Title: Long-day increase of *HvVRN2* expression marks the deadline to fulfill the vernalization requirement in winter barley

Running title: *HvVRN2* marks vernalization fulfillment end in winter barley

Highlight: In a climate change scenario, winter barley performance can be compromised by the expression of *HvVRN2* and *HvOS2*. *HvCO2* can accelerate flowering, like *HvFT3* after being effectively induced by cold.

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1 **Abstract**

2 Vernalization and photoperiod cues are integrated in winter barley plants to flower in the right
3 conditions. We hypothesize that there is a timeframe to satisfy the vernalization needs in
4 order to flower in the optimum moment. Growth and expression of different flowering
5 promoters (*HvVRN1*, *HvCO2*, *Ppd-H1*, *HvFT1*, *HvFT3*) and repressors (*HvVRN2*, *HvCO9*
6 and *HvOS2*) were evaluated in two winter barley varieties under: (1) natural increasing
7 photoperiod, without vernalization, and (2) under short day conditions in three insufficient
8 vernalization treatments. Here, we provide evidence of the existence of a day-length
9 threshold, around 12 h 30 min in our latitudes (Zaragoza, Spain, 41º43'N), marked by the rise
10 of *HvVRN2* expression, which defines the moment in which cold requirement must be
11 satisfied to acquire competency to flower. Before that, expression of *HvCO2* was induced and
12 might be promoting *HvFT1* in both inductive and non-inductive conditions. *HvFT3,* to be
13 effectively expressed, must receive induction of cold or plant development, through
14 downregulation of *HvVRN2* and *HvOS2*. We emphasize the contribution of *HvOS2*, together
15 with *HvVRN2*, in the delay of flowering in vernalization-responsive cultivars. Understanding
16 this complex mechanism of flowering might be useful for breeders to define varieties,
17 particularly in a climate change scenario.

18

19 Keyword index: barley, gene expression, *HvCO2*, *HvFT3*, *HvOS2*, *HvVRN1*, *HvVRN2*,
20 photoperiod, *Ppd-H1*, vernalization

21

Introduction

Tight coordination of flowering time to environmental conditions is crucial for crop reproductive success and has a major impact on yield (Campoli and von Korff, 2014; Digel *et al.*, 2015). Barley (*Hordeum vulgare* L.) and wheat (*Triticum* spp.) are long-day plants, flowering earlier under increasing day-lengths. Depending on their growth habit, cereals are classified as winter or spring. Winter cereals need a period of exposure to low temperature, a process called vernalization (Laurie *et al.*, 1995; Trevaskis *et al.*, 2003), which must be completed timely so the plant is prepared to take full advantage of the induction of flowering by long days (Trevaskis, 2010). This requirement could make winter barley and wheat more susceptible to climate change, since the probability of accumulating enough cold hours will decrease in warming winters. Winter barley varieties are sown in autumn, benefiting from the warmth of the soils and the humidity from autumn rains, which are essential at the beginning of the cycle. In the Mediterranean region, they have to survive a range of mild to harsh winters, and then flower sufficiently early in the spring to avoid the heat and drought of late spring or early summer.

The accepted gene model for vernalization-responsive varieties establishes that during winter, cold exposure upregulates the floral promoter *HvVRN1*, which is required to downregulate the flowering repressor *HvVRN2*, allowing expression of the flowering inducer *HvFT1* in leaves (Distelfeld *et al.*, 2009). *HvVRN2*, *ZCCT-H* gene and member of the *CONSTANS*-like gene family, delays flowering until plants have satisfied its cold needs (Yan *et al.*, 2004). In winter barleys is present in the dominant variant, whose expression is highly dependent on day-length, being induced in long days (Karsai *et al.*, 2005; Trevaskis *et al.*, 2006).

HvVRN1 encodes an AP1-like MADS-box transcription factor (Danyluk *et al.*, 2003; Trevaskis *et al.*, 2003; Yan *et al.*, 2003). It presents several alleles as a result of deletions or insertions in the first intron, associated with different degrees of vernalization requirement (Hemming *et al.*, 2009). In winter barley, *HvVRN1* is expressed after exposure to low-temperatures (von Zitzewitz *et al.*, 2005; Sasani *et al.*, 2009), although it can be activated by other pathways such as the developmental pathway, with a marked delay compared with induction by vernalization (Trevaskis *et al.*, 2006). Induction of *HvVRN1* is related to changes in the pattern of histone methylation, whose maintenance provides a memory of cold exposure in winter barley plants (Oliver *et al.*, 2009).

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53   Under short day (SD) conditions, *HvFT3*, a FT-like member of the PEBP family, and

54   candidate gene for *Ppd-H2* (Faure *et al.*, 2007; Kikuchi et al., 2009), has been described as

55   promoter of flowering (Laurie *et al.*, 1995). Two allelic variants for this gene are known: a

56   dominant one, with a functional copy of the gene, and a recessive allele, with most of the gene

57   missing and nonfunctional (Kikuchi *et al.*, 2009). Its presence caused differences in heading

58   date in SD (Boyd *et al.*, 2003; Faure *et al.*, 2007). A QTL for heading date co-locating with

59   this gene was identified in autumn sowings (Cuesta-Marcos *et al.*, 2008; Borràs-Gelonch *et*

60   *al.*, 2012), showing a remarkable importance for adaptation under Mediterranean conditions

61   (Casao *et al.*, 2011*b*), although it may have a negative impact for low temperature tolerance in

62   facultative genotypes (Cuesta-Marcos *et al.*, 2015, Rizza *et al*. 2016).

63   The sensitivity to long days (LD) is determined by *Ppd-H1* (Laurie *et al.*, 1995), candidate

64   gene for *HvPRR7*, pseudo-response regulator 7 (Turner *et al.*, 2005). The dominant allele

65   accelerates flowering mediating the induction of *HvFT1* through the activity of

66   *HvCO1/HvCO2* (Turner *et al.*, 2005). Recently, Mulki and von Korff (2016) have revealed a

67   possible role of *Ppd-H1* as repressor of flowering, mediating the induction of *HvVRN2* before

68   the vernalization

69   Another known repressor in the vernalization pathway is the monocot homolog of the

70   Arabidopsis *FLOWERING LOCUS C*, *ODDSOC2* (in barley *HvOS2*). It is a MADS-box

71   repressor of flowering, also downregulated by vernalization (Greenup *et al.* 2010; Ruelens *et*

72   *al.* 2013), which is affected by photoperiod and induced by high temperatures (Hemming *et*

73   *al.*, 2012).

74   VRN1 directly binds to the promoter regions of the repressor genes *HvVRN2* and *HvOS2*,

75   downregulating their expression, and also to the *HvFT1* promoter, enhancing its expression

76   (Deng *et al.*, 2015). These results explain why vernalization is a pre-requisite to promote

77   flowering under long-day (LD) in temperate cereals. However, it has been suggested that

78   other additional genes may be acting as regulators of *VRN2* when exposed to cold (Chen and

79   Dubcovsky, 2012; Sharma *et al.*, 2017).

80   Two closely related *CO* genes, *CO1* and *CO2*, are present in the temperate cereals. Both are

81   LD-flowering promoters modulated by circadian clock and day-length (Griffiths *et al.*, 2003;

82   Nemoto *et al.*, 2003). CO2 competes with VRN2 for interactions with a common protein, NF-

83   Y, in a mechanism to integrate environmental cues through regulation of *HvFT1* (Li *et al.*,

84   2011). Overexpression of *HvCO2*, induced the expression of *HvFT1* in spring barley but it

85   caused up-regulation of the repressor *HvVRN2*, in winter barley, resulting in reduced

86   expression of *HvFT1* and delayed flowering (Mulki and von Korff, 2016). Other member of

87   the *CONSTANS*-like family, *HvCO9* (*HvCMF11*, Cockram *et al.* (2012)) is a negative

88   regulator of flowering, paralogue of *HvVRN2* (Higgins *et al.*, 2010). It is expressed under

89   non-inductive SD conditions, correlating with *HvFT1* and *HvFT2*, but no relationship was

90   found between *HvCO9* and *HvFT3* (Kikuchi *et al.*, 2012).

91   Crop modelling studies have determined that barley ideotypes, for future Boreal and

92   Mediterranean climatic zones in Europe, should have appropriate vernalization and

93   photoperiod responses finely tuned to the needs of each specific region (Tao *et al.*, 2017). One

94   future avenue for plant breeding will be to use elite germplasm coming from regions that have

95   experienced the foreseen conditions (Atlin *et al.*, 2017). For example, transferring cultivars

96   adapted to Mediterranean conditions, which possess a strategy based on scape to drought, to

97   more northern latitudes. To achieve that goal, the responses to photoperiod should be

98   modified accordingly to avoid yield penalties (Dawson *et al.*, 2015). For this reason,

99   comprehensive studies on the effect of photoperiod on major flowering genes are called for.

100   This study focuses on the repression of flowering, under non-inductive conditions, in winter

101   barley. Previous studies have demonstrated that *HvVRN2* expression needs induction by long

102   days (Trevaskis *et al.*, 2006). But, how long? As studies have been performed under fixed

103   photoperiods in growth chambers, the actual day-length threshold to induce *HvVRN2* is

104   unknown. There is indication that this gene has no effect below 12 h (Karsai *et al*. 2006). This

105   question is relevant from the agronomic point of view. We hypothesize that there is a

106   vernalization window for satisfying the cold requirement, in order to make the plant

107   competent to flower at the right time and achieve a good yield. In these experiments, the goal

108   was to determine the day-length threshold leading to induction of the repressor *HvVRN2*,

109   assuming that this is the end of that window. Additionally, we want to further characterize the

110   role of *HvFT3* in the promotion to flowering in winter barley. Here, we investigate the effects

111   of photoperiod on the transcript levels of selected genes in winter barley, by examining

112   photoperiod responses in the medium-long term (21 - 90 days). The results provided may help

113   to understand the complex mechanism of flowering in suboptimal conditions, and facilitate

114   breeding for present and future climate conditions in Europe and elsewhere.

Materials and methods

Plant materials

Two French winter barley varieties, 'Hispanic' (two-rowed, 'Mosar' x ('Flika' x 'Lada')) and 'Barberousse' (six-rowed, ('Hauter' x ('Hatif de Grignon' x 'Ares')) x 'Ager') were selected. They have the same allelic combination in *HvVRN1* (winter allele, same first intron length), *HvVRN2* (all *ZCCT-H* genes present), and *Ppd-H1* (dominant, long photoperiod responsive), but differ in *HvFT1* and *HvFT3* (*Ppd-H2*, present in 'Hispanic', defective in 'Barberousse') (Loscos *et al.*, 2014).

Plant growth, phenotyping and sampling

Experiment 1 – Sowings under increasing natural photoperiod

For each variety, we used two 1L-pots at each sowing time (standard substrate made of peat, fine sand and perlite, from a mix with 46 kg, 150 kg and 1L, respectively). Pots were sown with 7 seeds once a week, sequentially, from Feb 11th until April 8th 2015, in a glasshouse in Zaragoza (41°43'N, 00°49'W) under natural photoperiod (Fig. 1) and controlled temperature (22±1°C day / 18±1°C night). Unless specified, plants were not vernalized (NV). Spatial homogeneity in irradiance was obtained rotating the plants each week. As vernalized control, three pots of each variety were sown on Feb 11th. They were grown during 7 days (until germination) under glasshouse conditions, and then were vernalized (VER) under short photoperiod (8 h light) and 6±2°C for 49 days. After the cold treatment, plants were transferred to the same glasshouse on April 8th, when natural photoperiod was 13 h. Duration of daylight at sowing and sampling dates was gathered from http://www.timeanddate.com/sun, taking sunrise and sunset as the times when the upper edge of the Sun's disc touches the horizon.

For gene expression, the last expanded leaf of three 21-day-old plants (3-leaf stage) was sampled 8 h after dawn, frozen in liquid nitrogen, homogenized (Mixer Mill model MM301, Retsch) and conserved at -80°C until RNA isolation. In all the experiments, sampling time was chosen to capture high expression moments of almost of the genes involved, taking into account their circadian rhythms.

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143   On a fixed date (19th May, day-length 15 h, 97 days after the first sowing), we took a cross-

144   sectional sample across sowing events. The last expanded leaf of each weekly-sown plant was

145   sampled 12 h after dawn for RNA isolation. Then, dissection of the plants (all stems of each

146   plant) was made in order to determine the development of the apex (with naked eye,

147   reproductive apex was equivalent to more than 3 mm).

148   **Experiment 2 – Growth chamber, 12 h light**

149   Seventy-two seeds of each variety were sown in 12-well trays (650 cc) and allowed to

150   germinate during 7 days in a growth chamber at 12 h light, 20°C/12 h dark, 16°C, 65% HR

151   and light intensity of 300 µmol m^{-2}s^{-1} PAR. Then, the trays were divided in three groups that

152   received the following treatments: (A) NV, (B) 14-days VER and (C) 28-days VER. Group A

153   stayed at the growth chamber while B and C were transferred to a vernalization chamber, 8 h

154   light/16 h night and constant temperature (6 ± 2°C). Groups B and C were returned to the

155   growth chamber after 14 and 28 days of cold treatment, respectively. After forty days at the

156   growth chamber, 3 plants of each variety and treatment were transferred to a 1L pots to let

157   them grow until flowering.

