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11 # The genetic control of reproductive development under high
12 # ambient temperature

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25 Summary: *PHOTOPERIOD1* (*Ppd-H1*) and *VERNALIZATION1* (*HvVRN1*) interact to control
26 reproductive development under high ambient temperature in barley.

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34 **Abstract:**

35 Ambient temperature has a large impact on reproductive development and grain yield in
36 temperate cereals. However, little is known about the genetic control of development under
37 different ambient temperatures. Here, we demonstrate that in barley high ambient
38 temperatures accelerate or delay reproductive development depending on the photoperiod
39 response gene *Ppd-H1* and its upstream regulator *EARLY FLOWERING 3 (HvELF3)*. A
40 natural mutation in *Ppd-H1* prevalent in spring barley delayed floral development and
41 reduced the number of florets and seeds per spike, while the wild-type *Ppd-H1* or a mutant
42 *Hvelf3* allele accelerated floral development and maintained the seed number under high
43 ambient temperatures. High ambient temperature delayed the expression phase and reduced
44 the amplitude of clock genes and repressed the floral integrator gene *FLOWERING LOCUS*
45 *T1* (*HvFT1*) independently of the genotype. *Ppd-H1* dependent variation in flowering time
46 under different ambient temperatures correlated with relative expression levels of the
47 *BARLEY MADS*-box genes *VERNALIZATION1 (HvVRN1), HvBM3* and *HvBM8* in the leaf.
48 Finally, we show that *Ppd-H1* interacts with regulatory variation at *HvVRN1*. *Ppd-H1* only
49 accelerated floral development in the background of a spring *HvVRN1* allele with a deletion
50 in the regulatory intron. The full-length winter *Hvvrn1* allele was strongly downregulated and
51 flowering was delayed by high temperatures irrespective of *Ppd-H1*. Our findings
52 demonstrate that the photoperiodic and vernalization pathways interact to control flowering
53 time and floret fertility in response to ambient temperature in barley.

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55 **Introduction:**

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

61 Temperature-dependent flowering is regulated by the vernalization and ambient
62 temperature pathways. Whereas vernalization requires long periods of cold during the winter,
63 the ambient temperature pathway modulates flowering in response to short-term temperature
64 changes (Wigge, 2013). Research in the model plant *Arabidopsis thaliana* (Arabidopsis), a
65 facultative long day plant, has demonstrated that the temperature and photoperiod pathways
66 interact to control reproductive development. For example, high temperature accelerates
67 flowering and overcomes the delay in flowering commonly observed under short
68 photoperiods in Arabidopsis (Balasubramanian et al., 2006). Early flowering in response to
69 high temperature was correlated with an increase in the expression of the floral pathway
70 integrator gene *FLOWERING LOCUS T* (*FT*) independently of day length (Halliday et al.,
71 2003; Balasubramanian et al., 2006). The FT protein acts as a long-distance signal (florigen)
72 that conveys the information to induce flowering from leaves to the shoot meristem
73 (Corbesier et al., 2007; Jaeger and Wigge, 2007; Kobayashi and Weigel, 2007; Mathieu et al.,
74 2007; Tamaki et al., 2007). In addition, recent studies have identified *EARLY FLOWERING 3*
75 *(ELF3)*, a repressor of light signals to the circadian clock as an essential component of
76 ambient temperature response (Thines and Harmon, 2010). *ELF3* forms together with *EARLY*
77 *FLOWERING 4 (ELF4)* and *LUX ARRHYTHMO (LUX)*, the so-called 'evening complex'
78 (EC) that functions as a night-time repressor of gene expression in the circadian clock of
79 Arabidopsis (Nusinow et al., 2011; Herrero et al., 2012). The circadian clock is an
80 autonomous oscillator that produces endogenous biological rhythms with a period of about 24
81 hours and consists of at least three interlocking feedback loops. The core loops comprise (a)
82 the inhibition of EC genes by CIRCADIAN CLOCK ASSOCIATED1 (CCA1) and LATE
83 ELONGATED HYPOCOTYL (LHY) late at night, (b) the inhibition of *PSEUDO*
84 *RESPONSE REGULATOR* genes by the EC early at night, and (c) the inhibition of
85 *LHY*/*CCA1* by TIMING OF CAB EXPRESSION1 (TOC1/PRR1) in the morning (Pokhilko
86 et al., 2012).

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87 Several independent studies have recently found that elevated temperatures,
88 specifically during dark periods, inhibit the activity of the EC by an unknown mechanism
89 (Box et al., 2014; Mizuno et al., 2014; Thines et al., 2014; Raschke et al., 2015) leading to
90 increased expression of *PHYTOCHROME-INTERACTING FACTOR 4 (PIF4)* (Koini et al.,
91 2009). PIF4 binding to the promoter of *FT* and consequent transcriptional activation of *FT* is
92 promoted by an improved chromatin accessibility through temperature dependent histone
93 modifications at the *FT* promoter (Kumar and Wigge, 2010, Kumar et al, 2012). However,
94 high temperature also accelerated flowering in *pif4* mutants under long photoperiods,
95 suggesting that a *PIF4*-independent thermoresponsive flowering pathway acts through
96 components of the photoperiod pathway (Koini et al., 2009; Press et al., 2016).
97 In addition, the MADS-box genes *SHORT VEGETATIVE PHASE* like (*SVP*), *FLOWERING*
98 *LOCUS C* (*FLC*) and *FLOWERING LOCUS M* (*FLM*; *MAF1*) play a role in the
99 thermosensory regulation of flowering in Arabidopsis (Balasubramanian et al., 2006; Lee et
100 al., 2007; Gu et al., 2013). Loss of function of either *SVP* or *FLM* results in partial
101 temperature-insensitive early flowering (Balasubramanian et al., 2006; Lee et al., 2007; 2013;
102 Posé et al., 2013). Moreover, *FLM* is subject to temperature dependent alternative splicing
103 (Balasubramanian et al., 2006, Sureshkumar et al., 2016) resulting in two major splice forms,
104 that either facilitate or inhibit *SVP* dependent repression of *FT*, and the floral homeotic genes
105 *SUPPRESSOR OF OVEREXPRESSION 1* (*SOC1)* and *SEPALLATA (SEP3)* (Posé et al.,
106 2013). Interestingly, a structural polymorphism in the first intron of *FLM* affects its
107 expression, splicing and also regulates flowering predominantly at lower ambient
108 temperatures (Lutz et al., 2015). Such structural polymorphisms within the first intron are
109 typical within the family of MADS-box transcription factor genes and play an important role
110 for expression variation and possibly adaptation to different environments across different
111 species (Hong et al., 2003; Distelfeld et al., 2009; Schauer et al., 2009; Yoo et al., 2011).

112 While flowering time control in response to temperature is well described in Arabidopsis,
113 little is known about the genetic determinants of ambient temperature response in cereal
114 grasses (Bullrich et al., 2002; Appendino and Slafer, 2003; Lewis et al., 2008; Hemming et
115 al., 2012). In barley, a complex interplay between day length and temperature in the
116 regulation of flowering has been reported. Under long-day (LD) conditions, barley plants
117 accelerated reproductive development at 25 °C compared with 15 °C, whereas the opposite
118 was the case in SDs (Hemming et al., 2012). In contrast to Arabidopsis, the transcript level of
119 the barley homolog of *FT* was not influenced by temperature and no clear candidate genes for

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120 the integration of thermal signals into the flowering time pathways have been identified so far
121 (Hemming et al., 2012). Barley is a facultative long day plant and is characterized by winter
122 and spring growth habits as determined by natural variation at the two vernalization genes
123 *HvVRN1* (*HvBM5a*) and *HvVRN2* (Yan et al., 2003; Yan et al., 2004; Trevaskis et al., 2006).
124 Winter types accelerate flowering after a prolonged period of cold (vernalization), whereas
125 spring barley does not respond to vernalization. The MADS-box gene *HvVRN1* is
126 characterized by a series of different deletions and insertions in the first regulatory intron
127 which has been linked to differences in vernalization response and flowering behavior
128 (Hemming et al., 2009). Photoperiod response, rapid flowering under long days is determined
129 by natural variation at the *PHOTOPERIOD-H1* (*Ppd-H1)* gene, which is homologous to the
130 *PSEUDO RESPONSE REGULATOR* genes of the circadian clock in Arabidopsis (Turner et
131 al., 2005). The wild-type allele is prevalent in winter barley, while a natural mutation in the
132 conserved CCT domain of *Ppd-H1* causes a delay in flowering under LDs and is predominant
133 in spring barley from cultivation areas with long growing seasons (Turner et al., 2005; von
134 Korff et al., 2006;2010; Wang et al., 2010) *Ppd-H1* induces flowering under LDs by
135 upregulating *HvFT1*, the barley homolog of FT in Arabidopsis (Turner et al., 2005; Campoli
136 et al., 2012). *Ppd-H1* is repressed during the night by *HvELF3*, *HvLUX1,* and
137 *PHYTOCHROME C*, and mutations in these genes result in a day-neutral upregulation of
138 *HvFT1* and early flowering (Faure et al., 2012; Zakhrabekova et al., 2012; Campoli et al.,
139 2013; Pankin et al., 2014). Consequently, the major vernalization and photoperiod response
140 genes are known in barley, if these also play a role for thermoresponsive flowering is not
141 known.

142 In Arabidopsis, commonly used macroscopic indicators of reproductive phase change or
143 floral transition are time to bolting or rosette leaf number under the first open floral bud
144 (Pouteau and Albertini, 2009). Under optimal conditions, floral transition, bolting and
145 flowering are well correlated in Arabidopsis. In barley, most stages of reproductive
146 development including flowering occur within the leaf sheath and can therefore only be
147 scored upon dissection of the shoot. Waddington et al. (1983) developed a quantitative scale
148 for barley and wheat development based on the morphogenesis of the shoot apex and carpels.
149 This scale is based on the progression of the most advanced floret primordium and carpel of
150 the inflorescence. The enlargement of the apical dome at Waddington stage (W) 1.0
151 represents an apex that is transitioning to a reproductive state and indicates the end of the
152 vegetative phase. The emergence of the first floret primordia on the shoot apex at the double

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153 ridge stage (W2.0) specifies a reproductive shoot apical meristem (SAM). At the stamen
154 primordium stage (W3.5), the first floral organ primordia differentiate, and stem elongation
155 initiates. In barley, the induction of floret primordia on the inflorescence continues until the
156 awn primordium stage (W5.0). Anthesis and pollination of the most advanced floret occurs at
157 the last stage of the Waddington scale (W10.0). This last step can be scored macroscopically
158 because it is marked by the emergence of awn tips from the top of the leaf sheath (heading).
159 Most commonly, flowering is scored as heading in barley. However, the different phases of
160 shoot apex development differ in their sensitivity to environmental cues and are controlled by
161 different genetic factors, so that floral transition and flowering may not be correlated and
162 separated in time by many weeks (Digel et al. 2015). Variation in the timing of different
163 developmental phases in turn affects the number of floret primordia, fertile flowers and seeds
164 per spike (Digel et al. 2015). To better understand the effects of temperature on development
165 it is therefore important to investigate the effects of environmental and genetic variation on
166 individual phases of shoot apex development.

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

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176 **RESULTS**

177 **High ambient temperature delays reproductive development and reduces seed set in**
178 **spring barley**

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

191 The effect of increased temperature on floral development was evaluated by monitoring the
192 progression of the main shoot apex (MSA) in Bowman and Scarlett plants grown at 20/16°C
193 and 28/24°C according to the Waddington scale (W, Waddington et al., 1983). Microscopic
194 dissection of the main shoot apex (MSA) revealed that high temperature did not have a strong
195 effect on floral transition (W2.0), but greatly delayed the late reproductive phase of
196 inflorescence development (after W3.5) both in Bowman and Scarlett (Fig. 2A, C). In
197 summary, high ambient temperature primarily delayed inflorescence development and
198 reduced the number of seeds per spike in the spring barley genotypes Bowman and Scarlett.

