-U3). The apex was dissected with a microsurgical stab knife (5 mm blade at 15° [SSC#72-1551], Sharepoint, Surgical Specialties) under the stereo microscope to confirm the developmental stage of each harvested MSA. In addition, morphological phenotypes of the main shoot, i.e. heading date (at Z49; Zadoks et al., 1974), the number of florets per spike, and the number of grains per spike, were recorded during development and at plant maturity for 20 plants per genotype.

Leaf Sampling, RNA Extraction, and Gene Expression Analysis

For the analysis of diurnal expression variation in clock and flowering-time genes in Scarlett, S42-IL107, S42-IL176, Bowman, and Bowman(*eam8.w*), plants were grown in 96-well trays (Einheitserde) under day/night temperatures of 20°C/16°C or 24°C/28°C, a light intensity of ~300 μ M and long photoperiods (LD; 16 h light/8 h dark). Leaf samples were harvested 21 DAE at 2 h intervals starting from the onset of light (ZT0) to the end of the night (T22). For all genotypes and treatment conditions, three biological replicates of two pooled plants were sampled per time point. Total RNA extraction, cDNA synthesis, and quantitative real-time-PCRs using gene-specific primers as detailed in Supplemental Table S2 were performed as explained in Campoli et al. (2012a, 2012b). The expression of target genes was normalized against the geometric mean of the three internal controls *HvACTIN*, *HvGAPDH*, and *Hv β TUBULIN* (Supplemental Table S1). Two technical replicates were used for each sample, and each data point was quantified based on the titration curve for each target gene and normalized against the geometric mean of the three housekeeping genes using the LightCycler 480 Software (Roche; version 1.5).

Statistical Analysis

Significant differences in flowering time, floret, and seed number were calculated with a two-factorial ANOVA with the factors genotype and temperature treatment. In addition, least square means for each gene by temperature combination were calculated followed by a Tukey's multiple comparison test. Significant differences in *HvVRN1* expression were calculated with an ANOVA including temperature treatment, *HvVRN1*, and *Ppd-H1* genotype and all possible interaction effects. Statistical differences in the MSA development between temperature regimes were calculated using a polynomial regression model at 95% confidence interval (Loess smooth line).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. *HvVRN1* affects reproductive development in response to ambient temperature after floral transition.

Supplemental Figure S2. Diurnal expression of circadian clock genes *HvPRR73* and *HvPRR95* in Scarlett, S42-IL107, and S42-IL176 under control and high ambient temperatures.

Supplemental Figure S3. Diurnal expression of circadian clock genes *HvPRR73* and *HvPRR95* in Bowman and Bowman(*eam8*) under control and high ambient temperatures.

Supplemental Figure S4. High ambient temperature down-regulates the expression of the flowering-time gene *HvCO1* in Scarlett, S42-IL107, and S42-IL176.

Supplemental Figure S5. Effect of high ambient temperature on diurnal expression of the flowering-time gene *HvCO1* in Bowman and Bowman(*eam8*).

Supplemental Table S1. Two-factorial ANOVA and least square means for heading date, floret, and seed number.

Supplemental Table S2. List of primers used in this study.

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