158   For gene expression the last expanded leaf of four plants was sampled 14, 28, 35 or 49 days

159   after germination (A) or after the end of the VER treatment (B and C), 10 hours into the light

160   period.

161   Number of leaves, tillers, development at Zadoks scale (Zadoks *et al.*, 1974) were recorded

162   along the experiment every 3-5 days. LSD multiple comparisons were obtained for each trait.

163   Also apex dissections were carried out at selected time points to establish the Waddington

164   developmental stage (Waddington and Cartwright, 1983). The experiment ended 136 days

165   after sowing.

166   **Vernalization response of 'Hispanic' and 'Barberousse'**

167   In the course of earlier experiments, carried out in the Phytotron of Martonvásár (Hungary),

168   both varieties were exposed to different VER treatments (0, 15, 30 or 45 days, 4°C, 8 h light),

169   and then transferred to a growth chamber 16 h day-length, 18°C and light intensity of 340

170   µmol m^{-2} s$^-$1. Flowering date was recorded at each treatment (Fig. S1).

171   **Gene expression analysis**

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172   Three individual plants were sampled at each time point per genotype. RNA extraction was
173   carried out using Nucleospin RNA Plant Kit (Macherey-Nagel, Germany) following
174   manufacturer instructions. Total RNA (1µg) was employed for cDNA synthesis using
175   SuperScript III Reverse Transcriptase (Invitrogen) and oligo $(dT)_{20}$ primer (Invitrogen). Real-
176   time PCR quantification (ABI 7500, Applied Biosystems) was performed for samples from
177   each time point from NV plants and for VER plants as control treatment. . Three biological
178   repeats and two technical repeats were performed per sample and pair of primers (*HvVRN1*,
179   *HvVRN2*, *Ppd-H1*, *HvCO2*, *HvCO9*, *HvOS2, HvFT1*, and *HvFT3*). Primer sequences and
180   conditions are specified in Table S1. Expression levels were normalized to *Actin* expression,
181   taking into account primer efficiencies.

182   **Statistical analysis**

183   Statistical analysis was carried out in R software (R Core Team, 2017). Multiple comparisons
184   were obtained by Fisher's protected Least Significant Differences (LSD) with the R package
185   'agricolae' (de Mendiburu, 2016). For gene expression results, the mean of two technical
186   replications of ΔCt (Ct actin – Ct target) was used as unit. Analyses of variance for each gene
187   and experiment were performed considering all factors as fixed. Pearson correlations were
188   carried out with 'cor' function.

189   # Results

190   **Gene expression under increasing photoperiod conditions (experiment 1)**

191   Natural day-length at sampling increased from ∼11 h 30 min at the first sowing to ∼14 h at
192   the 9th sowing event, and also for the VER control (Fig. 1).

193   Leaves were sampled 21 days after each sowing or 21 days after the end of the VER
194   treatment. Expression levels of *HvCO2*, *HvCO9*, *HvFT1*, *HvFT3*, *HvOS2*, *HvVRN1*, *HvVRN2*
195   and *Ppd-H1*, were analysed by qRT-PCR. *HvVRN1* expression was detected only in VER
196   plants (Fig. 2), in concordance with the cold treatment received. *HvVRN2* expression was
197   detected at all time points, although expression was lower in plants from the first four
198   sowings, grown under shorter photoperiods (Fig. 2). Concurrent higher levels of *HvCO2* were
199   detected in those same plants (Fig. 2). When the day-length reached 12 h 30 min (sowing
200   events 5-9), corresponding to 28th March in our latitudes, the expression of *HvCO2* decreased,
201   *Ppd-H1* increased in 'Hispanic' and the levels of *HvVRN2* increased in both genotypes.

202   Therefore, upregulation of *HvVRN2* in NV plants occurred under day-lengths longer than 12 h

203   30 min in 21 day-old plants, and was also detected in 14 day-old plants (Fig. S2).

204   Without vernalization, neither genotype showed expression of *HvFT3* (Fig. 2). This was

205   expected for 'Barberousse', as it has the null allele, but we could not anticipate this result for

206   'Hispanic'. In this genotype, the expression levels were below the detection limit, except for

207   VER plants.

208   In general, 'Barberousse' presented higher *HvOS2* expression levels than 'Hispanic', except

209   for the last samplings, when *HvOS2* expression was barely detectable in both genotypes.

210   Expression of *HvCO9* in 'Hispanic' was low and variable. Higher *HvCO9* expression was

211   observed in the last time points of 'Barberousse' (Fig. 2).

212   Differences between genotypes were also detected in VER plants. High expression of

213   *HvVRN1* and *HvFT3,* and barely any expression of *HvVRN2,* was seen in 'Hispanic'. On the

214   other hand, although *HvVRN1* was detected in VER plants from 'Barberousse', high

215   expression of *HvOS2* and *HvVRN2* was apparent (Fig. 2), suggesting a delay in development,

216   which was even more evident when assessing apex growth (Fig. 3).

217   **Reproductive stage and gene expression from medium to long term development at 15 h**

218   **day-length (experiment 1)**

219   Ninety-seven days since the beginning of the experiment, on **May 19th**, with 15 h light, the

220   number of apices at reproductive stage per plant was recorded (Fig. 3). Among NV plants,

221   only the second sowing event of 'Hispanic' reached the stage Z49 (first awns visible) at the

222   end of the experiment (83 days after sowing; no data available for the first sowing, whose

223   plants were dissected earlier and showed reproductive apices after 72 days). VER 'Hispanic'

224   and 'Barberousse' also showed apices at reproductive stage, 'Barberousse' more delayed than

225   'Hispanic'.

226   Expression levels on this same date were also analysed (Fig. 4). Under NV conditions,

227   flowering promoters (*HvVRN1*, *HvFT1* and *HvFT3*) were induced only in 'Hispanic' at the

228   first point available (sowing event 2), and were absent in 'Barberousse'. Accordingly,

229   *HvVRN2*, *HvCO9* and *HvOS2* were repressed in 'Hispanic', and induced in 'Barberousse'.

230   *Ppd-H1* was expressed at higher levels in 'Hispanic' and only *HvCO2* was equally expressed

231   in both varieties at this point.

232   For the rest of NV plants (Fig. 4, sowing events 3 to 10), no expression of *HvVRN1*, *HvFT1* or

233   *HvFT3* and high expression of *HvVRN2* and *HvOS2* was detected. Differences between

234   varieties were found in *HvCO2* and *HvCO9* expression, being low in 'Barberousse' from

235   sowing events 3 to 10, while levels increased from sowing event 6 in 'Hispanic', revealing a

236   common effect of development and variety for these two *CONSTANS*-like genes. Contrasting

237   with this, VER plants did not show differences in transcript levels, except for *HvCO2* and

238   *HvFT1*, which were more expressed in 'Hispanic', and less in 'Barberousse'.

Responses to 12h photoperiod after increasing vernalization treatments (experiment 2)

240   Experiment 1 made evident that gene expression was dependent of the plant's developmental

241   stage (Fig. S2). Two weeks after sowing was not enough to observe differences, but 3 weeks

242   was (Fig. 2). Therefore, for some genes, induction was dependent on plant age. A second

243   experiment was conceived, to assess the relevance of other factors on gene expression,

244   namely day-length, plant age and degree of vernalization. Thus, we set the day-length at 12h,

245   representative of day-length around the start of stem elongation in natural conditions in our

246   region, and short enough not to elicit LD responses. This was combined with insufficient

247   vernalization.

248   Flowering time was advanced in an inversely proportional manner to the duration of the VER

249   treatment (Fig. 5). Under 12 h of light, and NV, 'Hispanic' reached awn tipping (DEV49)

250   after 124 days, whereas 'Barberousse' did not reach that stage during the entire experimental

251   period (136 days). Two or four weeks of VER decreased markedly the time to DEV49 for

252   both genotypes. Plants from both VER treatments reached this stage before the NV plants did.

253   Most of this shortening occurred in the period until first node appearance (DEV31), although

254   some additional acceleration was observed between DEV31 and DEV49. Under the

255   conditions of experiment 2, 'Hispanic' had clearly higher total and reproductive tillers than

256   'Barberousse' (Fig. 5), increasing with the length of the VER treatment.

Gene expression under photoperiod of 12 h affected by vernalization and plant age

258   Expression analysis showed higher *HvVRN1* induction with the VER duration in both

259   varieties (Fig. 6). Concurrently to the larger expression of *HvVRN1*, *HvVRN2* was repressed,

260   as expected. Expression of *HvCO9* and *HvOS2* was also reduced with increasing VER. These

261   three repressors showed higher levels in 'Barberousse' than in 'Hispanic' (Fig. 6), which were

262   correlated with the delayed flowering of 'Barberousse' (Fig. S3a). Transcript levels of *Ppd-*

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263   *H1* were similar between treatments. Only 'Hispanic' V28 and 'Barberousse' V0 showed

264   differences between sample points. Expression of *HvCO2* was clearly related to that of

265   *HvFT1* in both genotypes, showing 'Barberousse' earlier induction but lower expression

266   levels (Fig. 6). Such decrease in 'Barberousse' is simultaneous with an increased expression

267   of *HvVRN2*, *HvOS2* or *HvCO9*. *HvFT3* transcript levels were present in 'Hispanic', only after

268   plants where 28-days VER, and concurrent with a total absence of *HvVRN2*. The decreased

269   expression of *HvCO2* at the last sampling point was inversely related with the longer VER

270   treatment and the early flowering (Fig. S3b).

271   The increased expression levels of the flowering promoters and the decreased levels of the

272   flowering repressors (Fig. 6) agree with the transition from vegetative to reproductive stage

273   (from W2 to W3; Fig. 7), which was observed only in 'Hispanic' VER 28 days. In contrast,

274   'Barberousse' apices only reached this stage unless vernalized and later in time (Fig. S4).

Discussion

276   Our results shed further light on the functioning and integration of the vernalization and

277   photoperiod pathways in barley. Although the experiments were performed under controlled

278   conditions, these were established bearing in mind their representativeness of natural

279   conditions. The combined results of the two experiments led us to put forward several new

280   hypotheses to explain the dynamics of vernalization in winter barleys. This and other studies,

281   show that the vernalization process involves more genes than previously thought. The

282   complexity found is challenging but, on the other hand, widens the catalogue of genes

283   amenable for breeding.

284   **Expression of *HvVRN2* is upregulated beyond 12 h 30 min natural daylight in absence of**

285   **vernalization**

286   Under typical autumn sowings, winter barley is capable of responding to long photoperiods

287   only after fulfilling a variety-specific low temperature requirement. The model described by

288   Distelfeld *et al.* (2009) and Trevaskis (2010) suggests that, flowering of autumn-sown cereals

289   is delayed before winter because neither day-length nor vernalization response pathways are

290   active. Under SD and low temperature conditions, *HvVRN1* is gradually induced and

291   represses *HvVRN2* to promote flowering. There is also evidence that expression of *HvVRN2* is

292   upregulated in LD (16 h light) and downregulated in SD (8 h light) to almost complete

293   repression (Trevaskis *et al.*, 2006) but, the environmental threshold that induces expression of

294   *HvVRN2* was still undefined. Karsai *et al.* (2006) found a QTL co-locating with *HvVRN2* in

295   vernalized plants when day-length was over 12 hours. Therefore, the limit is similar,

296   irrespective to vernalization.

297   We found expression of *HvVRN2,* even if at low levels, in NV plants under natural SD

298   (sowings 1-4 in experiment 1). Gene expression remained low until a sudden surge around

299   sowing event 5 (Fig. 2), coincident with an increase of natural daylight above 12 h 30 min

300   (~28th March). We propose that this rise marks the day-length threshold, defining the moment

301   in which cold needs must be satisfied, to acquire competency to flower timely, or else high

302   *HvVRN2* levels will delay flowering beyond agronomically acceptable. This hypothesis

303   should be put to test with specific field experiments.

304   **Expression of *HvVRN2* does not cause genotypic differences in earliness among two**

305   **winter genotypes**

306   The comparison of the two winter cultivars clearly revealed a faster early development of

307   'Hispanic', although they are similarly responsive to VER (Fig. S1). Differences in *HvVRN2*

308   expression cannot be the cause of earliness differences, as the early genotype has larger

309   transcript amounts of the repressor. This indicates that there are additional earliness factors

310   differentiating 'Hispanic' and 'Barberousse', affecting apex development. Such factors would

311   be needed to counteract the repressing effect caused by upregulated *HvVRN2* expression

312   levels. We have explored the possibility that this factor is *HvFT3*, candidate for *Ppd-H2*, the

313   gene affecting short photoperiod sensitivity (Laurie *et al.*, 1995).