199

200 **High ambient temperature accelerates flowering time in genotypes with a non-**
201 **functional *Hvelf3* allele and a dominant *Ppd-H1* allele**

202 In Arabidopsis, the circadian clock and photoperiod pathways modulate ambient temperature
203 responses to regulate flowering. Therefore, we further characterized reproductive
204 development in introgression lines with a non-functional *Hvelf3* or dominant *Ppd-H1* alleles
205 under control and high ambient temperatures. *HvELF3* is a component of the evening
206 complex in Arabidopsis and represses *Ppd-H1* expression in the night in barley (Faure et al.

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207 2012). Therefore, the barley *Hvelf3* mutant plant is characterized by high expression of *Ppd-*
208 *H1* during the night (Faure et al. 2012). The introgression line (IL) Bowman(*eam8*) carrying a
209 non-functional *Hvelf3* allele in the background of Bowman and the ILs S42-IL107 and
210 Bowman(*Ppd-H1*) with the wild-type *Ppd-H1* gene in the background of Scarlett and
211 Bowman were analyzed along with the parental genotypes for flowering time, floret fertility
212 and seed set. In addition, the microscopic development of the MSA was evaluated in Scarlett,
213 Bowman, S42-IL107 and Bowman(*eam8*) under control and high ambient temperatures.

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

218 Since *HvELF3* might control flowering time through its downstream target *Ppd-H1*, we
219 evaluated if variation at *Ppd-H1* mediated the flowering response under high ambient
220 temperature. In contrast to the parental lines, S42-IL107 and Bowman(*Ppd-H1*) plants
221 flowered on average 7 and 2 days earlier under high ambient compared to control
222 temperatures (Fig. 2D). The dissection of the MSA in Scarlett and S42-IL107 revealed that
223 high ambient temperature accelerated in particular the phase of stem elongation and
224 inflorescence development (Fig. 2D). In addition, the analysis of variance for floret and seed
225 number revealed a significant interaction between *Ppd-H1* and *ELF3* with temperature
226 (Supplementary Table 1). High ambient temperatures caused a larger reduction in floret and
227 seed number in Bowman and Scarlett than in the ILs Bowman(*eam8*), S42-IL107 and
228 Bowman(*Ppd-H1*) (Fig.1B, C, Supplementary Table 1).

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

232 **Variation at *HvVRN1* affects reproductive development under high ambient**
233 **temperature**

234 Natural variation in the length of the first regulatory intron of *HvVRN1* has a strong effect on
235 vernalization response in barley. Therefore, we examined whether this variation also affected
236 the response to ambient temperature variation in barley. For this purpose, we compared the
237 development of Scarlett with that of S42-IL176. Scarlett carries a spring *HvVRN1* allele with

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238 a deletion in the first regulatory intron, S42-IL176 carries an introgression of the full-length
239 winter *Hvvrn1* allele. Although high ambient temperature delayed reproductive development
240 in both genotypes, the effect was more pronounced in S42-IL176 which did not undergo
241 floral transition and did not flower until 160 days after emergence (DAE) when the
242 experiment was stopped (Fig. 2F, 1A). Consequently, the full-length intron of *Hvvrn1* was
243 correlated with a strong delay in floral transition under high ambient temperatures. In order
244 to assess if variation at *HvVRN1* was also associated with inflorescence development in
245 response to ambient temperature, we shifted Scarlett and S42-IL176 plants from 20/16°C to
246 28/24°C only after floral transition (W2.0). Under these conditions, the IL with the winter
247 *Hvvrn1* allele also showed a strong delay in inflorescence development under high ambient
248 temperatures compared to control conditions (Supplementary Figure 1B). Flowering was
249 delayed by about two weeks under 28/24°C compared to 20/16°C. However, S42-IL176
250 plants were able to produce flowers and seeds, when the temperature treatment was started
251 after floral transition (Supplementary Figure 1C).

252 **High ambient temperature affects the expression of clock genes**

253 To further characterize the *Ppd-H1*, *HvELF3,* and *HvVRN1* dependent effects of high
254 temperature on barley development, we analyzed the expression of barley genes from the
255 circadian clock, photoperiod and vernalization response pathways in the parental and
256 introgression lines. Because the barley clock is plastic under abiotic stresses, we first tested
257 the effects of high ambient temperature on variation in the diurnal pattern of clock gene
258 expression. Under control conditions, the circadian clock genes showed a diurnal pattern of
259 expression with clock genes peaking at different times of the day corroborating previous
260 results (Campoli et al., 2012; Habte et al., 2014). The expression phase of clock genes did not
261 differ between the parental lines Scarlett, and the ILs S42-IL107 and S42-IL176, suggesting
262 that *Ppd-H1* and *HvVRN1* did not affect diurnal clock oscillations. By contrast, the
263 expression phase and shape of clock genes were significantly different between Bowman and
264 Bowman(*eam8*). The expression phase of the clock genes in Bowman(*eam8*) was advanced
265 by two hours. The expression peaks were less defined and broader in Bowman(*eam8*) than in
266 Bowman. Moreover, Bowman(*eam8*) exhibited higher levels of *Ppd-H1* expression at most
267 time points during the day compared to Bowman. Consequently, the loss of function mutation
268 in *HvELF3* affected the diurnal pattern of clock gene expression and caused a strong increase
269 in *Ppd-H1* expression independent of the ambient temperature.

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270 High ambient temperatures caused a decrease in the expression of clock genes as seen for
271 most clock genes in Scarlett and for *HvCCA1* and *HvPRR1* in Bowman (Fig.3, 4). In
272 addition, the expression phase of clock genes was delayed by four hours under high ambient
273 temperature compared to control conditions in Scarlett and Bowman. This reduction in
274 expression amplitude and the shift in the expression phase were also observed in all ILs
275 suggesting that temperature affected the phase of clock gene expression independently of the
276 genotype.

277 **High ambient temperature reduces expression of flowering time genes**

278 As the clock genes are putative upstream regulators of flowering time genes, we investigated
279 whether the temperature dependent changes in clock gene expression correlated with changes
280 in the expression of flowering time genes. As observed for the clock genes, most flowering
281 time regulators showed a significantly lower expression under high ambient temperature.
282 *Ppd-H1* exhibited a reduction in expression in Scarlett, Scarlett derived ILs and
283 Bowman(*eam8*), but not in Bowman under high ambient temperature. The expression levels
284 of *HvCO1*, the barley homolog of the major Arabidopsis photoperiod response gene
285 CONSTANS, were reduced and the peak expression was delayed by approximately four
286 hours under high ambient temperature in Scarlett and Scarlett derived ILs (Supplementary
287 figure 4). While in Bowman *HvCO1* expression peaked at dusk (T16) under control
288 temperature, it showed an expression peak in the night at T20 under high ambient
289 temperature (Supplementary figure 5). This suggested that *HvCO1* expression was controlled
290 by the clock and a temperature dependent phase shift of clock genes. However, no consistent
291 changes in the level and peak time of *HvCO1* expression were observed in Bowman and
292 Bowman(*eam8*) (Supplementary figure 5).

293 The expression levels of the *HvFT1*, a putative target of *Ppd-H1*, were significantly
294 downregulated under high temperature in all genotypes. In addition, *HvFT1* expression levels
295 were overall significantly different between genotypes with higher transcript abundance in
296 S42-IL107 and Bowman(*eam8*) and lower transcript levels in S42-IL176 compared to the
297 parental lines (Fig. 5, 6).

298 The MADS-box genes *HvVRN1*, *HvBM3* and *HvBM8* were also strongly downregulated
299 under high versus control temperatures. In S42-IL176, the expression levels of the winter
300 *Hvvrn1* allele were 90-fold lower, while the expression levels of the spring *HvVRN1* allele in
301 Scarlett were only 2-fold lower under high ambient compared to control temperature (Fig. 5).

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302 This suggested that the winter allele of *HvVRN1* was repressed by high ambient temperatures
303 (Fig.5). The expression patterns of *HvBM3* and *HvBM8* were comparable to those of
304 *HvVRN1* with a stronger temperature dependent downregulation in S42-IL176 compared to
305 Scarlett. In contrast, S42-IL107 with a dominant *Ppd-H1* allele exhibited an upregulation of
306 *HvVRN1*, *HvBM3,* and *HvBM8* under high compared to control temperatures (Fig.5). In
307 Bowman(*eam8*) expression levels of the *HvBM* genes were approximately 10-fold higher
308 compared to Bowman under control and high ambient temperature conditions. In addition,
309 *HvVRN1* and *HvBM3* were only slightly downregulated under high versus control
310 temperatures, while expression of *HvBM8* was not significantly different between control and
311 high-temperature conditions (Fig.6). *HvOS2*, a repressor of flowering and homolog of the
312 major Arabidopsis vernalization gene *FLOWERING LOCUS C* (Greenup et al. 2010, Ruelens
313 et al. 2013), was upregulated under high versus control temperatures and was controlled by
314 *Ppd-H1*, *HvELF3,* and *HvVRN1*. Expression levels of *HvOS2* were upregulated under high
315 ambient temperature in Scarlett and Bowman, but very low during the day in S42-IL107 and
316 Bowman(*eam8*) under both temperatures. *HvOS2* expression levels were further increased
317 under high temperatures in S42-IL176 with the winter *Hvvrn1* allele and no detectable
318 expression of *Hvvrn1*. *HvOS2* expression levels were consequently negatively correlated with
319 *HvVRN1* expression and controlled by ambient temperature.

320 Variation at *Ppd-H1* and *HvELF3*, therefore, correlated with the temperature-dependent
321 regulation of the MADS-box transcription factor genes. It is interesting to note, that in S42-
322 IL107 the expression patterns of *HvFT1* and the *HvBM* genes were not correlated under the
323 different temperature regimes, as the *HvBM* genes were upregulated, but *HvFT*1 was
324 downregulated under high compared to control temperatures. The expression patterns of the
325 *HvBM* genes, but not of *HvFT1,* correlated with the differential flowering time in response to
326 high ambient temperatures. Low expression of the *HvBM* genes under high temperatures in
327 Scarlett and Bowman coincided with a delay in reproductive development, while accelerated
328 inflorescence development in S42-IL107 correlated with an upregulation of the *HvBM* genes
329 under high ambient versus control temperatures. In Bowman(*eam8*) with accelerated
330 development under high temperatures, the expression of *HvBM3* and *HvBM8* was strongly
331 increased compared to Bowman and not very different between temperature regimes. In S42-
332 IL176 with a winter *Hvvrn1* allele, a complete downregulation of *HvFT1* and *HvBM* genes
333 correlated with a strong delay in reproductive development as this genotype did not undergo
334 floral transition under high temperatures.

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335 Taken together, the wild type *Ppd-H1* and a loss-of–function *Hvelf3* allele correlated with an
336 accelerated development under high compared to control temperatures and a higher
337 expression of *HvBM* genes under high compared to control temperatures. In addition,
338 variation in the regulatory region of the first intron in *HvVRN1* controlled the expression of
339 *HvVRN1* itself, of the related *HvBM* genes and reproductive development under high ambient
340 temperatures.