314   ***HvFT3* expression needs induction by cold and plant development, through**

315   **downregulation of flowering repressors, under non-inductive conditions.**

316   The two varieties differ (among others) in the presence/absence of *HvFT3*, which could be the

317   key factor that differentiates them. This gene bears particular agronomic relevance for

318   Mediterranean environments, as it stands at the peak of flowering time QTL and grain yield

319   QTLxEnvironment peaks in several populations (Cuesta-Marcos *et al.*, 2008, 2009; Karsai *et*

320   *al.*, 2008; Francia *et al.*, 2011; Tondelli *et al.*, 2014). A supporting role for promotion to

321   flowering in winter cultivars, receiving less than enough vernalization under field conditions,

322   was proposed for *HvFT3* (Casao *et al.* 2011*b*). Its expression is usually reported in SD,

323   although it is also found in LD conditions (Kikuchi *et al.*, 2009; Casao *et al.*, 2011*a*). In our

324   experiments, under natural photoperiod, *HvFT3* transcripts were only detected: (a) after full or

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325   partial vernalization, in early-medium development (Figures 2 and 6), and (b) in absence of

326   vernalization, in rather late developmental stages, and only in plants sown under shortest day-

327   lengths (Figure 4). We expected expression of *HvFT3* at least in the earliest sowings,

328   experiencing the shortest photoperiods. Instead, it was effectively repressed, either by the low

329   but always presence of *HvVRN2*, or by other repressors. Under constant photoperiod of 12h,

330   *HvFT3* was detected in 'Hispanic' only after four weeks VER (2 weeks were insufficient) and

331   5 weeks in growth chamber (Figure 6). Thus, *HvFT3* is expressed in a winter cultivar only

332   after there has been some cold exposure, and increasingly with plant age. It is particularly

333   remarkable that the expression of *HvFT3* was correlated with earlier flowering, although it

334   was detected only after the transition from vegetative to reproductive apex had occurred (Fig.

335   S3a and S4). This late effect on development is consistent with findings in spring wheat

336   varieties (Halliwell *et al.*, 2016), and in a GWAS study in barley (Alqudah *et al.*, 2014). This

337   last study associated polymorphisms at the *HvFT3* region with the time to tipping and the sub-

338   phase awn primordium-tipping.

339   The induction of *HvFT3* in sowing event 2 (cross-sectional sampling in experiment 1),

340   together with the progressive increase of the transcripts after 28-days VER, when *HvVRN2* is

341   not detected, are consistent with the antagonistic role between *HvVRN2* and *HvFT3* revealed

342   by Casao *et al.*, (2011a). However, there were samples in which the absence of *HvVRN2* did

343   not spur the expression of *HvFT3*, showing that the antagonistic relationship is not perfect.

344   These findings overall support that *HvVRN2* absence allows induction of *HvFT3,* but also

345   indicate that it is not sufficient to ensure *HvFT3* expression, hinting at the possible

346   involvement of other repressors. *HvOS2* showed an inverse relationship with *HvVRN1* and

347   *HvFT1* in the cross-sectional sampling (sowings 2, 10 and V in Fig. 4). This finding

348   highlights the interest of further studying the role of *HvOS2*, and its possible relationship with

349   *HvFT3* (already pointed out by Cuesta-Marcos *et al.*, 2015).

350   *HvFT3* expression was paralleled by that of *HvVRN1* and *HvFT1*. Previous reports agree with

351   our observation. Lv *et al.*, (2014) reported in *Brachypodium* and wheat, that developmental

352   changes regulated by *FT1* were related to transcript levels of other *FT*-like genes, as *FT3*.

353   Under LD, these authors only found upregulation of *FT3* when *FT1* was upregulated,

354   similarly to our findings. In this respect, Li *et al.*, (2015) demonstrated different interactions

355   between FT1 and other FT-like proteins, including FT3, with FD-like and wheat and barley

356   14-3-3 proteins, all components of the florigen activation complex (FAC). Indeed, they

13

357   showed a high specificity in the interaction of the protein HvFT3 with Ta14-3-3C, revealing

358   its importance in flowering.

359   **Coordinated expression of photoperiod and vernalization intermediaries *HvCO2* and**

360   **_HvVRN2_**

361   *HvVRN2* expression usually occurs in LD. Under SD conditions (8-9 h), the repression of

362   *HvVRN2* is controlled by components of the circadian clock (Turner *et al.*, 2013), although

363   expression under SD, due to the overexpression of *HvCO2*, has been reported (Mulki and von

364   Korff, 2016). Our findings suggest an apparent coordination of *HvCO2* and *HvVRN2*

365   responses in three-week old plants in experiment 1. Up to sowing event 4, the expression of

366   both genes was low, albeit gradually increasing in both cultivars. After that, expression of

367   *HvCO2* dropped dramatically, concurrent with *HvVRN2* raise at sowings 5 and 6. This pattern

368   is consistent with the reported competition between these proteins for binding to NF-Y

369   proteins (Li *et al*. 2011), and also with the feedback loop proposed by Mulki and von Korff

370   (2016). At some point between sowing events 4 and 5, there is a tipping point in expression,

371   possibly related to the dynamics of these two proteins, which could shift the balance of the

372   feedback loop towards higher expression of *HvVRN2*. Then, at event 8 and on, the

373   relationship between the expressions of these two genes seems less tight. Also, at later date,

374   (under 15 h), the relationship presented clear genotypic differences. At that moment, *HvVRN2*

375   expression remained relatively strong (in absence of vernalization); *HvCO2* expression,

376   however, showed a strong recovery after sowing event 4 in 'Hispanic', whereas 'Barberousse'

377   steadily showed low expression. Therefore, there is a clear shift in the balance of these two

378   genes when day-length is longer than 12h 30min. From that point on, the two genotypes

379   present different patterns.

380   The control of these two genes has been linked to *Ppd-H1*. Mulki and von Korff (2016)

381   presented evidence of another feedback loop, between *HvVRN2* and *Ppd-H1*, whereas the

382   induction of *HvCO2* by *Ppd-H1*, proposed in the past (as reviewed in Campoli *et al*. 2014), is

383   currently questioned (Chen *et al.*, 2014; Song *et al.*, 2015). In any case, it is clear that both

384   *HvVRN2* and *HvCO2* respond to day-length, either directly through *PHYTOCHROME C*

385   (*PhyC)*, or having *Ppd-H1* as an intermediary (as reviewed by Song *et al.*, 2015). *Ppd-H1*

386   (*HvPRR37* as reported by Campoli *et al.*, 2012) is the long photoperiod sensitivity gene in

387   barley, and its expression is under circadian control, with a broad expression peak around 12

388   h of light in LD (Turner *et al.*, 2005; Campoli *et al.*, 2012). Consequently, its maximum

14

389   expression levels require days of 12 h or longer. Although sampling times do not match that

390   peak, we can observe and effect over the expression of *HvCO2* and *HvVRN2*, which gradually

391   increased under longer days. The tipping point at 12 h 30 min actually agrees with the date

392   when natural day-length surpasses the maximum expression threshold for *Ppd-H1*. Recently,

393   it was demonstrated in *A. thaliana* that different PRRs not only induce *CO* transcription, but

394   also stabilize the CO protein during the day, enabling to accumulate under LD and initiate

395   floral transition (Hayama *et al.*, 2017). The role of these proteins in cereals should be further

396   clarified.

397   Mulki and von Korff (2016) proposed that, the dominant *Ppd-H1* could be acting as a

398   flowering repressor before vernalization is fulfilled, which usually takes place under non-

399   inductive photoperiods. We could say that it has a direct impact onto the vernalization

400   requirement of a genotype, as higher *HvVRN1* induction may be needed to down-regulate the

401   increased *HvVRN2* levels brought about by *Ppd-H1* induction, although a parallel mechanism

402   to explain *Ppd-H1* delaying effects in facultative barleys could exist.

403   **Photoperiod sensitivity through *Ppd-H1* delays field heading/flowering date irrespective**

404   **of the vernalization process**

405   The mechanism just described sheds light on a phenomenon repeatedly observed in field

406   conditions: flowering delay associated with the dominant (sensitive) allele at *Ppd-H1*, and

407   when field trials contrasted for flowering Julian date. A QTL peak for heading date locating

408   with *Ppd-H1*, with opposite effects in trials, was found in different biparental populations

409   (Ponce-Molina *et al.*, 2012; Mansour *et al.*, 2014). At the earliest trials, the sensitive *Ppd-H1*

410   allele slightly delayed heading, whereas accelerated it in later trials. *HvVRN2* is present in

411   these two populations and, therefore, the delaying effect could be the result of *HvVRN2*

412   expression reinforcement (Mulki and von Korff, 2016). Similar findings were reported for

413   populations Dicktoo × Morex (Pan *et al.*, 1994) and Steptoe × Morex (Borràs-Gelonch *et al.*,

414   2012), in which *HvVRN2* is absent. A parallel mechanism is needed to explain this effect. In

415   all four populations, the sensitive *Ppd-H1* allele was responsible for delaying heading or

416   flowering dates when it occurred in environments with short day-lengths. It is expected, given

417   the locations used, that in most of these experimental situations there was no lack of natural

418   vernalization. Yet, it is possible that there was a window of opportunity for *Ppd-H1*-dominant

419   genotypes to induce expression of *HvVRN2*, or other repressors, at higher levels than would

420   occur in *Ppd-H1*-recessive genotypes, thus causing its delaying effect.

15

Transition to reproductive stage can be achieved through several ways

Traditionally, the transition to the reproductive stage has been associated to expression of *HvFT1*, whose protein is translocated from the leaves to the apices (Song *et al.*, 2015). In our experiments, under lesser-inductive conditions, *HvFT1* expression was not always paralleled by expression of *HvVRN1* (experiment 2). We observed *HvCO2* expression concurrently with induction of *HvFT1*, even in absence of vernalization and, remarkably, without detectable expression of other promoters, such as *HvVRN1* or *HvFT3*. These results agree with the transcriptome data reported by Digel *et al.* (2015), who showed expression of *HvFT1* and *HvCO2*, dependent on *Ppd-H1* in LD, in leaves. These authors found that expression in leaves was higher when the apices had passed the double ridge stage (Waddington stage > 2.0), which could explain the increased transcript levels we found after 49 days under controlled conditions.

Our results suggest that *HvCO2* contributes to *HvFT1* induction, similarly to results reported for wheat (Chen *et al.*, 2014), and is downregulated after vernalization as shown in *B. distachyon* (Huan *et al.*, 2013). Li *et al.* (2011) found that both wheat VRN2 and CO2 interacted with the same set of HAP/NF-Y inducer proteins and suggested that both play a role integrating environmental signals for transcriptional regulation of *FT1*. In Arabidopsis, *CO* induces *FT* in LD, and consequently flowering, probably through interaction with its promoter (Andrés and Coupland, 2012). In the winter barley varieties employed in this study, under non-inductive LD conditions (Experiment 1, points 6-10, Fig. 2 and Fig. 4), the expression profile of both *CONSTANS* genes (*HvCO2*, *HvCO9*), was rather similar, suggesting a common regulation, possibly in an age or photoperiod dependent-manner.

HvCO9 is another CCT domain gene, like *HvCO2* or *HvVRN2* (Higgins *et al.*, 2010; Cockram *et al.*, 2012; Kikuchi *et al.*, 2012). Kikuchi et al. (2012) found higher expression of *HvCO9* under SD (12 h light), in spring barleys lacking *HvVRN2*. Our study, using winter barley genotypes, also suggest a repressor role of this gene, stronger in 'Barberousse' than in 'Hispanic' under SD conditions, related to development and amount of vernalization.

HvOS2 could explain differences in development among winter varieties

In this study, striking differences between genotypes have been observed under non-inductive conditions (no or reduced vernalization and SD). 'Hispanic' flowered always earlier than 'Barberousse', as also evidenced by the expression of all flowering promoters in the first and

16

452 high expression of the repressors in the latter. The results illustrate the different behavior of

453 these winter genotypes.

454 The third repressor of flowering time studied, *HvOS2,* showed slight differences between NV

455 and VER plants in early development. The difference became more evident in older plants.

456 Expression of *HvOS2* was highly correlated with the absence of *HvVRN1,* being low in plants

457 which flowered, as was observed in barley, wheat and *Brachypodium* (Greenup *et al.*, 2010;

458 Sharma *et al.*, 2017). Deng *et al.*, (2015) showed that the protein VRN1 binds to the

459 promoters of *VERNALIZATION2* and *ODDSOC2*. By now, there is enough evidence

460 substantiating that expression of *OS2* genes in winter cereals is suppressed by cold. For

461 *Brachypodium*, it has been proposed that *BdODDSOC2* "plays a role in setting the length of

462 the vernalization requirement in a rheostatic manner, i.e. higher *ODDSOC2* transcript levels

463 before cold result in a longer cold period needed to saturate the vernalization requirement"

464 (Sharma *et al*., 2017), although its specific role in the vernalization response is not clear.

465 In our results, variety 'Barberousse' showed higher levels of *HvOS2* transcripts than

466 'Hispanic' at most sampling times. This higher expression in 'Barberousse' is consistent with

467 its delay in development compared to 'Hispanic'.

468 **_HvVRN1_ expression in winter barley under non-inductive conditions occurred after the**

469 **apex transition**

470 'Hispanic' and 'Barberousse' showed differences in development in response to photoperiod

471 and vernalization. The overall expression patterns for flowering genes were in line with

472 expectations for winter varieties. Commonly, winter varieties need to undergo a specific

473 number of cold hours before flowering. The varieties used in this study carry *vrn1* allele, with

474 a full-length intron and present a strong vernalization need (Fig. S1). In absence of

475 vernalization, there is no expression of *HvVRN1* and consequently, flowering is delayed, as

476 expected (Trevaskis *et al.*, 2003; Yan *et al.*, 2003; von Zitzewitz *et al.*, 2005). We did not

477 observe expression of *HvVRN1* in NV 21-day-old plants at any point. Only plants from

478 sowing event 2, 13 weeks after sowing, showed *HvVRN1* expression. This result agrees with

479 previous reports of the induction of *HvVRN1* by development after 10-12 weeks in

480 unvernalized winter plants, under LD (Trevaskis *et al.*, 2006). The lack of *HvVRN1*

481 expression in non-vernalized plants is related to the presence of *HvVRN2* and *HvOS2*

482 transcripts, as was evidenced by other authors (Dubcovsky *et al.*, 2006; Trevaskis *et al.*, 2006;

483   Greenup *et al.*, 2010; Deng *et al.*, 2015; Sharma *et al.*, 2017). When transcripts of these
484   flowering repressors were present, the flowering promoters *HvVRN1*, *HvFT1* and *HvFT3*
485   were directly or indirectly downregulated, and flowering was delayed (Fig. 4, Fig. 6). Indeed,
486   Deng *et al.* (2015) showed that, in winter, VRN1 controls directly *HvFT1* levels binding to its
487   promoter, and indirectly through *HvVRN2* and *HvOS2* down-regulation. This repression could
488   also be stimulating *HvFT3* and expression of other flowering promoters (Cuesta-Marcos *et*
489   *al.*, 2015).

490   We observed a more loose relationship between *HvVRN1* and *HvFT1* than expected. Some
491   apices developed to reproductive stage (W2, Fig. S4), even though this was not paralleled by
492   induction of *HvVRN1* in the leaves at the same time in both experiments (Fig.4, Fig.6).
493   Indeed, the peak of expression of *HvVRN1* seems to be related with the appearance of the
494   floret primordium (W3.5). While this result could be in conflict with the essential role of
495   *HvVRN1* in the initiation of reproductive phase described before (Trevaskis *et al.*, 2003), data
496   by Digel *et al.* (2015) can clarify our observations. These authors showed that *HvVRN1,*
497   together with other MADS box transcription factors, was upregulated in the leaves and shoot
498   apices during pre-anthesis, but the transcript levels of *HvVRN1* were first induced in the shoot
499   apices. This gap would explain why we did not detect *HvVRN1* expression in the leaves
500   although some apices had already progressed in their development.

501   **Conclusion**

502   The use of different sowing events, under natural increasing photoperiod corroborate that the
503   expression of *HvVRN2* is highly dependent on day-length, and we provide evidence of the
504   threshold, around 12 h 30 min, above which this expression rises markedly and affects most
505   plant development. This experiment also highlighted the importance of completing the
506   vernalization requirement before a certain day-length threshold, in order to promote flowering
507   in optimum conditions. *HvFT3*, a central gene for winter barley performance in Southern
508   Europe, is not induced just by short days. In winter cultivars with dominant *Ppd-H1*, it must
509   receive additional induction through either the autonomous pathway, and/or a cold period, to
510   be effective in reducing time to flowering.

511   In winter barleys, *HvVRN2* transcript levels are always present, but we propose that its
512   activity (and that of *HvOS2*) must be below a functional threshold to allow timely flowering,
513   which will not occur in absence of vernalization. Here, we emphasize the importance of
514   *HvVRN2* in the promotion to flowering, but also the role of *HvOS2* and *HvCO2* in

515   vernalization-responsive cultivars. *HvOS2* seems to contribute to *HvVRN2* function in the

516   delay of flowering, while *HvCO2* might be promoter of *HvFT1* in both inductive and non-

517   inductive conditions, being affected by those two repressors and *HvCO9*.

518   The photoperiod conditions of the experiments here described, correspond to a wide range of

519   late spring sowings for winter barley in the Mediterranean area. The genetic mechanisms and

520   the environmental controls involved in this study will be useful to define both varieties and

521   agronomics best suited for current and future climate conditions.

522

Supplementary data

524   Supplementary Table 1. Primer sequences.

525   Supplementary Figure S1. Flowering date under different vernalization treatments in the

526   preliminary experiment.

527   Supplementary Figure S2. Gene expression in 2-week-old plants sown under natural and

528   increasing photoperiods (without vernalization and control).

529   Supplementary Figure S3. Associations between gene expressions and heading date (DEV49)

530   in experiment 2.

531   Supplementary Figure S4. Apex development in experiment 2 (growth chamber, 12 h day).

Acknowledgements

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26

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Figure legends

Figure 1. Experiment planning. Each sowing and its sampling are represented. Yellow bars show the time that plants were kept under non-vernalized conditions. Blue bar shows the time spent in the vernalization chamber. X-axis represent dates of start of experiment and sampling date (three weeks after sowing). The second numbers inside the yellow bars are the day-length at sampling date (HH:MM). The first numbers in italics represent day-length at sowing day, and underlined numbers are day-length in the shift day (vernalized plants were transferred to glasshouse). Sunrise and sunset are the times when the upper edge of the Sun's disc touches the horizon.

Figure 2. Gene expression three weeks after sowing. X-axis represent the successive sowings, from 11th February until 8th April. Unvernalized plants (sowings 1 to 9) and vernalized control (V) of 'Hispanic' (blue) and 'Barberousse' (yellow) are plotted. Mean of 3 biological replicates. Error bars are SEM. ND, Not detected. *HvFT1* expression is not reported as it was null for all non-vernalized samples. For each variety, bars with the same letter are not significantly different at P<0.05 (LSD test).

Figure 3. Percentage of reproductive apices regarding the total (vegetative and reproductive) after 100 days of the experiment. Mean of 10-12 plants. Error bars are SD. For each variety, bars with the same letter are not significantly different at P<0.05 (LSD test).

Figure 4. Cross-sectional gene expression under 15 h of natural daylight of the sequential sowings under natural photoperiod experiment. X- upper axis represent the weeks after sowing of unvernalized plants. Control plants (V) were maintained under natural photoperiod for 6 weeks after 49 days of vernalization. Enlarged view of the *HvFT1* expression is shown. Mean of 3 biological replicates. Error bars are SEM. For each variety, bars with the same letter are not significantly different at P<0.05 (LSD test).

Figure 5. Phenotype of plants growing under controlled conditions (12 h daylight) after different vernalization treatments. Mean of 3 plants. Error bars are SD. ND, no detected (discarded after 130 days without flowering). For each variety, bars with the same letter are not significantly different at P<0.05 (LSD test).

Figure 6. Gene expression under 12 h daylight in growth chamber. X-axis represent days of vernalization chamber. Increasing grey scale is the days after the end of the vernalization treatment when leaves were sampled (14, 28, 35 or 49 days). Mean of 3 biological replicates. Error bars are SEM. For each variety, bars with the same letter are not significantly different at $P<0.05$ (LSD test).