341 ***Ppd-H1* and *HvVRN1* interact to control inflorescence development under high ambient**
342 **temperatures**

343 Our results showed that variation at *Ppd-H1* was correlated with the expression of *HvBM*
344 genes including *HvVRN1* under high relative to control temperatures. Therefore, we
345 examined if *Ppd-H1* and *HvVRN1* interacted to control reproductive development under
346 different ambient temperatures. For this purpose, we analyzed MSA development and gene
347 expression of *HvVRN1* in F_3 families selected from a cross between the winter barley variety
348 Igri and the spring barley variety Golden Promise. The F3 families segregated for variation at
349 *Ppd-H1* and *HvVRN1* but were fixed for the spring alleles at the other major flowering loci
350 *HvVRN2* and *HvFT1*. Reproductive development was delayed under high ambient
351 temperatures in F_3 plants with a spring *ppd-H1* allele irrespective of the *HvVRN1* allele as
352 seen for Scarlett and S42-IL176. In addition, under high ambient temperature, the dominant
353 *Ppd-H1* allele accelerated development in the background of a spring *HvVRN1* allele as
354 observed for S42-IL107. F_3 plants carrying a winter *Hvvrn1* allele and a wild-type *Ppd-H1*
355 allele exhibited a delay in MSA development under high ambient temperature compared to
356 control conditions (Fig. 7A). Consequently, *Ppd-H1* interacted with *HvVRN1* to control the
357 development under high temperatures, where only plants with a dominant *Ppd-H1* and a
358 spring *HvVRN1* allele showed an accelerated development under high versus control
359 temperatures. Gene expression analysis showed that the spring *HvVRN1* allele was not
360 affected in the presence of dominant *Ppd-H1* allele under high ambient versus control
361 temperatures. However, the winter *Hvvrn1* allele was downregulated in the *Ppd-H1* and *ppd-*
362 *H1* backgrounds under high compared to control temperature (Fig. 7B). The winter *Hvvrn1*
363 allele was stronger downregulated than the spring *HvVRN1* allele as shown for Scarlett and
364 S42-IL176. These results indicated that *Ppd-H1* interacts with *HvVRN1*, where a dominant
365 *Ppd-H1* allele only accelerated floral development under high ambient temperature in the
366 background of a spring *HvVRN1* allele.

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367 ## Discussion

368 Understanding how ambient temperature controls plant development and eventually grain
369 yield in crop plants is gaining importance in the light of a predicted increase in average global
370 temperatures. The circadian clock influences plant adaptation to different abiotic stresses and
371 controls many different output traits including plant development. Furthermore, the circadian
372 clock itself is altered in response to changing environmental conditions. For example,
373 osmotic stress increased the amplitude and advanced the expression phase of clock genes in
374 barley, and high salinity resulted in a lengthening of the circadian period in wheat (Erdei et
375 al., 1998; Habte et al., 2014). We found that an increase in ambient temperature from
376 20/16°C to 28/24°C decreased expression levels and delayed the phase of clock gene
377 expression. Although the clock is temperature-compensated and maintains a ca. 24h period
378 over a range of ambient temperatures (Pittendrigh, 1954; Gould et al., 2006; Salomé et al.,
379 2010), previous studies have reported changes in the expression phase and amplitude of
380 oscillator components under different temperatures. For example, in Arabidopsis peak
381 expression levels *CCA1* and *LHY* RNA rhythms increased in amplitude as temperatures
382 decreased from 17 to 12°C (Gould et al., 2006; Mizuno et al., 2014). Temperatures of above
383 30°C are considered as heat stress for temperate cereals (Barnabas et al. 2007). However, an
384 induction of a stress response when increasing the temperature from 20°C to 28°C cannot be
385 excluded. Therefore, the observed changes in clock oscillations in this work may be related to
386 changes in the level of stress response hormones. In Arabidopsis, application of the stress
387 hormone abscisic acid (ABA) lengthened the period of the Arabidopsis clock (Hanano et al.,
388 2006), probably through evolutionary conserved ABREs present in the promoters of *TOC1*,
389 LHY, and CCA1 (Bieniawska et al., 2008; Spensley et al., 2009; Picot et al., 2010; Habte et
390 al., 2014). In addition, the heat shock transcription factor *HsfB2b* repressed transcription of
391 *PSEUDO-RESPONSE REGULATOR 7 (PRR7)* at high temperatures and in response to
392 drought (Kolmos et al., 2014). (Salomé et al., 2010; Kolmos et al., 2014) found that the *PRR*
393 genes are important for the temperature compensation of the clock in Arabidopsis, as high
394 temperature led to overcompensation and lengthening of the period in a *HsfB2b*
395 overexpression line or double *prr7/9* mutant. In our study, the changes in clock gene
396 expression under high ambient temperature were also observed in S42-IL107 and
397 Bowman(*eam8*) suggesting that these temperature mediated changes of the clock were not
398 controlled by the *PRR* homolog *Ppd-H1* or its upstream regulator *HvELF3*. In addition, the
399 downregulation of all *PRR* genes under high ambient temperature suggested that the

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400 repressive evening complex (EC) consisting of *HvELF3*, *HvELF4* and *HvLUX1* was not
401 reduced in its activity under high temperature in barley as demonstrated for Arabidopsis
402 (Mizuno et al., 2014).

403 Although the function of clock plasticity under different environmental conditions is not well
404 understood, it may affect the expression of different clock output genes and corresponding
405 traits. We observed that the altered clock expression patterns correlated with changes in the
406 diurnal expression patterns of flowering time genes. Similar to the reduction in the expression
407 amplitudes of clock genes, the expression levels of the majority of flowering time genes
408 including *Ppd-H1* and its downstream target *HvFT1* were strongly reduced under high
409 ambient temperatures. However, in contrast to the clock genes, temperature dependent
410 changes in the expression of flowering time genes were controlled by *Ppd-H1*, *HvELF3,* and
411 *HvVRN1*. *Ppd-H1* and *HvFT1* transcripts were reduced under high compared to the control
412 temperatures in all genotypes. In contrast, relative expression patterns of *BARLEY MADS-box*
413 (*BM*) genes *HvBM3*, *HvVRN1* (*HvBM5a*) and *HvBM8* were genotype and condition specific.
414 While in Scarlett *HvVRN1*, *HvBM3*, and *HvBM8* were downregulated, they were not
415 downregulated or even up-regulated under high versus control temperature in S42-IL107 and
416 Bowman(*eam8*). This indicated that *Ppd-H1* and *HvELF3* controlled the relative expression
417 levels of *BM* genes under different ambient temperature conditions. *HvBM3* and *HvBM8* are
418 known targets of *Ppd-H1* under long day conditions and their expression patterns correlate to
419 the development of the inflorescence (Digel et al., 2015; Digel et al., 2016). In the present
420 study, we show that the effect of *Ppd-H1* on *HvBM3* and *HvBM8* expression and flowering
421 time was temperature dependent. Scarlett and Bowman with a mutated *ppd-H1* allele showed
422 a relatively lower expression of *HvBM3*, *HvVRN1* and *HvBM8* and a delay in floral
423 development under high versus control temperatures. S42-IL107, with a wild type *Ppd-H1*
424 allele, exhibited a relatively higher expression of *HvBM3*, *HvVRN1,* and *HvBM8,* and was
425 characterized by a faster inflorescence development under high versus control temperatures.
426 Interestingly, functional variation at *Ppd-H1* also had a strong effect on the number of florets
427 and seeds per main spike under high ambient temperatures. While in Scarlett and Bowman
428 the mutated *ppd-H1* allele was correlated with a strong reduction of the number of seeds per
429 main spike, S42-IL107 did not show a significant reduction in seed number under high
430 temperatures. This suggested that *Ppd-H1* affected floret fertility and seed set under high
431 ambient temperatures, possibly controlling the rate of development of the inflorescence.

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

441 Among the *BM* genes, *HvVRN1* has been extensively characterized for its role in
442 vernalization response. The winter *HvVRN1* allele is upregulated by a prolonged exposure to
443 cold to allow flowering after winter. Our results suggest that *HvVRN1* expression is
444 negatively regulated by high ambient temperature and this downregulation of the winter
445 *Hvvrn1* allele correlated with a strong delay in reproductive development. The full-length
446 winter *Hvvrn1* allele in S42-IL176 was more strongly downregulated by high ambient
447 temperature compared to the spring *HvVRN1* allele with a deletion in the first intron.
448 Interestingly, a recent study has revealed that natural variation in the first intron of *MADS-*
449 *box* gene *FLM* was responsible for differential temperature response in Arabidopsis (Lutz et
450 al. 2015). Consequently, structural variation in related *MADS-box* transcription factors may
451 play a role in temperature adaptation across different species. In Arabidopsis, high ambient
452 temperature accelerates plant development and growth. However, different Arabidopsis
453 ecotypes show substantial variation in the thermosensitive response mediated by natural
454 variation at the vernalization gene and floral repressor *FLC*. High expression levels of *FLC* in
455 autonomous pathway mutants functioned as a potent suppressor of thermal induction
456 (Balasubramanian et al., 2006). *HvOS2*, the putative barley homolog of *FLC*, was
457 upregulated under high ambient temperature in a *HvVRN1* dependent manner. The barley
458 vernalization gene and floral inducer *HvVRN1*, in turn, was downregulated by high
459 temperature and this correlated with a downregulation of Hv*BM3* and *HvBM8* and a delay in
460 floral development. Different vernalization genes might, therefore, mediate thermosensitive
461 flowering across different species.

462 **Conclusion:**

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

467

468 Materials and Methods

469 Plant material, growth conditions, and phenotyping:

470 Flowering time, development of the shoot apical meristem (SAM), flower fertility, and seed
471 set were scored in the spring cultivars Bowman and Scarlett and four derived introgression
472 lines (ILs). Bowman and Scarlett are characterized by a mutation in the CCT domain of *Ppd-*
473 *H1* and the spring allele (*HvVRN1-1*, Hemming et al., 2009) at the vernalization response
474 gene *HvVRN1*. The introgression line Bowman(*eam8*.w) carries a base pair mutation leading
475 to a premature stop codon in *HvELF3*, orthologous to *ELF3* in Arabidopsis (Faure et al.,
476 2012). Bowman(*Ppd-H1*) carries an introgression of the dominant *Ppd-H1* allele from wild
477 barley (Druka et al., 2011). The Scarlett derived introgression lines S42-IL107 and S42-
478 IL176 carry a dominant allele of *Ppd-H1* and a recessive winter *Hvvrn1* allele, respectively,
479 both derived from wild barley (von Korff et al., 2006; Schmalenbach et al., 2008; Wang et
480 al., 2010). In addition, development of the main shoot apex and expression of *HvVRN1* were
481 analyzed in selected F_3 families derived from a cross between the winter barley Igri and the
482 spring barley Golden Promise. These F_3 families segregated for natural variation at *Ppd-H1*
483 and *HvVRN1* and were fixed for the spring alleles at *HvFT1* and *VRN-H2* (locus deleted).

484 For scoring of shoot apex development, flowering time, floret number, and seed number per
485 spike plants were stratified at 4°C for 5d for even germination followed by a transfer to
486 controlled growth chambers with day/night temperatures of 20/16°C or 24/28°C, a light
487 intensity of ~300uM and long photoperiods (LD, 16h light/8h dark). Light and temperature
488 were monitored throughout the experiments using WatchDog series 1000 light sensors. Plants
489 were fertilized once per week and trays were shuffled twice a week to normalize for position
490 effects. Plants were either shifted to high ambient temperatures after stratification or after
491 floral transition as determined by the formation of a double ridge SAM (Waddington et al.
492 1983). Experiments were replicated 2-3 time using different randomizations to minimize the
493 environmental effects.

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494 The main shoot apex (MSA) of three to four representative plants per genotype (Bowman, 495 Bowman(*eam8*), Scarlett, S42-IL107 and S42-IL176) and treatment were dissected every 496 three to seven days starting from the 3rd day after emergence (DAE) until 36 DAE. At each 497 time point, the developmental stage of the MSA was determined according to the quantitative 498 scale of Waddington et al. (1983), which rates the development of the most advanced floret 499 primordium. Images of apices were obtained using the Nikon imaging software and a stereo 500 microscope (Nikon SMZ18) equipped with a digital camera (Nikon digital sight DS-U3). 501 The apex was dissected with a microsurgical stab knife (5 mm blade at 15° (SSC#72-1551), 502 Sharepoint, Surgical Specialties) under the stereo microscope to confirm the developmental 503 stage of each harvested MSA. In addition, morphological phenotypes of the main shoot, i.e., 504 heading date (at Z49, ZADOKS et al., 1974), the number of florets per spike, and the number 505 of grains per spike were recorded during development at plant maturity for 20 plants per 506 genotype.