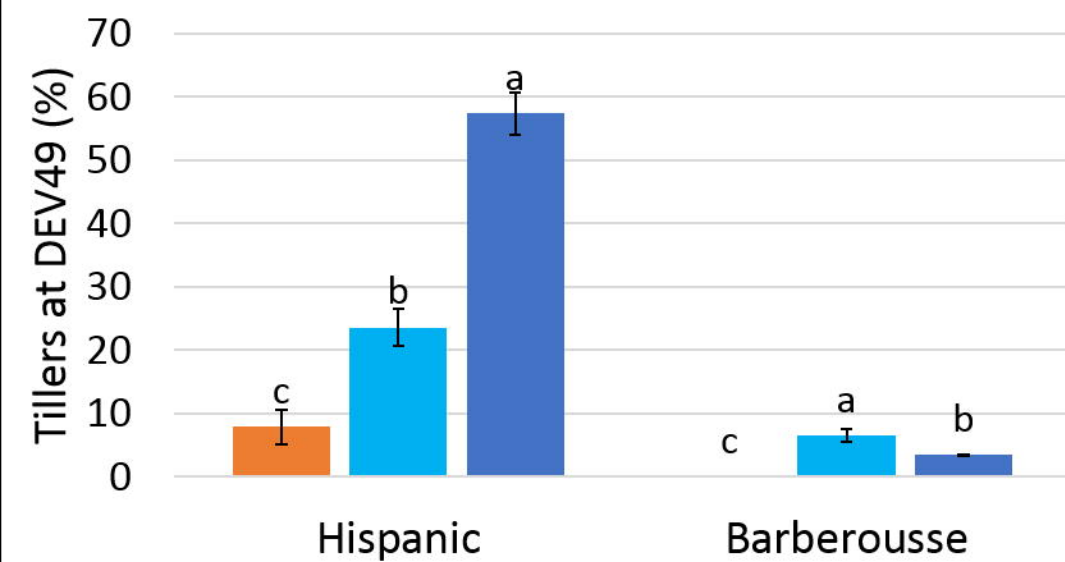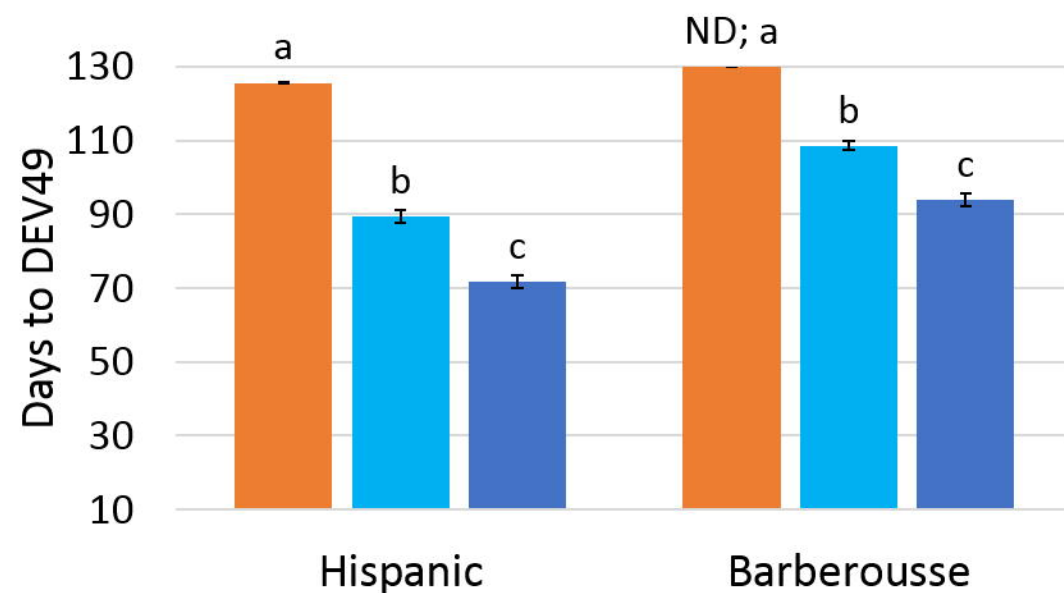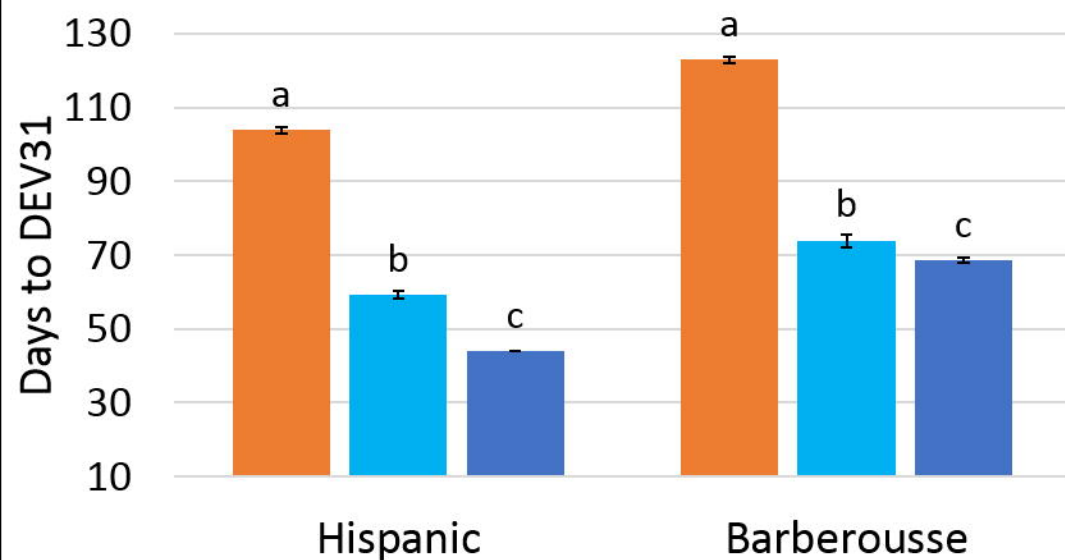Figure 7. Apex dissection of plants grown under 12h light, 4 weeks after each vernalization treatment. Red bar is 500 µm.
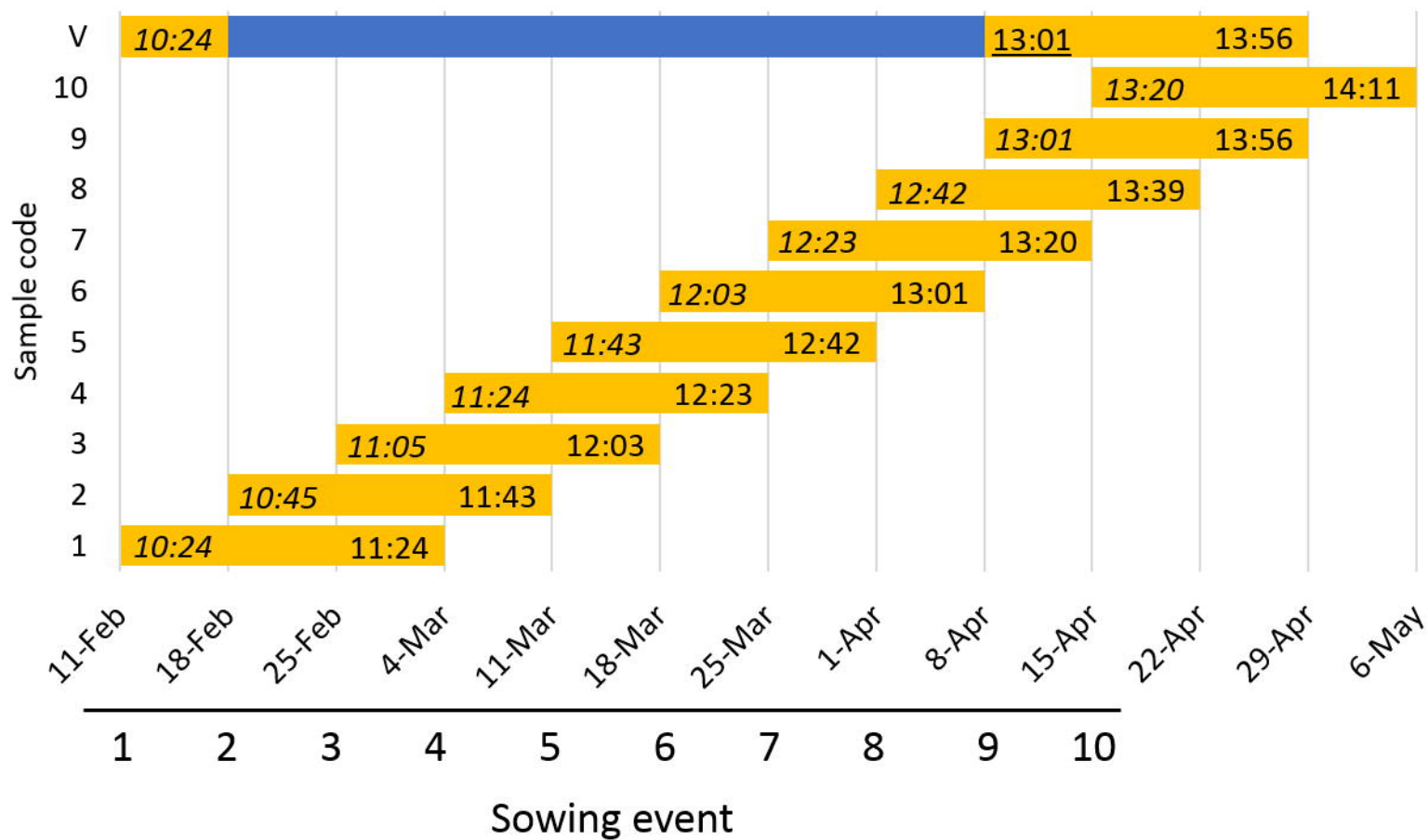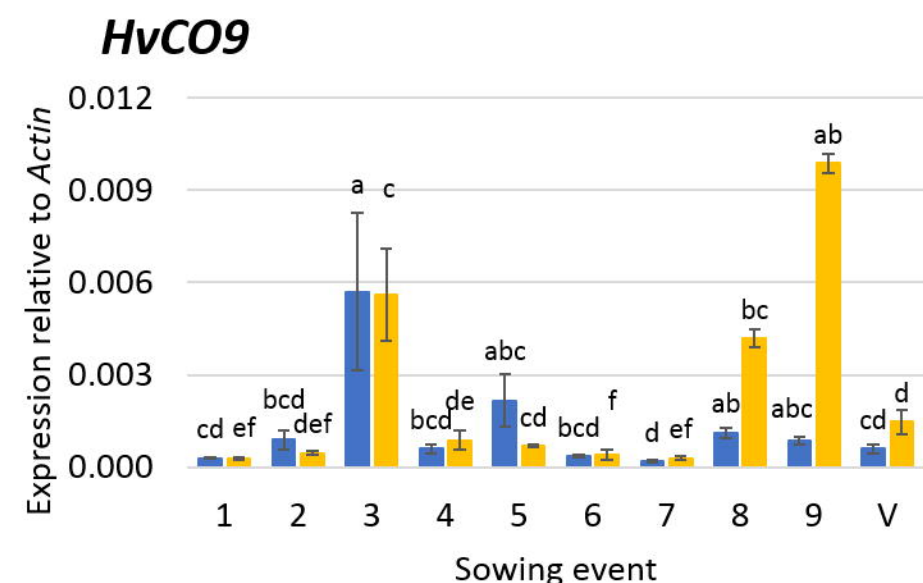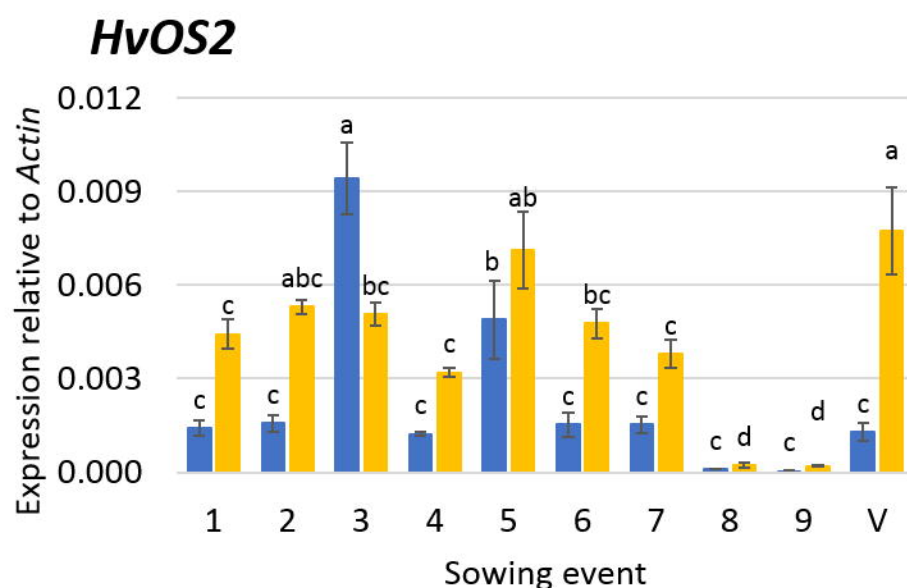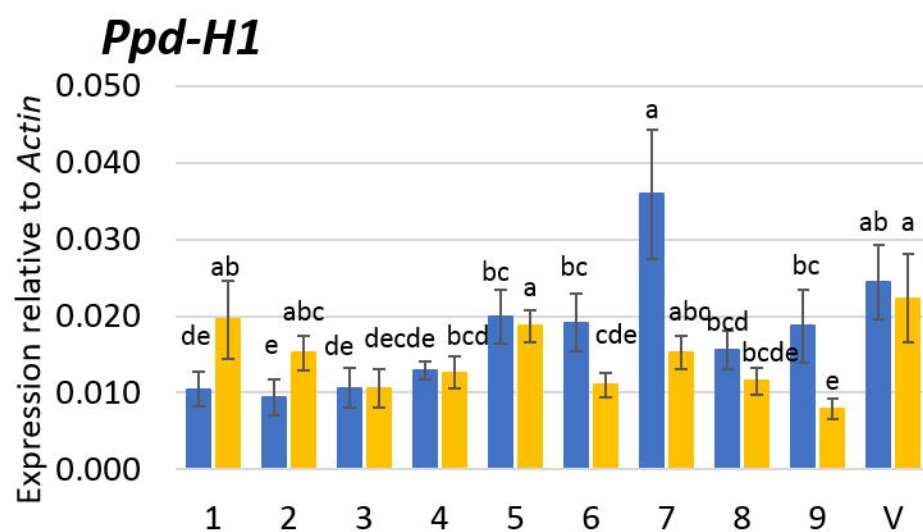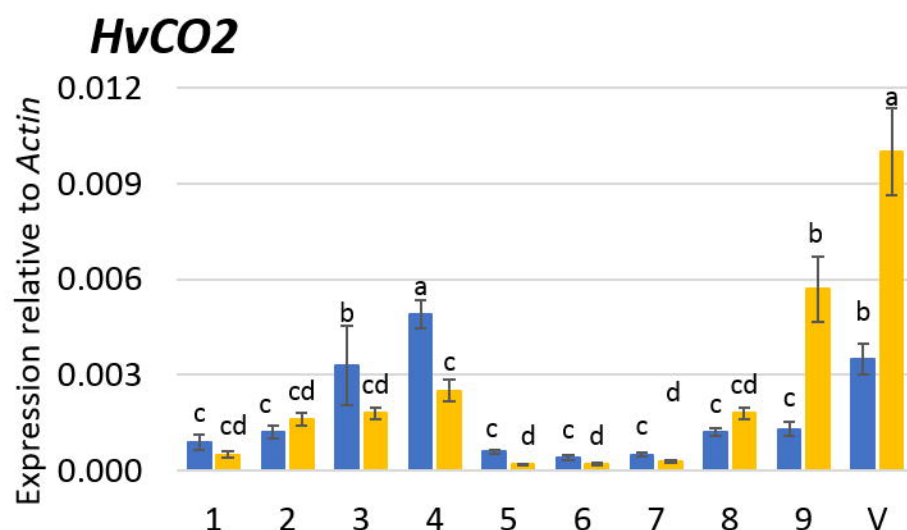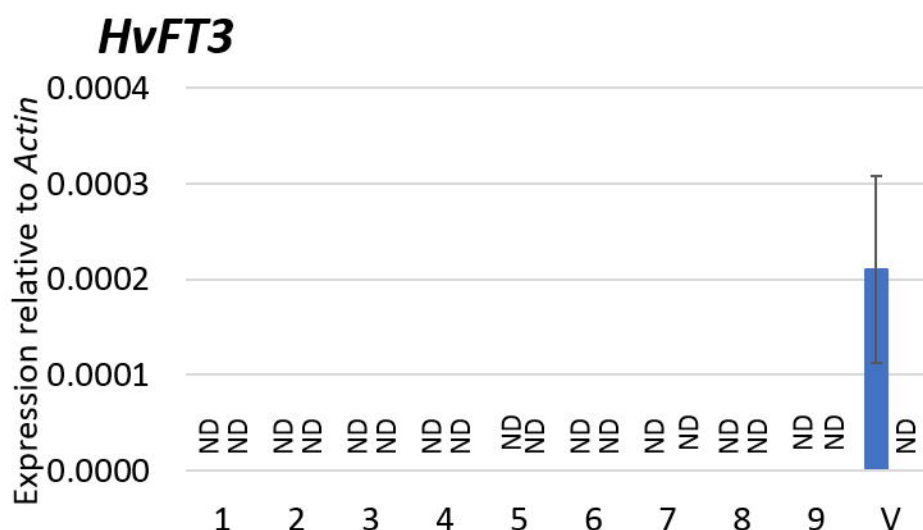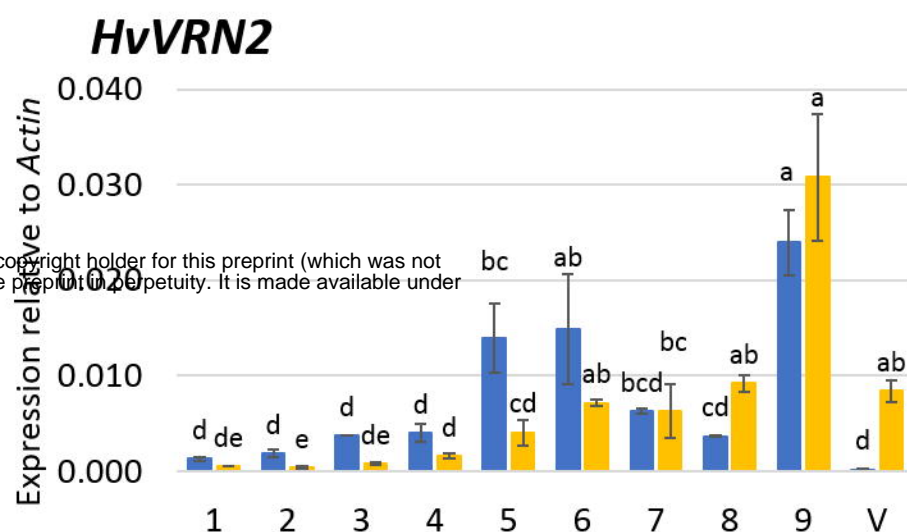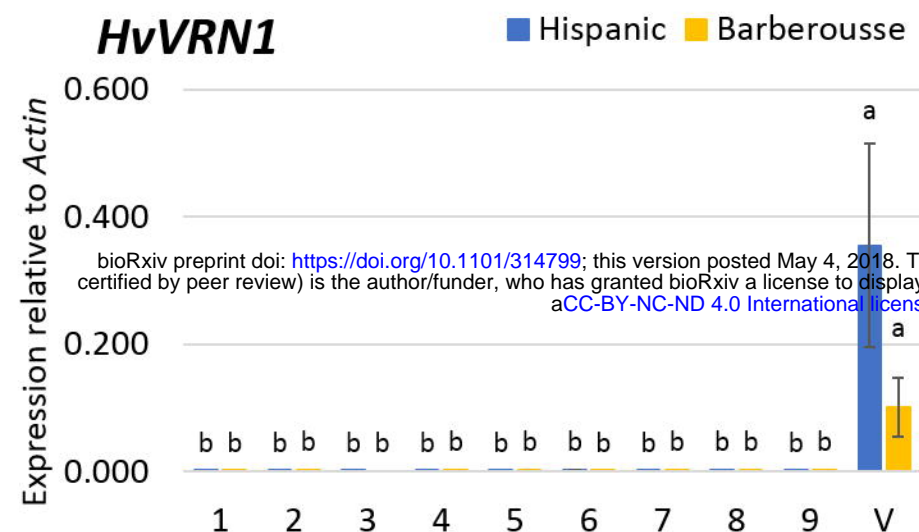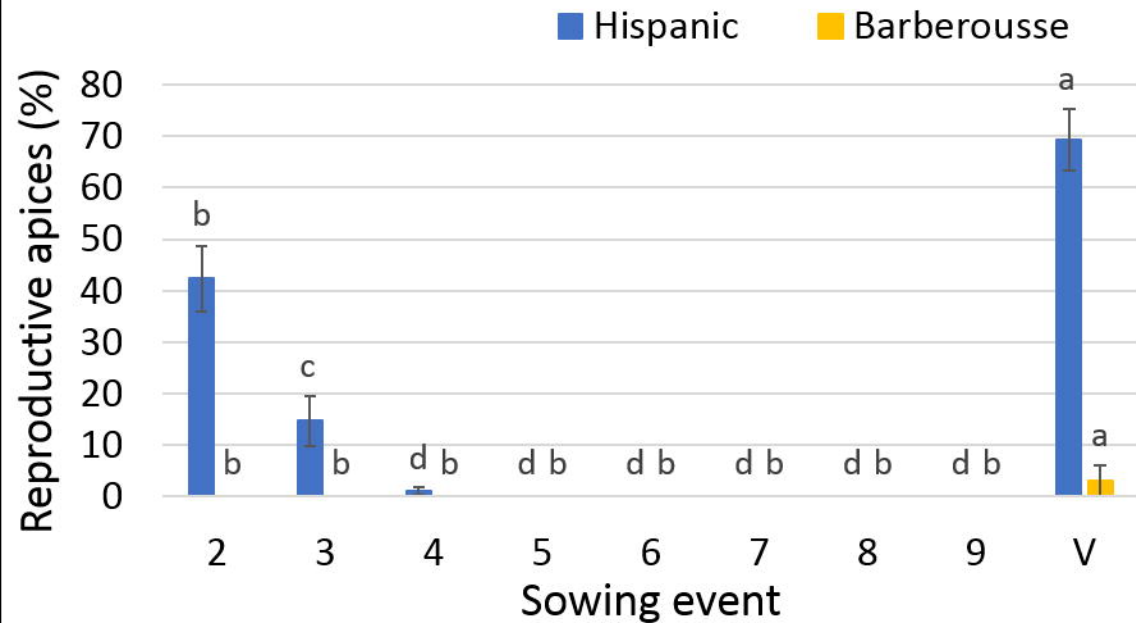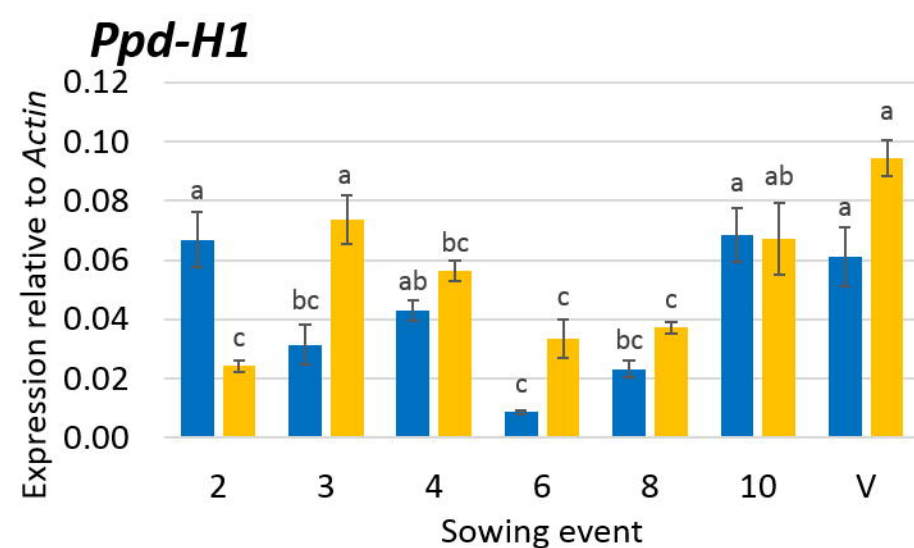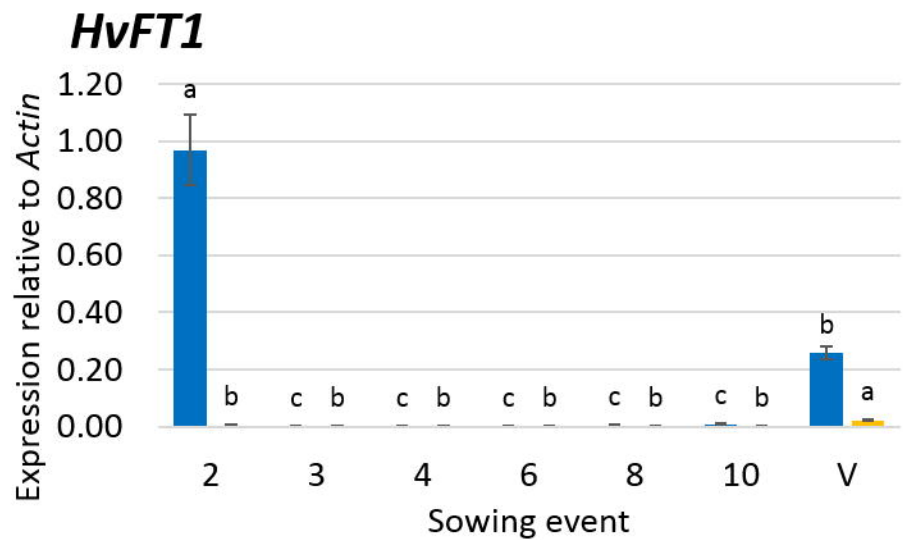