507 **Leaf sampling, RNA extraction and gene expression analysis**

508 For the analysis of diurnal expression variation in clock and flowering time genes in Scarlett, 509 S42-IL107, S42-IL176, Bowman and Bowman(*eam8*.w), plants were grown in 96-well trays 510 (Einheitserde) under day/night temperatures of 20/16°C or 24/28°C, a light intensity of 511 ~300uM and long photoperiods (LD, 16h light/8h dark). Leaf samples were harvested 21 512 DAE at 2h intervals starting from the onset of light (ZT0) to the end of the night (T22). For 513 all genotypes and treatment conditions, three biological replicates of two pooled plants were 514 sampled per time point. Total RNA extraction, cDNA synthesis, and qRT-PCRs using gene-515 specific primers as detailed in Supplementary Table 2 were performed as explained in 516 Campoli et al. (2012). The expression of target genes was normalized against the geometric 517 mean of the three internal controls *HvACTIN*, *HvGAPDH* and *HvβTUBULIN* (Supplementary 518 Table 1). Two technical replicates were used for each sample and each data point was 519 quantified based on the titration curve for each target gene and normalized against the 520 geometric mean of the three housekeeping genes using the LightCycler 480 Software (Roche; 521 version 1.5).

522 **Statistical analysis**

523 Significant differences in flowering time, floret, and seed number were calculated with a two-524 factorial ANOVA with the factors genotype and temperature treatment. In addition, least 525 square means for each gene by temperature combination were calculated followed by a

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526 Tukey's multiple comparison test. Significant differences in *HvVRN1* expression were
527 calculated with an ANOVA including temperature treatment, *HvVRN1* and *Ppd-H1* genotype
528 and all possible interaction effects. Statistical differences in the MSA development between
529 temperature regimes were calculated using a polynomial regression model at 95% confidence
530 interval (Loess smooth line).

531

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532

533 **Supplemental Material**

534

535 Figure S1: *HvVRN1* affects reproductive development in response to ambient temperature after floral
536 transition.
537 Figure S2: Diurnal expression of circadian clock genes *HvPRR73* and *HvPRR95* in Scarlett, S42-
538 IL107, and S42-IL176 under control and high ambient temperatures.
539
540 Figure S3: Diurnal expression of circadian clock genes *HvPRR73* and *HvPRR95* in Bowman and
541 Bowman(*eam8*) under control and high ambient temperatures
542
543 Figure S4: High ambient temperature downregulates the expression of flowering time gene *HvCO1* in
544 Scarlett, S42-IL107, and S42-IL176.
545
546 Figure S5: Effect of high ambient temperature on diurnal expression of flowering time gene *HvCO1*
547 in Bowman and Bowman(*eam8*).
548
549 Table S1. Two-factorial ANOVA and least square means for heading date, floret and seed number
550
551 Table S2. List of primers used in this study.
552
553

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556 assistance.

557 **Authors' Contribution**
558 M.E. and M.K. conceived and designed the experiments. M.E. carried out all the experiments and
559 analyzed the data. M.E. and M.K wrote the manuscript.

560 **Competing Interests**
561 The authors do not have any financial, personal or professional interests that have influenced this
562 present paper.

563

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564 **Figure Legends**

565 **Figure 1. High ambient temperature affects flowering time, floret, and seed number per main**
566 **spike in barley**. Days to flower A), the number of florets B), and the number of seeds per main spike
567 C) under control (blue, 20/16°C, day/night) and high ambient temperatures (pink, 28/24°C, day/night)
568 in the spring barley varieties Bowman and Scarlett and and the derived introgression lines
569 Bowman(*eam8*) (*Hvelf3*), Bowman(*Ppd-H1*), S42-IL107 (*Ppd-H1*), S42-IL176 (*Hvvrn1*). Flowering
570 time, floret, and seed number were recorded for 20 plants per genotype and treatment under long days
571 (16h light/8h night). N.F indicates non-flowering plants. Statistical differences were calculated by a
572 two-factorial ANOVA and a posthoc Tukey's multiple comparison test: *P < 0.05, **P < 0.01, ***P
573 < 0.001., n.s= non-significant.

574 **Figure 2**. **High ambient temperature affects shoot apex development (SAM) in barley**.
575 Microscopic development of the main shoot apex (MSA) was scored under control (blue, 20/16°C,
576 day/night) and high ambient (pink, 28/24°C, day/night) temperatures every three days according to
577 the Waddington scale (Waddington et al., 1983). MSA development was delayed under high
578 compared to control temperature in Bowman (A) and Scarlett (C, E), accelerated in Bowman(*eam8*)
579 (B) and S42-IL107 (D), and further delayed floral transition in S42-IL176 (F). 3-4 plants per genotype
580 were dissected at each time point in each treatment under long days (16h light/8h night). Statistical
581 differences (p<0.05) were calculated using a polynomial regression model at 95% confidence interval
582 (Loess smooth line).

583 **Figure 3. Diurnal expression patterns of circadian clock genes in Scarlett, S42-IL107, and S42-**
584 **IL176 under control and high ambient temperatures.** Diurnal expression of circadian clock genes
585 was assayed every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient
586 (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night). Grey boxes indicate
587 nights. Error bars indicate ±SD of three biological replicates.

588 **Figure 4. Diurnal expression of circadian clock genes in Bowman, and Bowman(*eam8*) under**
589 **control and high ambient temperatures.** Diurnal expression of circadian clock genes was assayed
590 every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink,
591 28/24°C, day/night) temperatures under long days (16h light/8h night) are shown. Grey boxes indicate
592 nights. Error bars indicate ±SD of three biological replicates.

593 **Figure 5. High ambient temperature affects the expression of flowering time genes in Scarlett,**
594 **S42-IL107, and S42-IL176 under control and high ambient temperatures.** Diurnal expression of
595 flowering time genes was assayed every two hours for 24 hours under control (blue, 20/16°C,
596 day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h
597 night) are shown. Grey boxes indicate nights. Error bars indicate ±SD of three biological replicates.

598 **Figure 6. Diurnal expression of flowering time genes in Bowman and Bowman(*eam8*) under**
599 **control and high ambient temperatures.** Diurnal expression of flowering time genes sampled every
600 two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C,
601 day/night) temperatures under long days (16h light/8h night) are shown. Grey boxes indicate nights.
602 Error bars indicate ±SD of three biological replicates.

603 **Figure 7. *Ppd-H1* and *HvVRN1* interact to control the development of the main shoot apex**
604 **(MSA) under different ambient temperatures**. Microscopic changes in MSA development were
605 scored under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night)
606 temperatures under long days (16h light/8h night) in F3 families derived from a cross between the
607 winter barley Igri and the spring barley Golden Promise. Selected F3 families segregated for *Ppd-H1*

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608 and *HvVRN1* and were fixed for the spring alleles at *HvVRN2* (deleted) and *HvFT1*. Early MSA
609 development was accelerated under high temperature in *Ppd-H1*/*HvVRN1* and delayed in *Ppd-*
610 *H1*/*Hvvrn1*, *ppd-H1*/*HvVRN1*, and *ppd-H1*/*Hvvrn1*. Significant differences were determined by a two-
611 way ANOVA and a Tukey HSD pairwise comparison test: *P < 0.05, **P < 0.01, ***P < 0.001., n.s=
612 non-significant.
613

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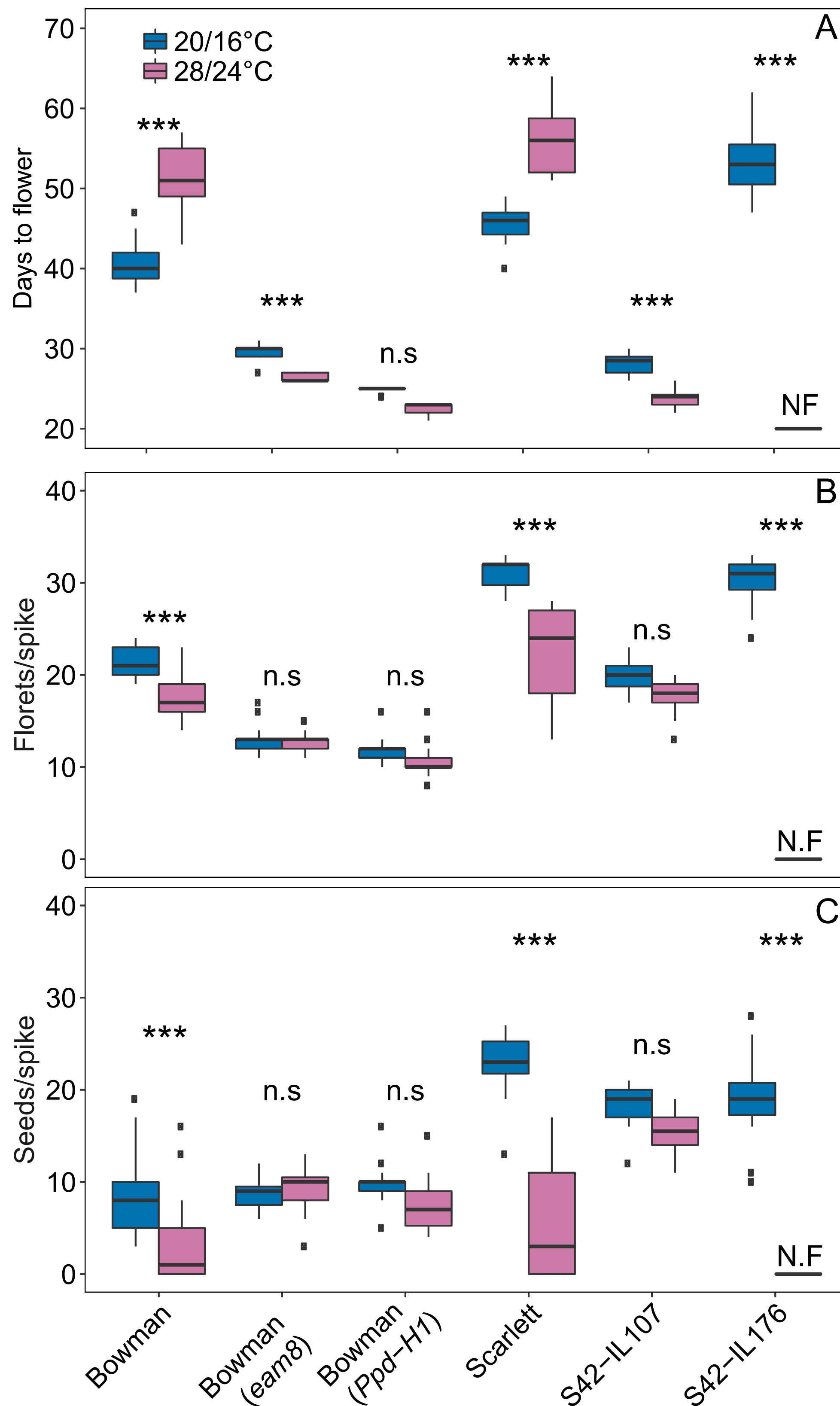

Figure 1: High ambient temperature affects flowering time, floret and seed number per main spike in barley. Days to flower A), the number of florets B), and the number of seeds per main spike C) under control (blue, 20/16°C, day/night) and high ambient temperatures (pink, 28/24°C, day/night) in the spring barley varieties Bowman and Scarlett and the derived introgression lines Bowman(*eam8*)(*Hvelf3*), Bowman(*Ppd-H1*), S42-IL107 (*Ppd-H1*), S42-IL176 (*Hvvrn1*). Flowering time, floret, and seed number were recorded for 20 plants per genotype and treatment under long days (16h light/8h night). N.F indicates non-flowering plants. Statistical differences were calculated by a multi-factorial ANOVA and a posthoc Tukeys HSD pairwise comparison test: *P <0.05, **P <0.01, ***P <0.001, n.s.= non-significant.

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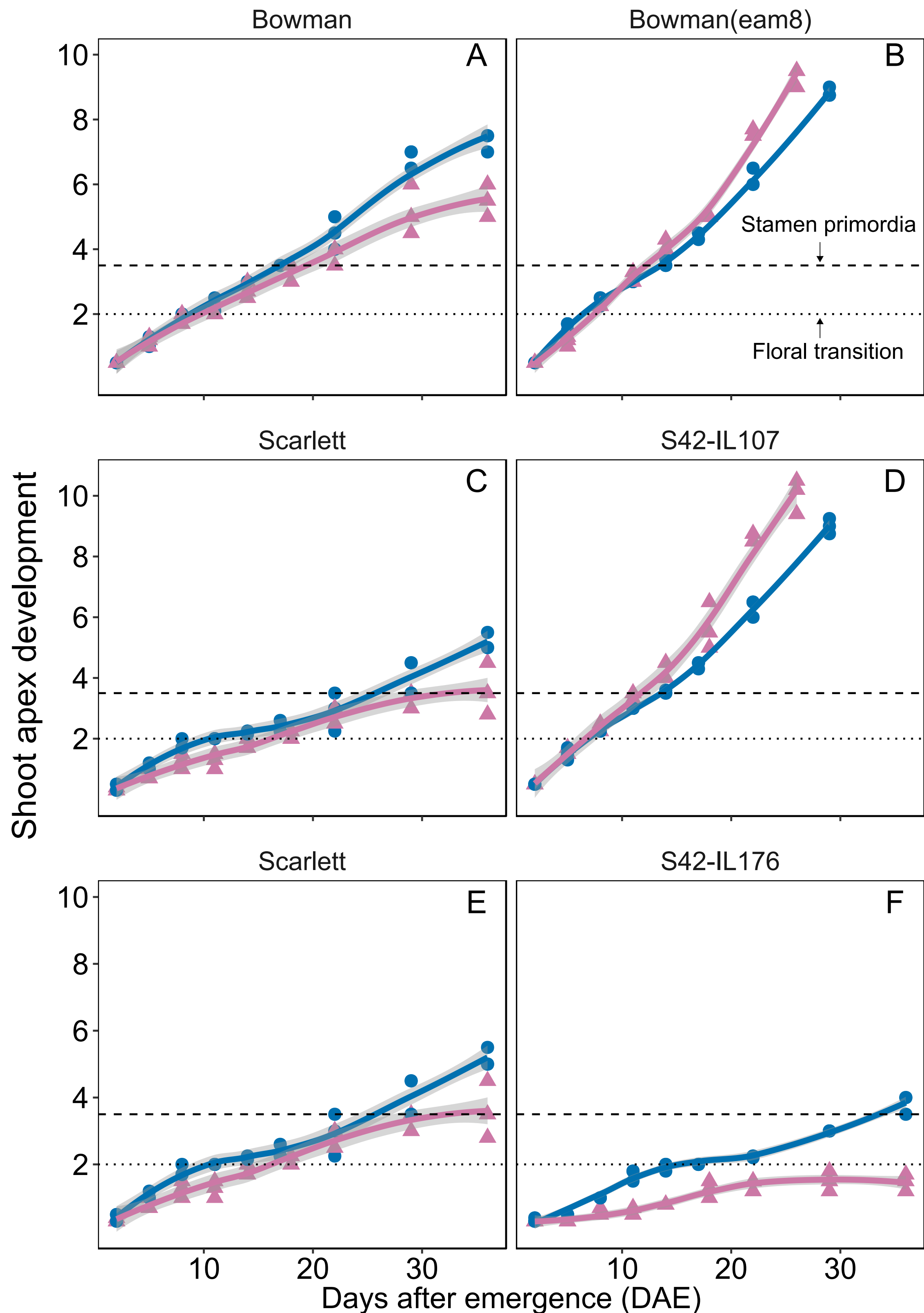

Figure 2: High ambient temperature affects shoot apex development in barley. Microscopic development of the main shoot apex (MSA) was scored under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures every three days according to the Waddington scale (Waddington et al., 1983). MSA development was delayed under high compared to control temperature in Bowman (A) and Scarlett (C, E), accelerated in Bowman(*eam8*) (B) and S42-IL107 (D), and further delayed floral transition in S42-IL176 (F). 3-4 plants per genotype were dissected at each time point in each treatment under long days (16h light/8h night). Statistical differences ($p<0.05$) were calculated using a polynomial regression model at 95% confidence interval (Loess smooth line).

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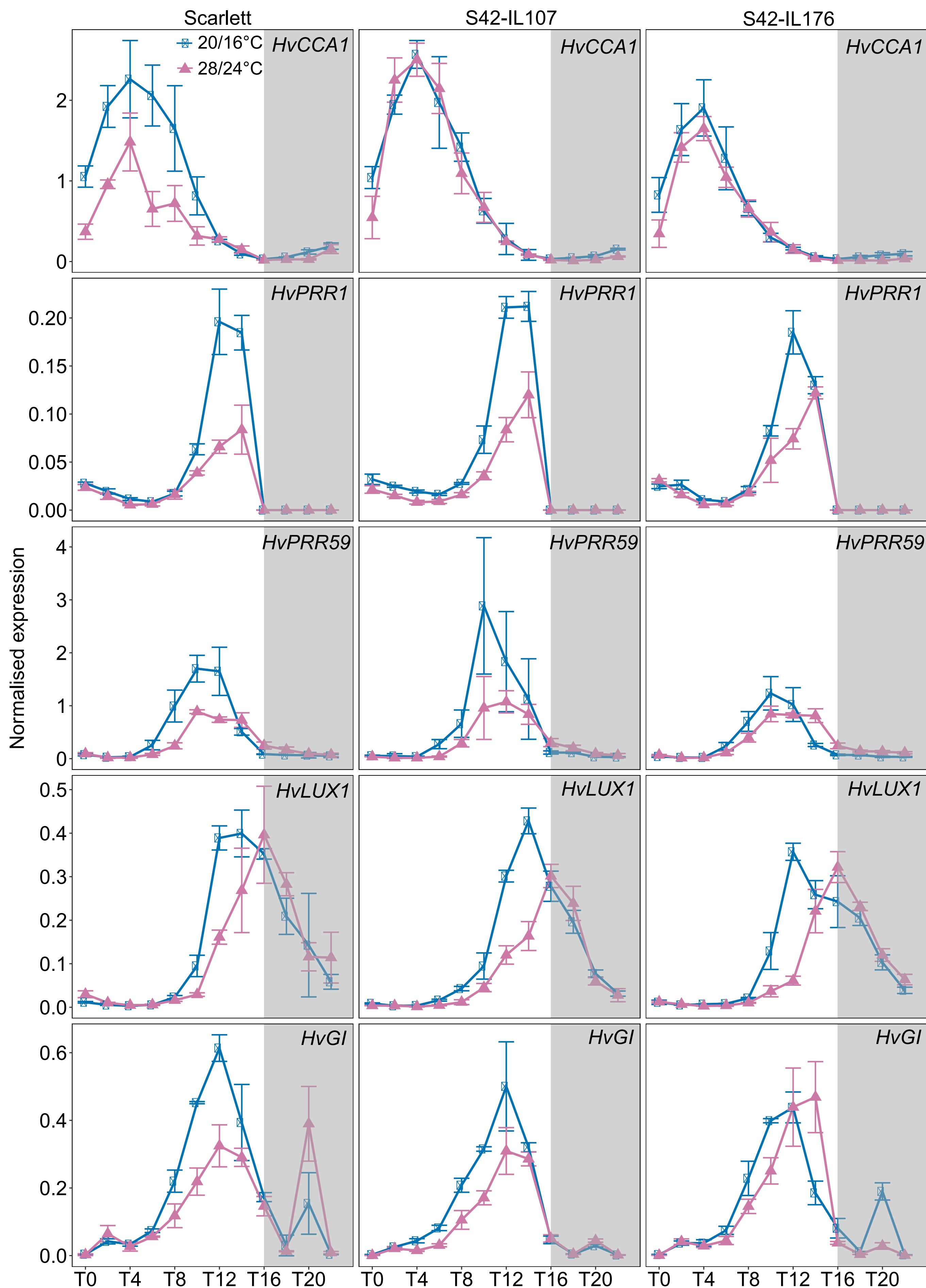

Figure 3: Diurnal expression patterns of circadian clock genes in Scarlett, S42-IL107, and S42-IL176 under control and high ambient temperatures. Diurnal expression of circadian clock genes was assayed every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night). Grey boxes indicate nights. Error bars indicate ±SD of three biological replicates.

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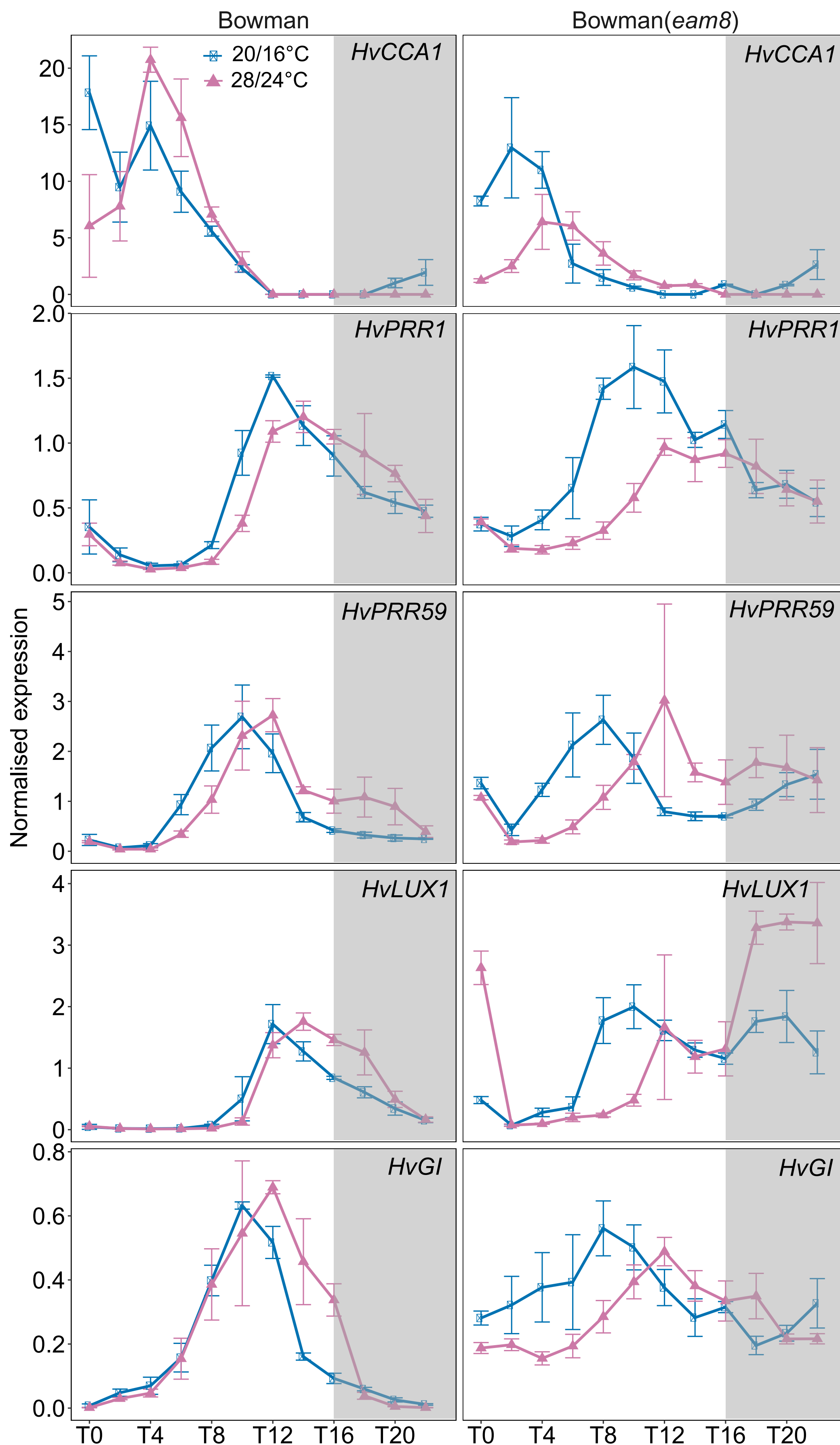

Figure 4: Diurnal expression of circadian clock genes in Bowman, and Bowman(*eam8*) under control and high ambient temperatures. Diurnal expression of circadian clock genes was assayed every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night). Grey boxes indicate nights. Error bars indicate ±SD of three biological replicates.