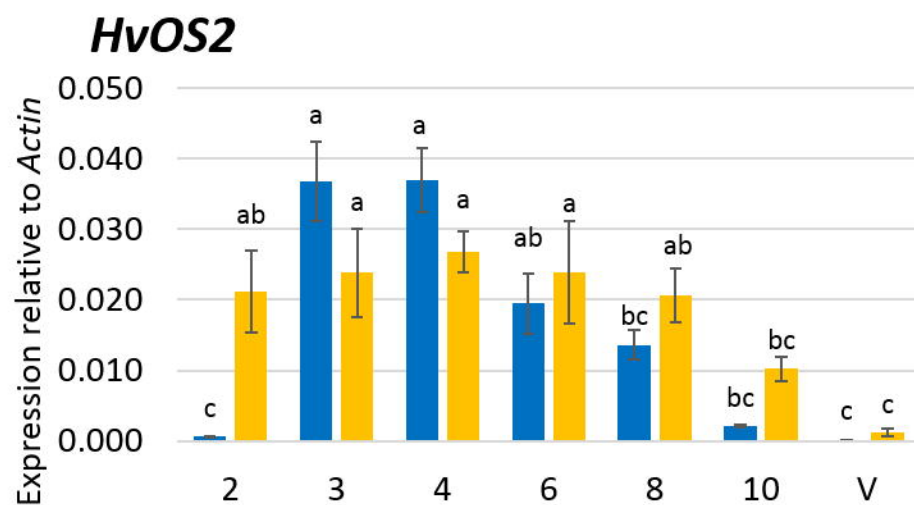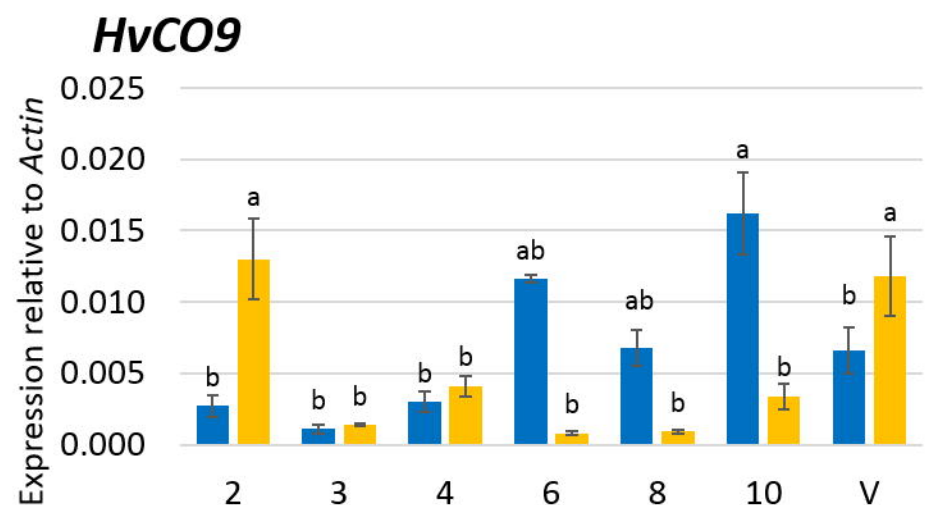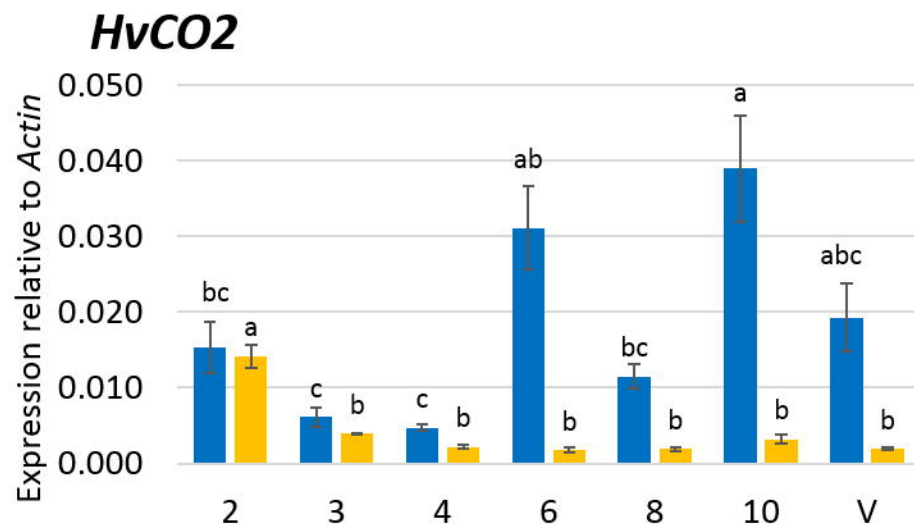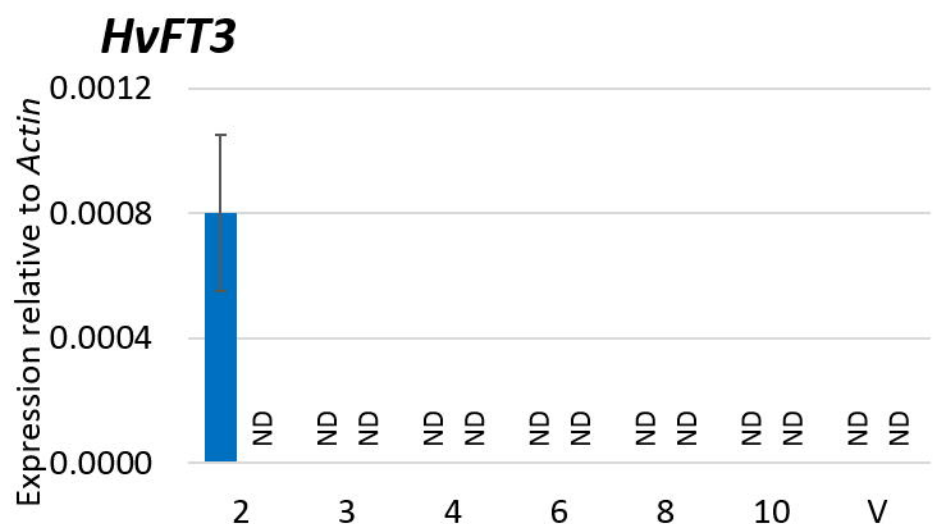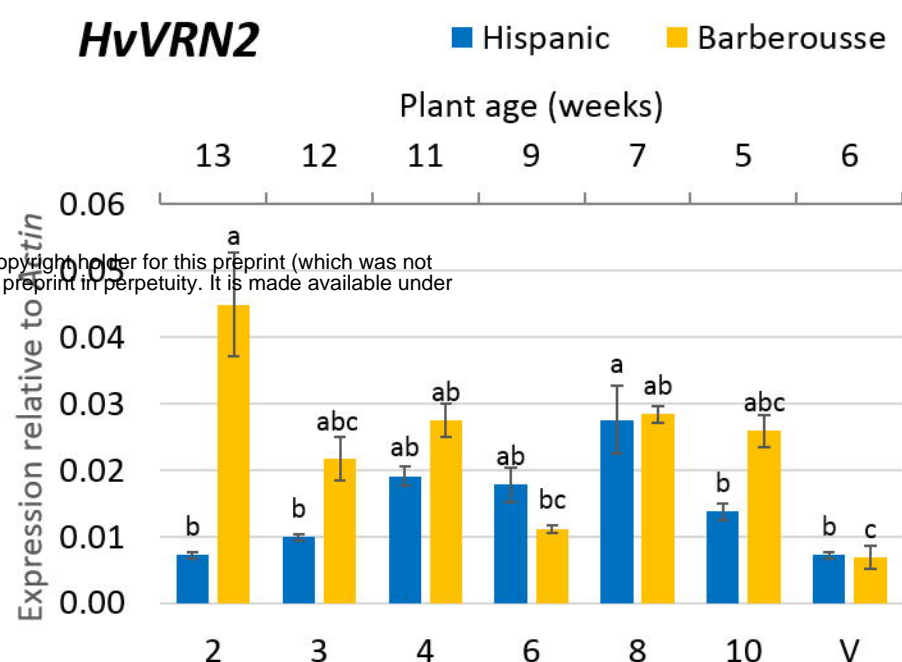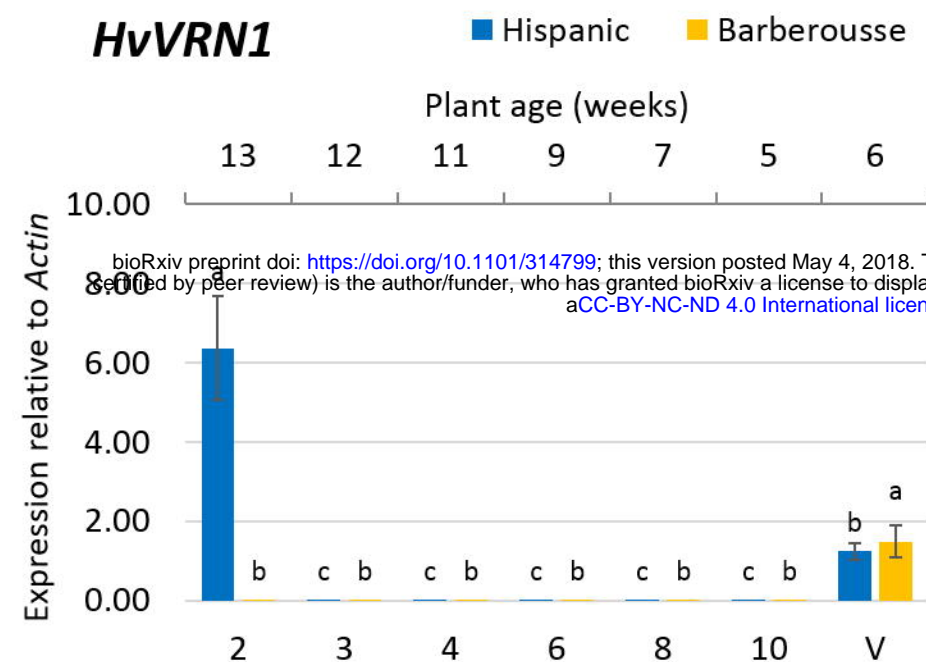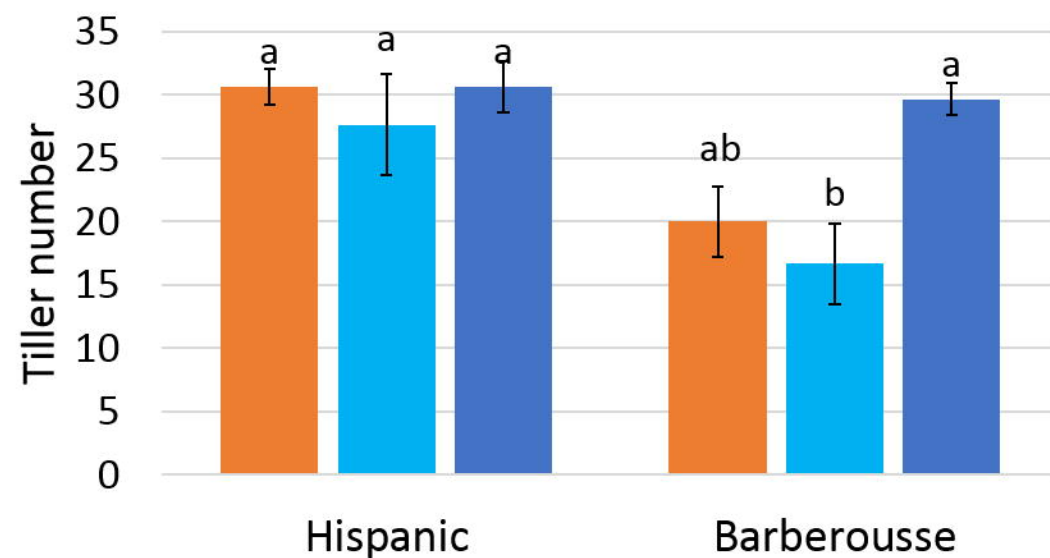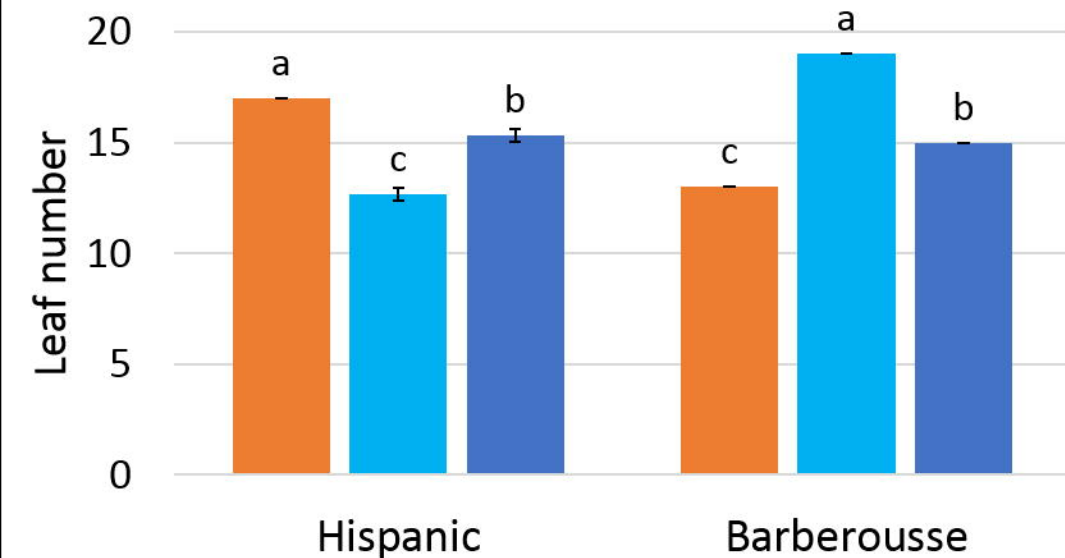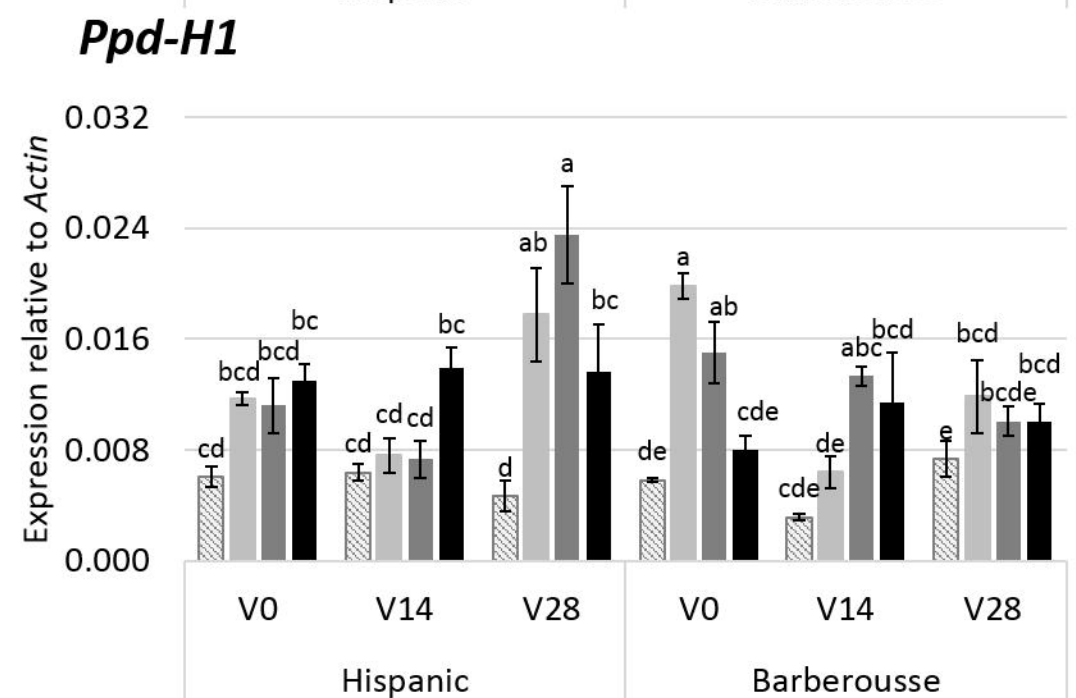