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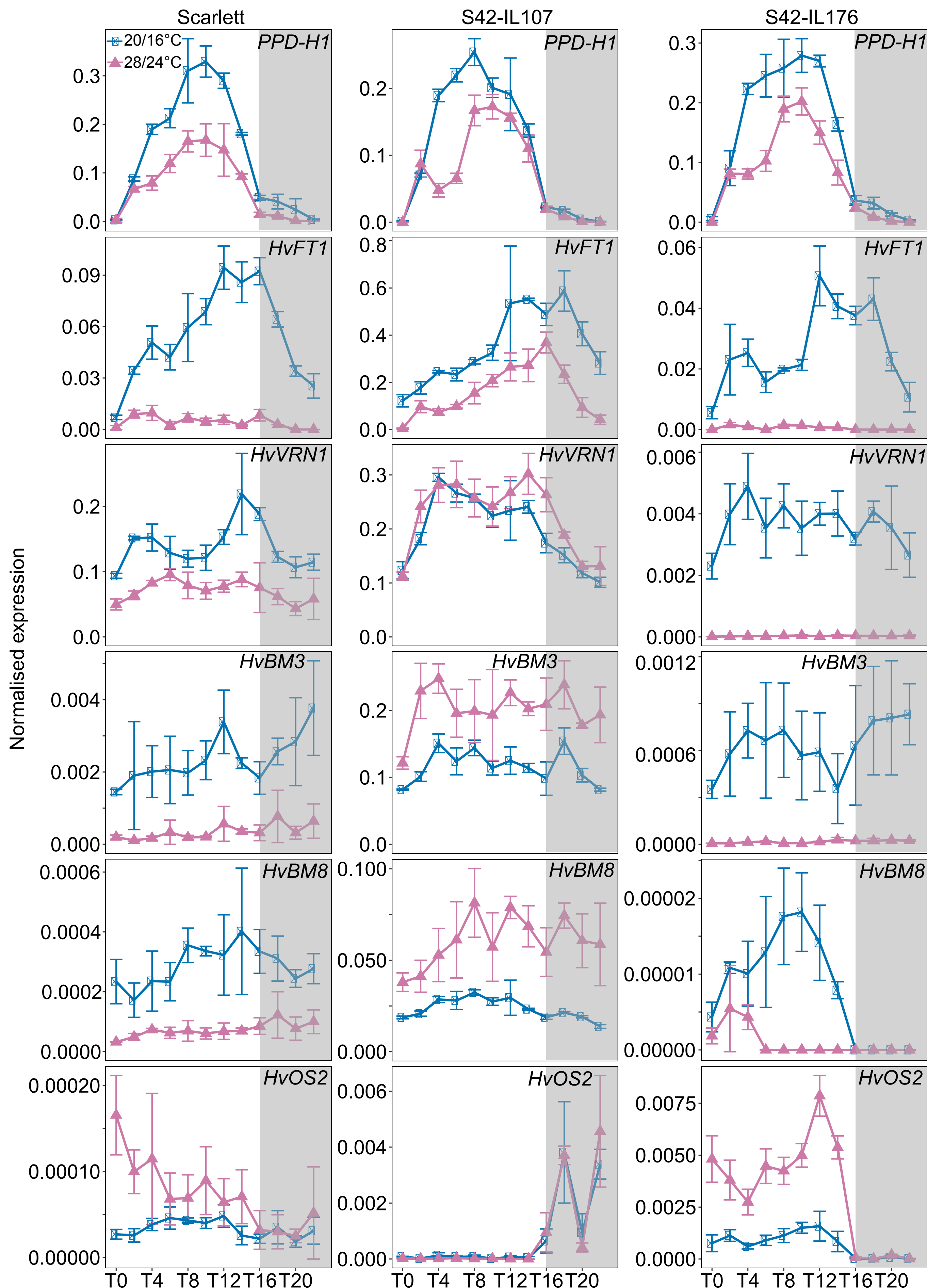

Figure 5: High ambient temperature affects the expression of flowering time genes in Scarlett, S42-IL107, and S42-IL176 Diurnal expression of flowering time genes was assayed every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night). Grey boxes indicate nights. Error bars indicate ±SD of three biological replicates.

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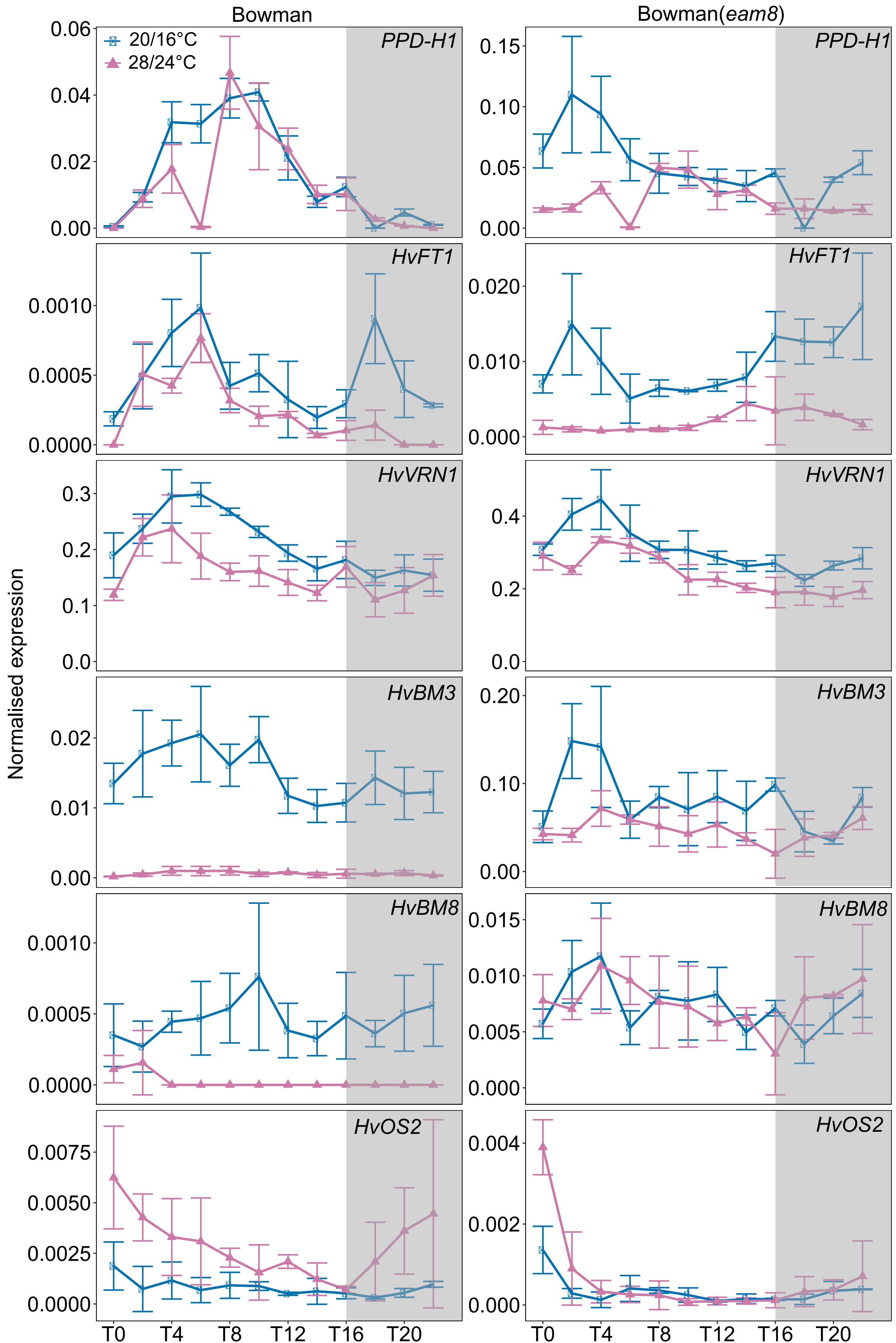

Figure 6: Diurnal expression of flowering time genes in Bowman and Bowman(*eam8*) under control and high ambient temperatures. Diurnal expression of flowering time genes was assayed every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night). Grey boxes indicate nights. Error bars indicate ±SD of three biological replicates.

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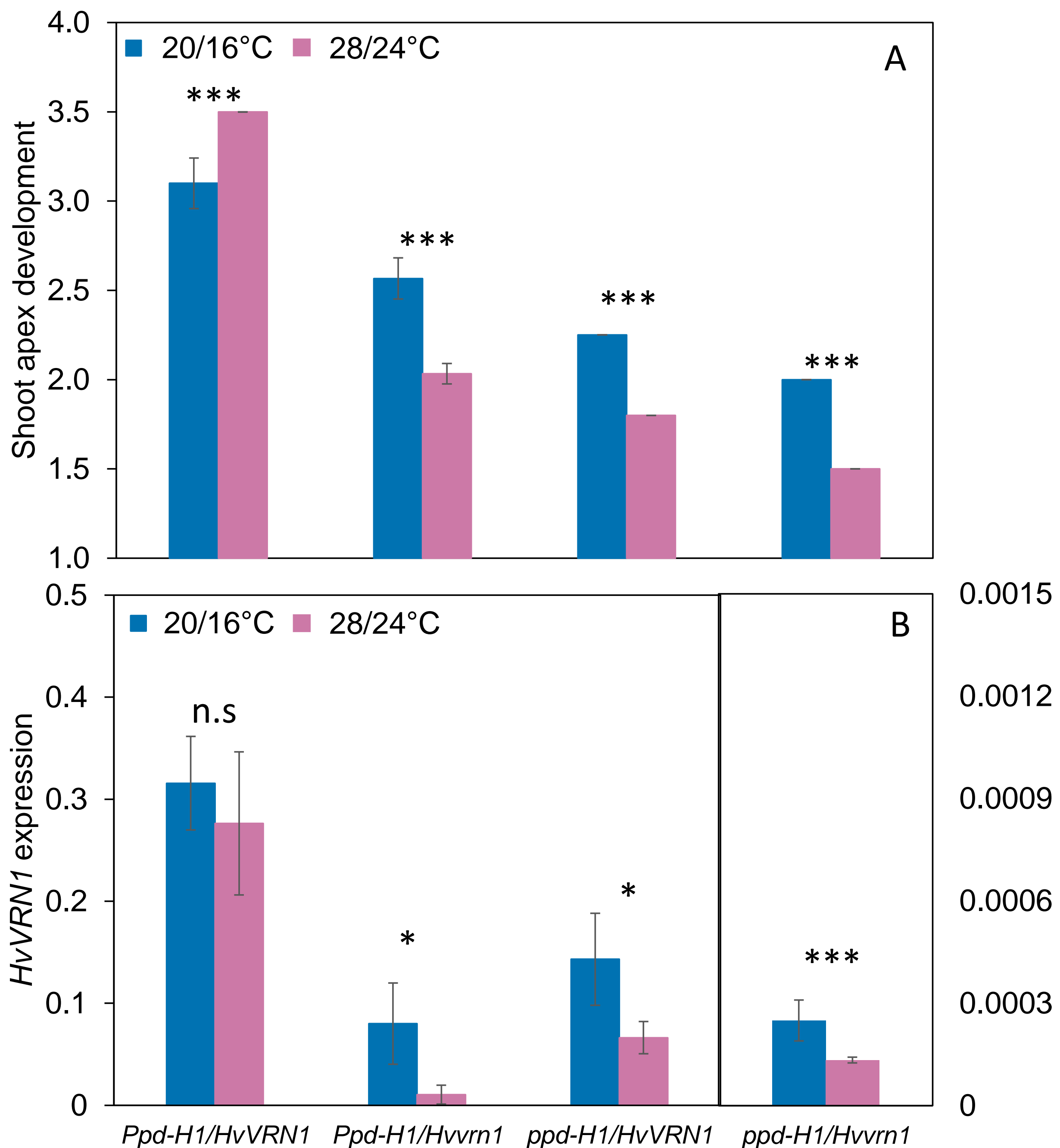

Figure 7: *Ppd-H1* and *HvVRN1* interact to control the development of the main shoot apex (MSA) under different ambient temperatures. Microscopic changes in MSA development were scored under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night) in F_3 families derived from a cross between the winter barley Igri and the spring barley Golden Promise. Selected F_3 families segregated for *Ppd-H1* and *HvVRN1* and were fixed for the spring alleles at *HvVRN2* (deleted), *HvFT1*, and *HvFT3*. 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 multi-factorial ANOVA and a Tukey HSD pairwise comparison test: *P <0.05, **P <0.01, ***P <0.001, n.s= non-significant.

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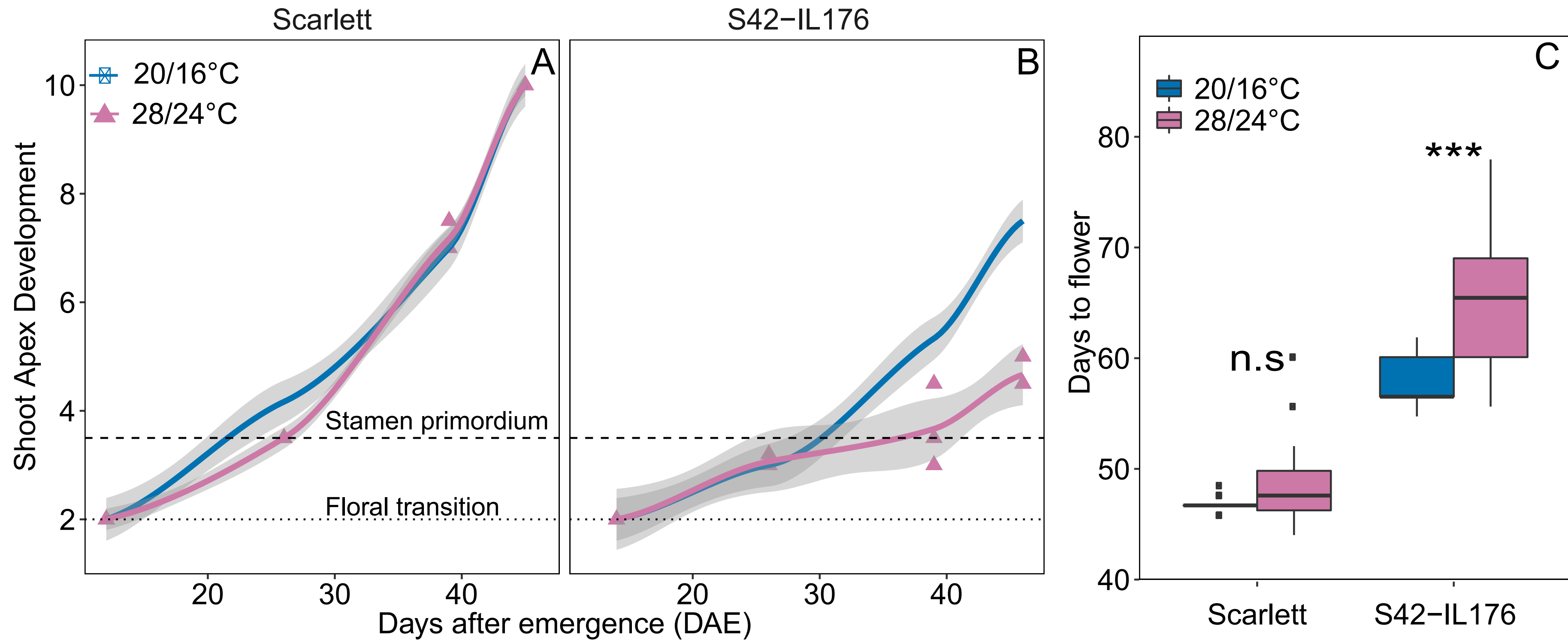
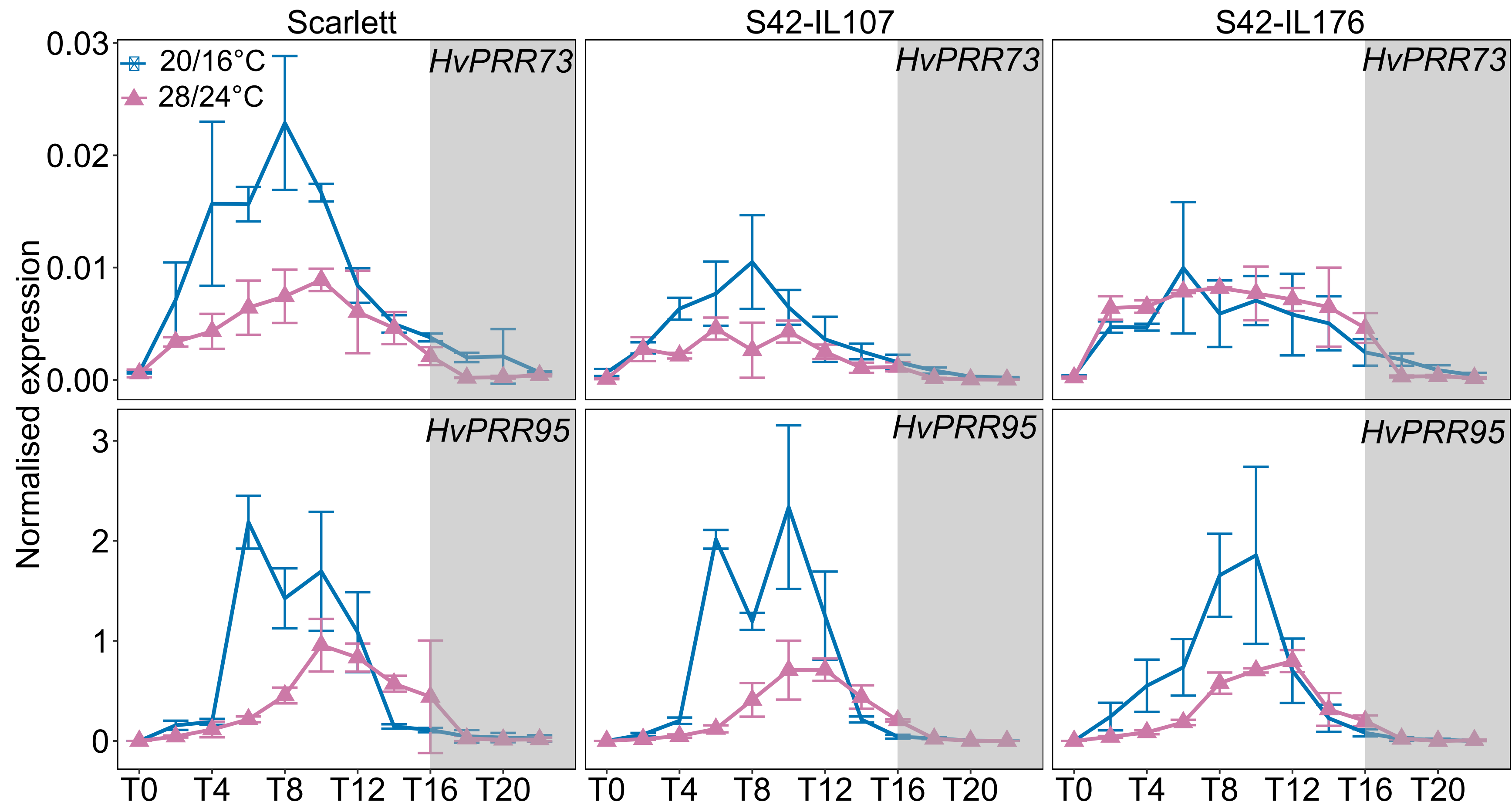
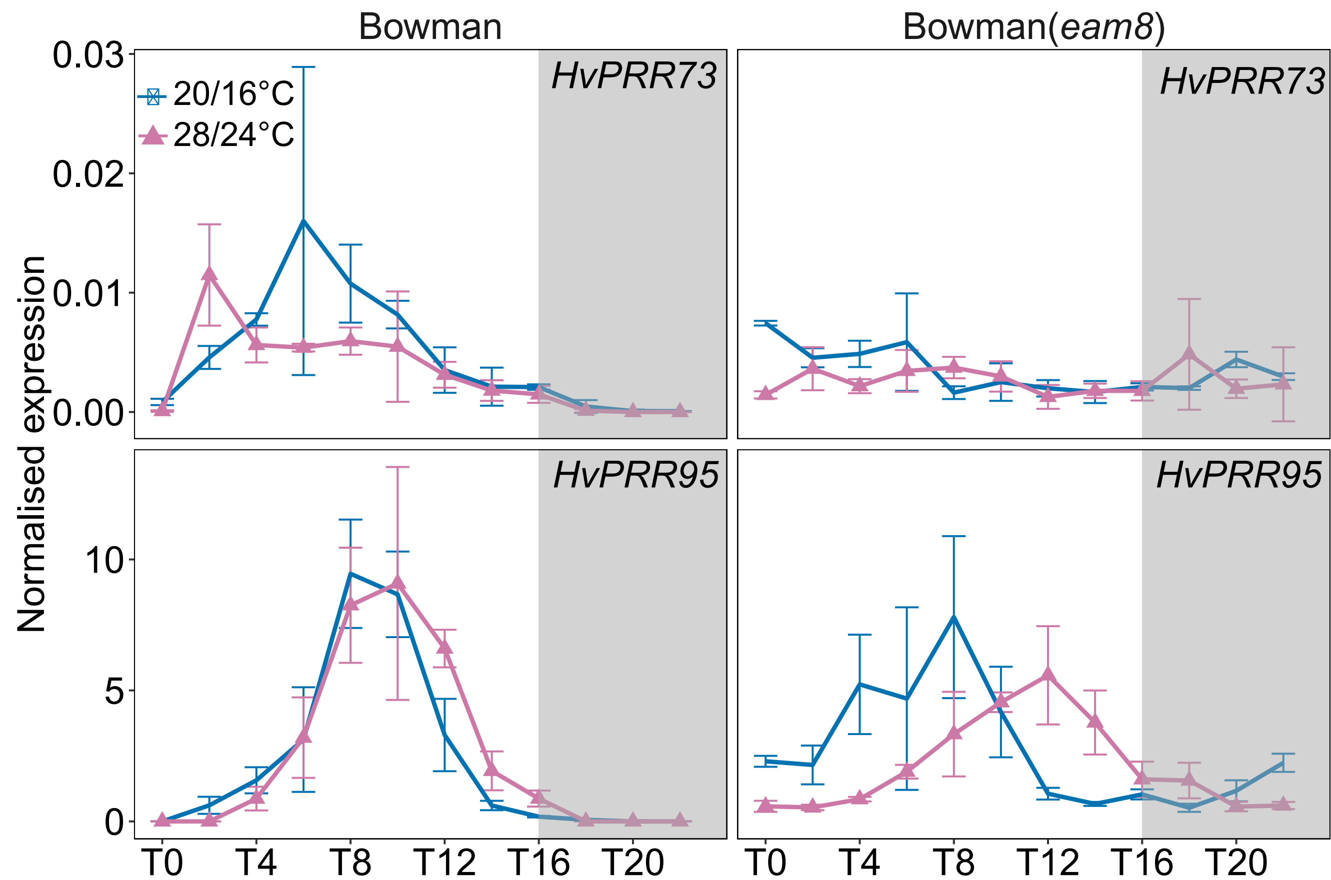