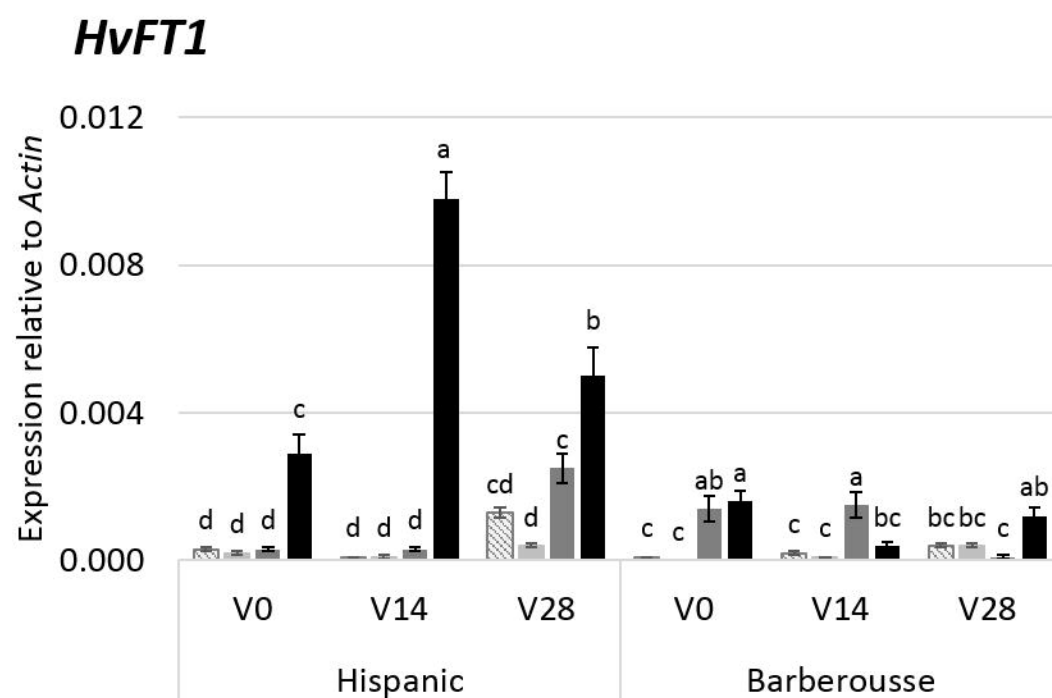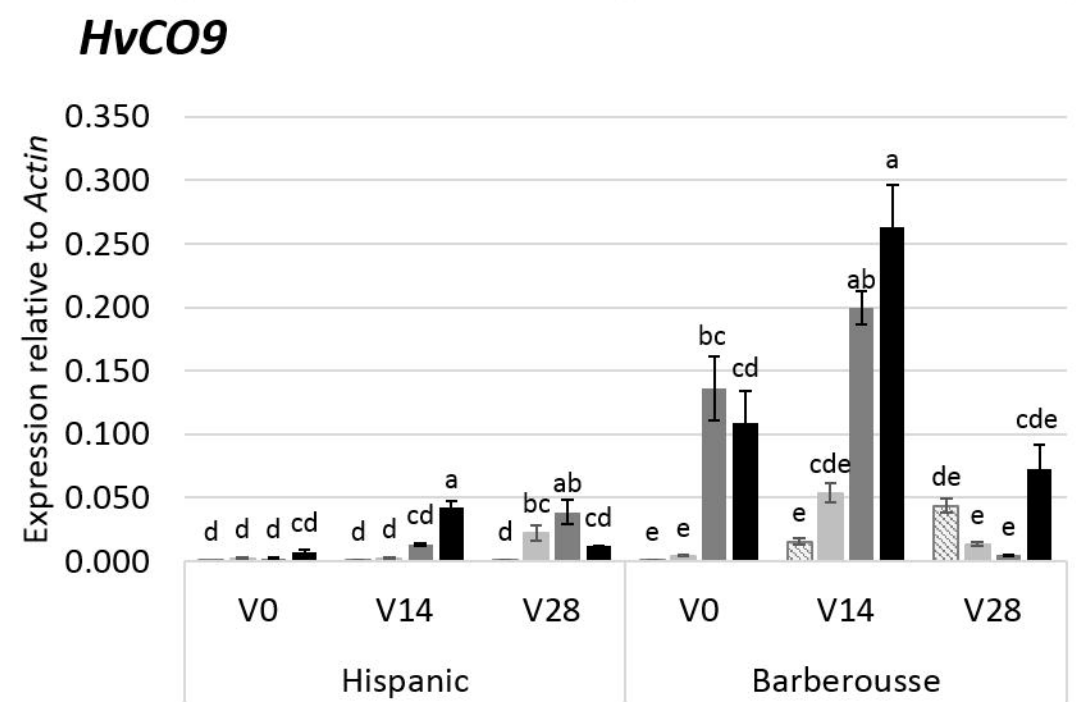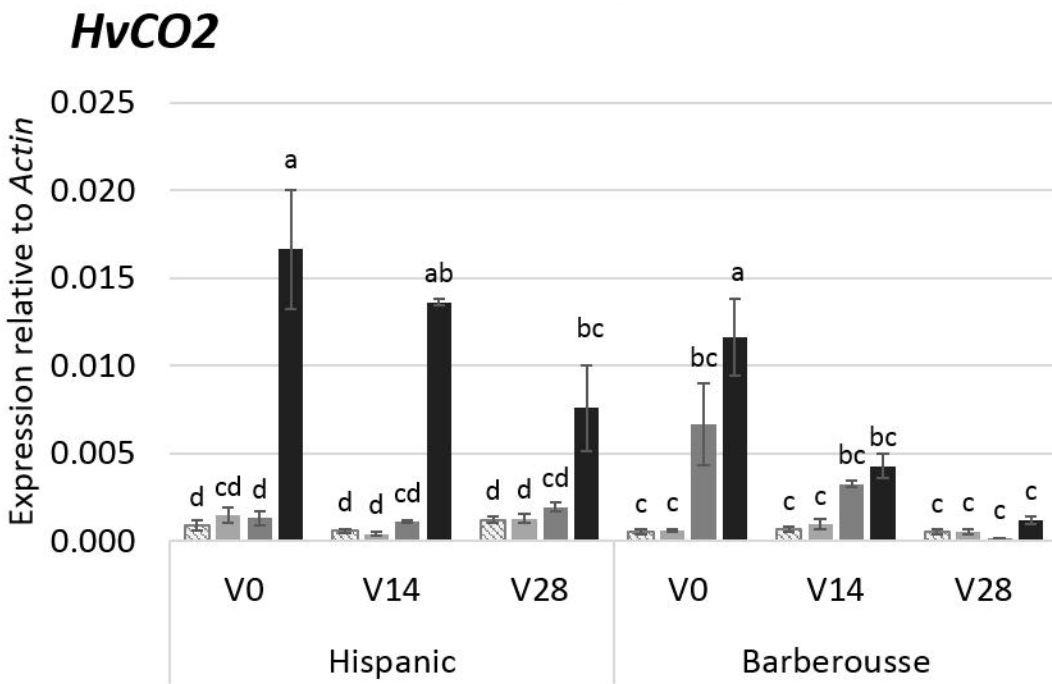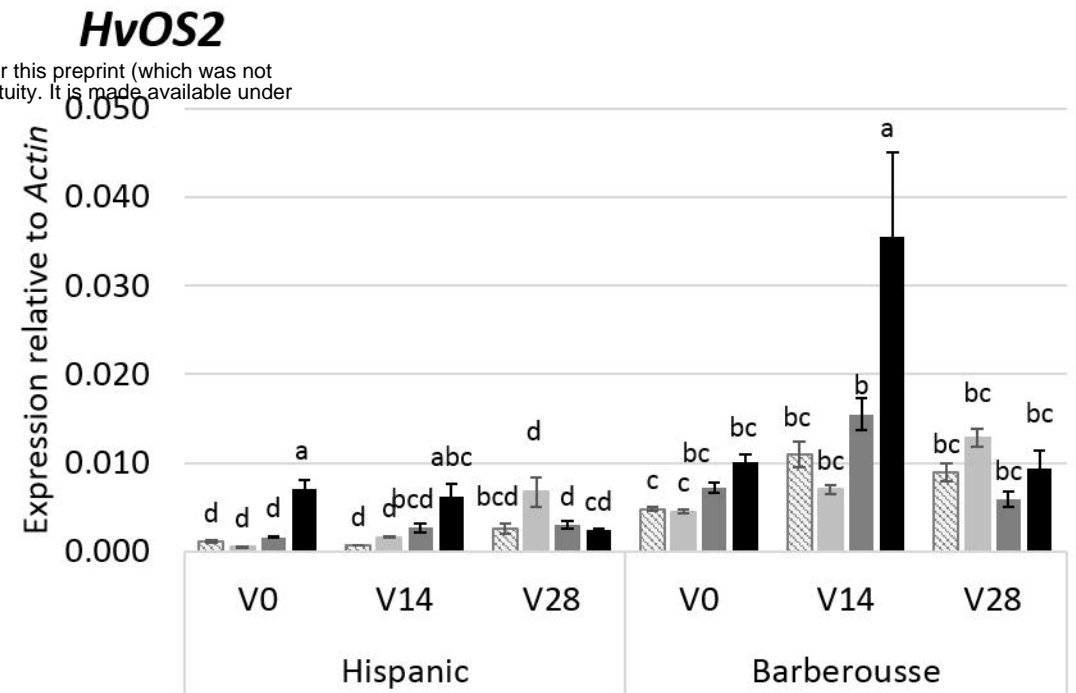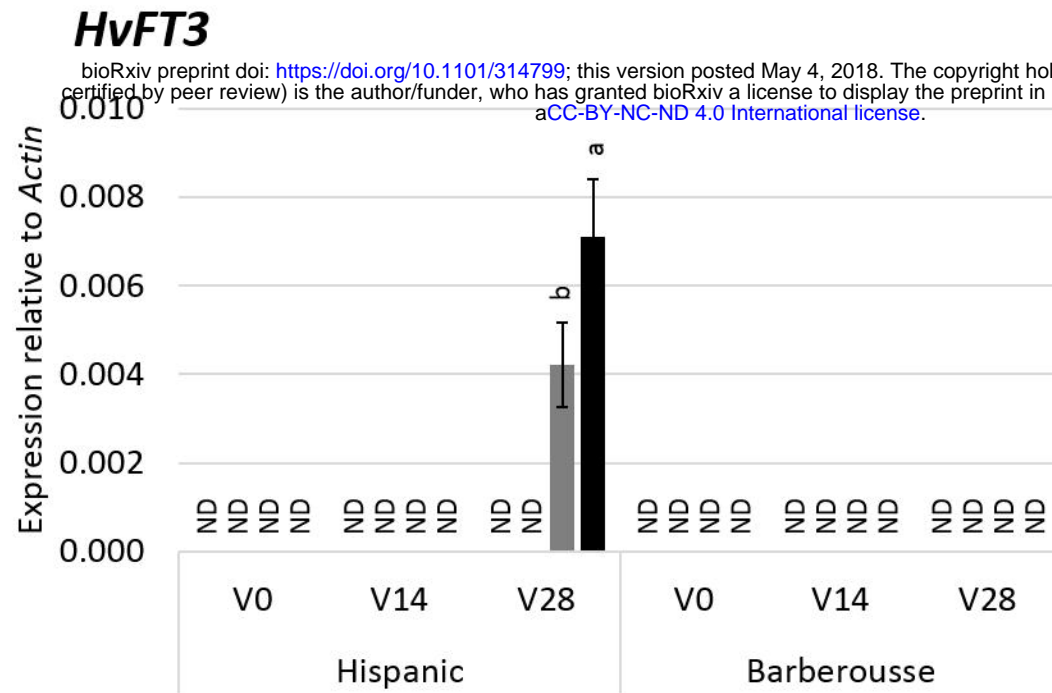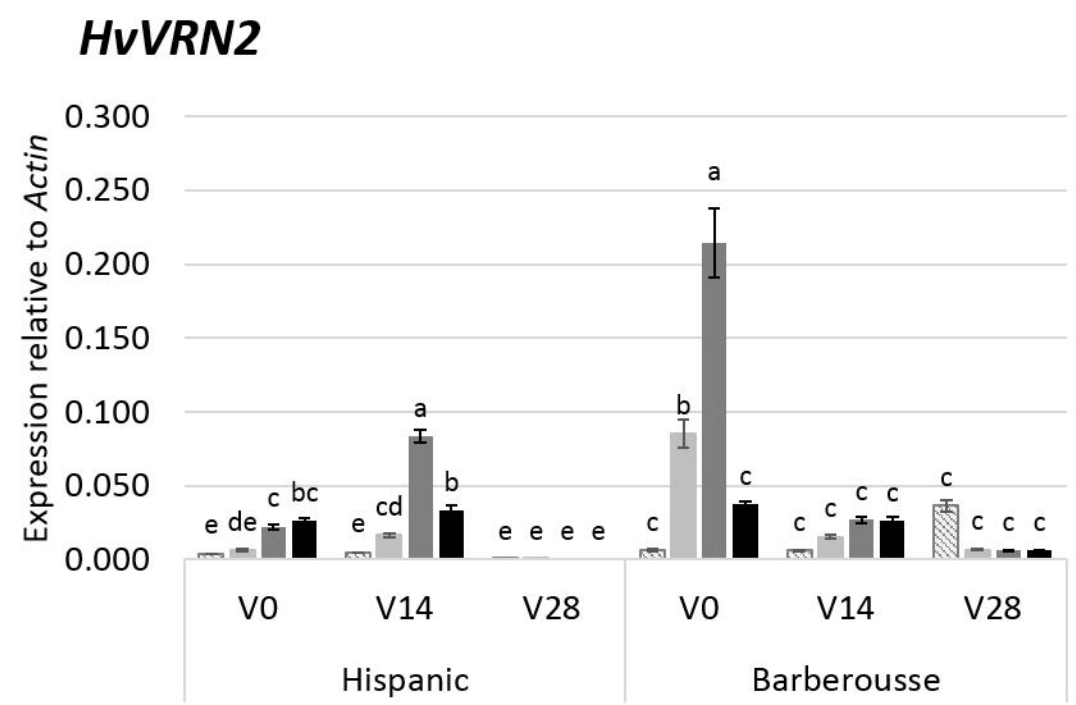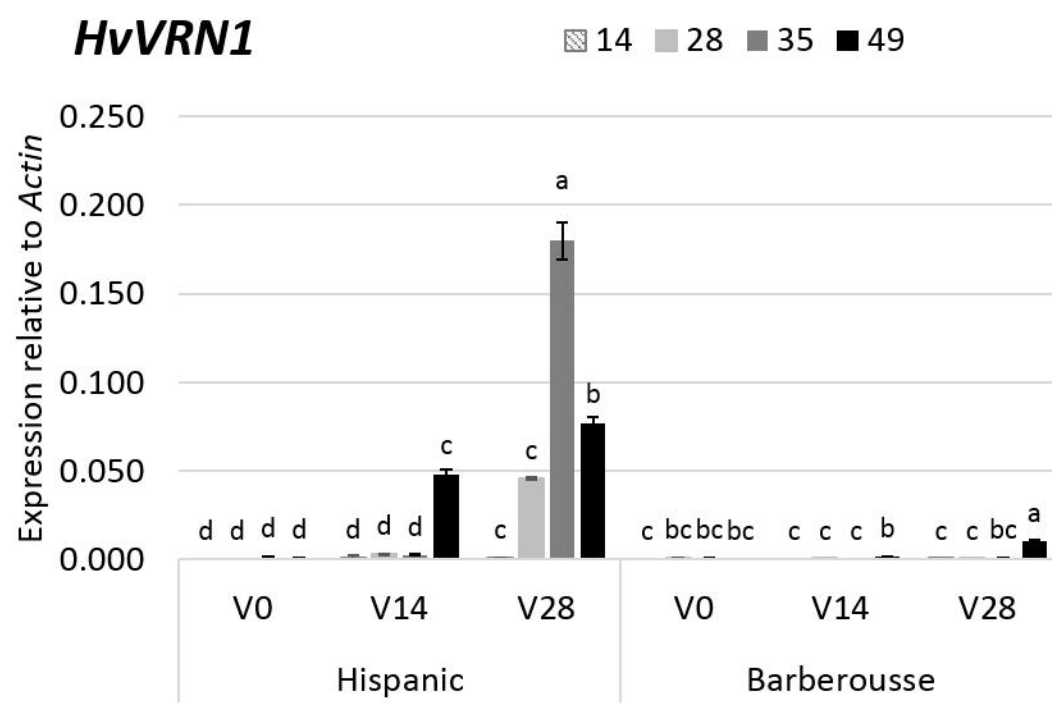
	V0	V14	V28
Hispanic	W 2	W 2.25	W 3.75
Barberousse	W 1.5	W 2	W 2.25