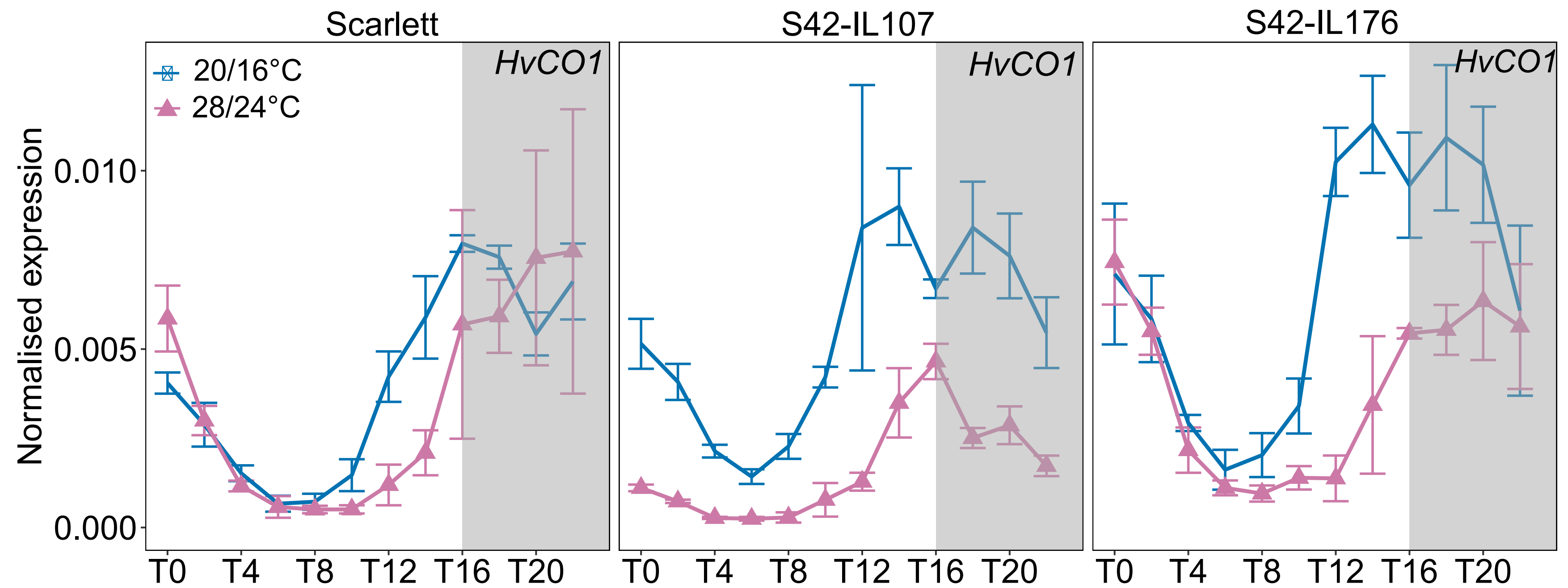
Supplementary Figure 1: *HvVRN1* affects reproductive development in response to ambient temperature after floral transition. Development of the main shoot apex (MSA) was scored under control (blue) and high ambient (pink) temperatures every ten days according to the Waddington scale (Waddington et al., 1983). MSA development was not affected under high compared to control temperatures in Scarlett (A) and delayed inflorescence development in the derived introgression line S42-IL176 (*Hvvrn1*) (B). Plants were grown at control temperature (blue, 20/16°C, day/night) and transferred to high temperature (pink, 28/24°C, day/night) at floral transition (W2.0). 3-4 plants per genotype were dissected at each time point in each treatment under long days (16h light/8h night). Statistical differences were calculated using a polynomial regression model at a 95% confidence interval (Loess smooth line). (C) Days to flowering of the MSA under control (blue, 20/16°C, day/night) and high ambient temperatures (pink, 28/24°C, day/night) in the spring barley variety Scarlett and the derived introgression line S42-IL176 (*Hvvrn1*). Flowering time was recorded for 6-8 plants per genotype and treatment. Statistical differences were calculated by an ANOVA and a posthoc Tukeys HSD pairwise comparison test: *P <0.05, **P <0.01, ***P <0.001, n.s=non-significant.



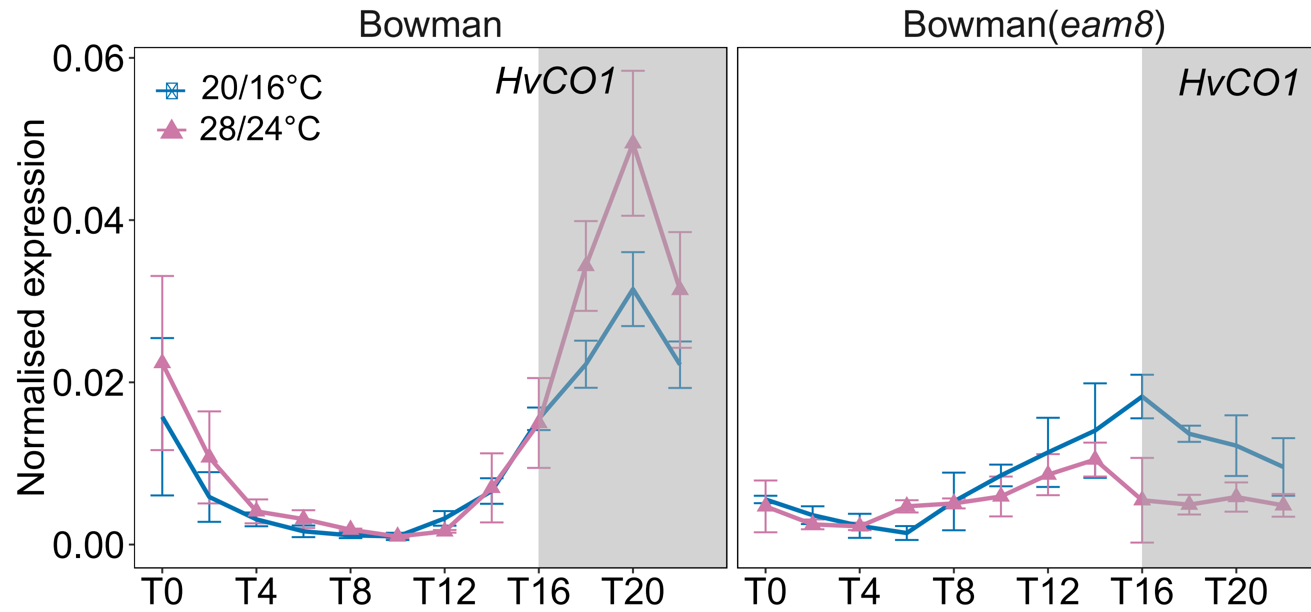
Supplementary Figure 2: Diurnal expression of circadian clock genes *HvPRR73* and *HvPRR95* in Scarlett, S42-IL107, and S42-IL176 under control and high ambient temperatures. Gene expression was assayed every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night). Grey boxes indicate nights. Error bars indicate ±SD of three biological replicates.



Supplementary Figure 3: Diurnal expression of circadian clock genes *HvPRR73* and *HvPRR95* in Bowman and Bowman(*eam8*) under control and high ambient temperatures Gene expression was assayed every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night). Grey boxes indicate nights. Error bars indicate ±SD of three biological replicates.



Supplementary Figure 4: High ambient temperature downregulates the expression of flowering time gene *HvCO1* in Scarlett, S42-IL107, and S42-IL176. Diurnal expression of *HvCO1* was assayed every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night). Grey boxes indicate nights. Error bars indicate ±SD of three biological replicates.



Supplementary Figure 5: Effect of high ambient temperature on diurnal expression of flowering time gene *HvCO1* in Bowman and Bowman(*eam8*). Diurnal expression of *HvCO1* was assayed every two hours for 24 hours under control (blue, 20/16°C, day/night) and high ambient (pink, 28/24°C, day/night) temperatures under long days (16h light/8h night). Grey boxes indicate nights. Error bars indicate ±SD of three biological replicates.

Supplementary Table 1: A) Two-factorial ANOVA, F values and significances (**p<0.01, *** p<0.001, ns = non-significant) and B) Least square means for heading date, floret and seed number for each genotype (P = Parental genotype, Scarlett or Bowman, V = Introgression line for *HvELF3*, *PPD-H1* or *HvVRN1*) by environment combination (C = Control, H= High ambient temperatures). Small letters indicate significant differences (p<0.05).

A

Factor	Heading	Floret number	Seed number
	F Value	F Value	F Value
HvELF3			
Temperature	44***	27***	10**
HvELF3	1102***	310***	15***
*HvELF3**Temp	178***	22***	15***
Ppd-H1			
Temperature	50***	35***	53***
PPD-H1	2098***	117***	12***
*PPD-H1**Temperature	189***	10***	19***
HvVRN1			
Temperature	6995***	732***	363***
HvVRN1	6131***	236***	23***
*HvVRN1**Temp	4617***	235***	1 ns

B

Factor	P/C	P/H	V/C	V/H
HvELF3				
Heading	40^{a}	51^{c}	29.8^{b}	26^{d}
Floret number	21^{a}	17c	13^{b}	13^{b}
Seed number	9^{a}	3^{b}	9^{a}	9^{a}
PPD-H1				
Heading	42^{a}	52^{b}	26^{c}	23^{d}
Floret number	26^{a}	19^{c}	16^{b}	14^{b}
Seed number	15^{a}	4^{c}	14^{ab}	11^{b}
HvVRN1				
Heading	46^{a}	56^{c}	52^{b}	$>106^{d}$
Floret number	30^{a}	22^{b}	30^{a}	0^{c}
Seed number	23^{a}	5^{c}	19^{b}	0^{d}

Supplementary Table 2: List of q-PCR primers used in this study.

Gene ID	Gene name	Forward primer sequence	Reverse primer sequence	Source
AY145451	*HvACTIN*	CGT GTT GGA TTC TGG TGA TG	AGC CAC ATA TGC GAG CTT CT	Campoli et al.2012a
AJ249143	*HvBM3*	GCC GTC ACC AGC ACA AGC AA	CCC CAT TCA CCC TGT AGC AAA GA	Digel et al. 2015
AJ249146	*HvBM8*	CCA CAG CAG CCG ACA CCT A	TGC CTT TGG GGG AGA AGA CG	Digel et al. 2015
JN603242	*HvCCA1*	CCT GGA ATT GGA GAT GGA GA	TGA GCA TGG CTT CTG ATT TG	Campoli et al.2012b
AF490468	*HvCO1*	CTG CTG GGG CTA GTG CTT AC	CCT TGT TGC ATA ACG TGT GG	Campoli et al.2012a
DQ100327	*HvFT1*	GGT AGA CCC AGA TGC TCC AA	TCG TAG CAC ATC ACC TCC TG	Campoli et al.2012a
AK362208	*HvGAPDH*	GTG AGG CTG GTG CTG ATT ACG	AGT GGT GCA GCT AGC ATT TGA GAC	unpublished
AY740524	*HvGI*	TCA GTT AGA GCT CCT GGA AGT	GGT AGT TTG GGC TTT GGA TG	Campoli et al.2012b
Hv.20312	*HvLUX1*	AAT TCA GTC CAC GGA TGC TC	CTT CAC TTC AGC TCC CCT TG	Campoli et al.2012
HM130525	*HvOS2*	CAA TGC TGA TGA CTC AGA TGC T	CGCTATTTCGTTGCGCCAAT	Green up et al. 2010
JN603243	*HvPRR1*	GAG CAT AGC ATG GCA CTT CA	TGT CTT TCC TCG GAA ATT GG	Campoli et al.2012b
AK361360	*HvPRR59*	GAA ATT CCG CAT GAA AAG GA	TTC CGC ATC TTC TGT TGT TG	Campoli et al.2012b
AK376549	*HvPRR73*	GCG CCG TAG AGA ATC AGA AC	CAT GTC GGG TAC AGT CAT CG	Campoli et al.2012b
AK252005	*HvPRR95*	CAG AAC TCC AGT GTC GCA AA	TGC TGT TGC CAG AGT TGT TC	Campoli et al.2012b
Y09741	*HvβTUBLIN*	GTG CAT GGT TCT TGA CAA CG	GCA TGT GAC TCC ACT CAT GG	unpublished
AY750995	*HvVRN1*	CTG AAG GCG AAG GTT GAG AC	TTC TCC TCC TGC AGT GAC CT	Campoli et al.2012a
AY970701	*PPD-H1*	GAT GGA TTC AAA GGC AAG GA	GAA CAA TTG GCT CCT CCA AA	Campoli et al.2012a